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**CHARACTERIZATION OF A NOVEL cdc2-RELATED KINASE
EXPRESSED IN THE NERVOUS SYSTEM**

By

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ABSTRACT

In order to identify a homologue of the cell division cycle regulatory kinase p34^{cdc2} that may function in the nervous system, a mouse brain cDNA library was screened at low stringency using a *cdc2* cDNA probe. A 2.5 kb cDNA was isolated that contains a 423-amino acid open reading frame encoding a novel *cdc2*-related protein kinase that was named PFTAIRE, because of the consensus PSTAIRE-like motif (single letter amino acid code) characteristic of *cdc2* kinase family. The novel PFTAIRE protein contains conserved kinase and non-kinase domains and phosphorylation sites like all other members of the *cdc2* kinase family, but is most homologous to the PCTAIRE class of *cdc2*-related kinases because of similar N- and C-terminal extensions that are lacking in many other *cdc2*-related kinases. Restriction fragment length polymorphisms were used to determine that the PFTAIRE gene (*Pftk1*) is positioned close to the centromere of mouse chromosome 5, proximal to another *cdc2*-related kinase gene, the *cdk5* gene.

Analysis of the tissue distribution and temporal expression pattern of PFTAIRE mRNA has demonstrated that PFTAIRE cDNA probes hybridize to a single mRNA transcript of approximately 5.0 kb, which is expressed predominantly in the nervous tissue of postnatal and adult mice. I have demonstrated by *in situ* hybridization and by indirect immunofluorescence that several discrete populations of terminally differentiated neurons and some glia express PFTAIRE mRNA and protein. In neurons, the PFTAIRE protein is localized primarily in the nucleus and cytoplasm of cell bodies but some populations of neurons also contain PFTAIRE in axons. The anatomical, cellular and ontogenic patterns of PFTAIRE expression in the nervous system differ from those of p34^{cdc2} and *cdk5*, which are expressed in nervous tissue and in several other mitotic tissues. The characterization of PFTAIRE mRNA and protein suggests that PFTAIRE may be the first member of the *cdc2* kinase family identified to date that is so specifically localized to the mammalian nervous system.

The biochemical characterization of PFTAIRE kinase activity has demonstrated that when immunoprecipitated from cytosolic protein preparations of adult mouse brain and transfected cells, PFTAIRE forms a complex with proteins of approximately 58-60 kDa that were specifically co-precipitated. These proteins also appear to be major endogenous targets of this kinase activity, but it is not known whether the associated proteins are regulators of PFTAIRE activity or substrate effector proteins involved in downstream events. The phosphorylation of these proteins was reduced in the presence of a PFTAIRE dominant negative mutant, generated by site-directed

mutagenesis of a residue required for the phosphate transfer reaction. This suggests that the phosphorylation of the associated proteins was due to PFTAIRE kinase activity. The biochemical characterization of PFTAIRE has also revealed that PFTAIRE itself is phosphorylated by a protein kinase in brain extract and that PFTAIRE may also autophosphorylate.

To address the possible function of PFTAIRE, PFTAIRE plasmids were transfected transiently in several proliferating fibroblast cell lines that expressed little or no endogenous PFTAIRE protein. Low transfection efficiencies and decreased cell viability were consistently observed. Cells overexpressing PFTAIRE showed morphological changes including decreased cell size, irregularly shaped nuclei, multiple or fragmented nucleoli and changes in the distribution of nuclear lamin proteins resembling that of cells arrested in the early G₁ phase of the cell cycle or cells undergoing apoptotic death. These results indicate that PFTAIRE overexpression in proliferating fibroblast cell lines was not well tolerated and suggests that inappropriate expression of PFTAIRE may interfere with cell cycle progression. Thus it is possible that the function of PFTAIRE in postmitotic neurons may be related to specialized properties acquired by cells that no longer progress through the cell cycle.

In summary, I have identified a novel member of the cdc2 family of protein kinases that is localized predominantly in the nervous system. The temporal and spatial expression patterns of PFTAIRE in the postnatal and adult nervous system suggest that PFTAIRE kinase activity may be associated with the postmitotic and differentiated state of cells in the nervous system, and that its function is likely to be distinct from p34^{cdc2} and cdk5.

ABRÉGÉ

Afin d'identifier dans le système nerveux un homologue de p34cdc2, une kinase régulant le cycle de division cellulaire, une banque d'ADNc préparée à partir de cerveau de souris a été criblée à faible stringence avec une sonde d'ADNc de cdc2. Un ADNc de 2.5 kb, ayant un cadre de lecture ouvert de 423 acides aminés codant pour une nouvelle protéine kinase apparentée à cdc2, a été isolé. Elle a été nommée PFTAIRE (code à une lettre pour les acides aminés) en raison de sa similitude au motif consensus PSTAIRE, caractéristique de la famille cdc2. Cette nouvelle protéine possède les domaines kinases et non-kinases conservés, de même que les sites de phosphorylation communs à tous les autres membres de la famille des kinases cdc2. L'homologie est toutefois plus élevée avec la classe des protéines kinases apparentées à cdc2 appelée PCTAIRE, en raison de leurs extensions amino et carboxy-terminales similaires, absentes chez les autres protéines kinases de la famille cdc2. La technique d'analyse du polymorphisme de fragments de restriction (RFLP) a permis de localiser le gène codant pour PFTAIRE (*Pftk1*) à proximité du centromère du chromosome 5 de souris, où est également situé le gène cdk5 codant pour une protéine kinase apparentée à la famille cdc2.

L'analyse temporelle de l'expression de l'ARNm de PFTAIRE dans différents tissus a montré que les sondes d'ADNc de PFTAIRE hybridaient à un produit unique de transcription, d'environ 5.0 kb, exprimé principalement dans les tissus du système nerveux chez la souris post natale et adulte. J'ai démontré par hybridation in situ, ainsi que par immunofluorescence indirecte, que plusieurs populations distinctes de neurones pleinement différenciés, ainsi que quelques cellules gliales, expriment l'ARNm et la protéine PFTAIRE. Dans les neurones, la protéine PFTAIRE est localisée principalement dans le noyau et le cytoplasme du corps cellulaire. Cependant, chez certains neurones, PFTAIRE est également présente dans l'axone. Les patrons d'expression anatomique, cellulaire et ontogénétique de PFTAIRE dans le système nerveux diffèrent de ceux de p34cdc2 et cdk5. Ces dernières sont exprimées dans le système nerveux et plusieurs autres tissus avec division mitotique. La caractérisation de l'ARNm et de la protéine PFTAIRE suggère que PFTAIRE pourrait être la première kinase de la famille de cdc2 à être spécifiquement localisée dans le système nerveux.

La caractérisation de l'activité protéine kinase de PFTAIRE a montré que des protéines d'environ 58-60 kDa sont spécifiquement co-précipitées lorsque PFTAIRE est immunoprécipitée d'une préparation de protéines cytosoliques de cerveau de souris adulte ou de cellules

transfectées. Ces protéines semblent être d'importantes cibles endogènes de l'activité protéine kinase. On ne sait toujours pas si ces protéines associées sont des régulateurs de l'activité protéine kinase de PFTAIRE ou des substrats-effecteurs impliqués dans les événements ultérieurs. La phosphorylation de ces protéines était réduite en présence d'un mutant PFTAIRE à action dominante négative résultant d'une mutagenèse dirigée sur un résidu nécessaire au transfert de phosphate. Ceci suggère que la phosphorylation des protéines associées est due à l'activité protéine kinase de PFTAIRE. La caractérisation biochimique de PFTAIRE a également permis de constater que PFTAIRE elle-même est phosphorylée par une kinase présente dans les extraits de cerveau et que PFTAIRE peut s'autophosphoryler.

Afin d'élucider la fonction de PFTAIRE, des plasmides codant pour PFTAIRE ont été transfectés transitoirement dans plusieurs lignées de cellules fibroblastiques en division exprimant peu ou pas de protéine PFTAIRE endogène. Une faible efficacité de transfection, de même que la viabilité réduite des cellules, étaient systématiquement obtenues. Les cellules surexprimant PFTAIRE présentaient des modifications morphologiques tel que: une réduction de la taille de la cellule, un noyau de forme irrégulière, des nucléoles multiples ou fragmentés et un changement au niveau des lamines qui ressemblait à ce qui est observé dans les cellules bloquées tôt dans la phase G₁ du cycle cellulaire ou en apoptose. Ces résultats indiquent que la surexpression de PFTAIRE dans les cellules fibroblastiques en division n'est pas bien tolérée et suggèrent qu'une expression inappropriée de PFTAIRE peut interférer avec la progression du cycle cellulaire. Il est donc possible que la fonction de PFTAIRE dans les neurones post-mitotiques soit reliée à certaines propriétés spécifiques acquises par les cellules qui n'empruntent plus la voie du cycle cellulaire.

En résumé, j'ai identifié un nouveau membre de la famille des protéines kinases cdc2 qui est localisé de façon prédominante dans le système nerveux. Les patrons d'expression spatiale et temporelle de PFTAIRE dans le système nerveux post-natale et adulte suggèrent que l'activité kinase de PFTAIRE peut être associée à l'état différencié et post-mitotique des cellules et que sa fonction est vraisemblablement différente de celles de p34^{cdc2} et cdk5.

*Dedicated to my husband and parents,
with all my love*

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Taken from the *Guidelines for Thesis Preparation*, Faculty of Graduate Studies and Research, McGill University.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

- 1) I have identified a cDNA in postnatal mouse brain encoding a novel cdc2-related protein kinase, named PFTAIRE, which shares approximately 50-55% amino acid identity with several other cyclin dependent kinases and cdc2-related kinases.
- 2) I have demonstrated that the PFTAIRE gene (*Pftk1*) is positioned close to the centromere of mouse chromosome 5.
- 3) I have shown by northern blot analysis that the PFTAIRE cDNA corresponds to a single 5 kb mRNA transcript, which is expressed predominantly in the postnatal and adult nervous system. I have also demonstrated by *in situ* hybridization that in the central nervous system PFTAIRE mRNA is localized in several neuronal populations and in some glial cells.
- 4) I have raised polyclonal antibodies against PFTAIRE that react specifically on immunoblots with a 52 kDa protein that is detected only in extracts of mouse nervous tissue.
- 5) I have demonstrated by immunoprecipitation-kinase assays that endogenous mouse brain PFTAIRE co-immunoprecipitates with and specifically phosphorylates substrate proteins of approximately 58-60 kDa.
- 6) I have generated a kinase-inactive mutant of PFTAIRE, D228N. I have demonstrated that phosphorylation of the 58-60 kDa substrates that associate with PFTAIRE is reduced in the presence of the kinase-inactive mutant, suggesting that phosphorylation of these proteins is due to PFTAIRE kinase activity.
- 7) I have demonstrated that transfection of the PFTAIRE cDNA in proliferating cell lines correlates with a change in cellular morphology that has features of cell cycle arrest in G₁ and apoptotic cell death.

Table of Contents

Abstract	i
Abrégé	iii
Dedication	v
Acknowledgements	vi
Contribution to Original Knowledge	viii
Table of Contents	ix
List of Tables	xv
List of Figures	xvi
Abbreviations	xix

Chapter 1

Introduction

1.1 Protein Phosphorylation and the Eukaryotic Cell Cycle	1
a. Protein kinases	1
b. The cell cycle is regulated by protein kinases	1
c. A cytoplasmic factor, maturation promoting factor (MPF), triggers key meiotic and mitotic events in frog oocytes and diverse eukaryotic species	3
d. Identification of genes that regulate yeast cell cycle	4
e. Identification of the protein components of MPF	6
1.2 A Family of Protein Kinases Related to p34 ^{cdc2} in Higher Eukaryotes	7
a. Several cdc2-related kinases are identified in mammals	7
b. The conserved features of CDKs and cdc2-related kinases	11
1.3 The Regulation of CDK Activity by Other Proteins and How Key Components of the Cell Cycle Engine May Regulate Cell Cycle Progression	15
a. Cyclin proteins are positive regulators of CDKs	16
b. The G ₁ CDK-cyclin complexes, restriction point and the G ₁ -S transition	17
c. CDK2-cyclin A and S phase	20

d. p34 ^{cdc2} kinase-cyclin B and the G ₂ -M transition	21
e. The protein kinases and phosphatases that regulate CDK activity	22
f. The CDK inhibitor proteins (CDKIs)	27
g. The proteins that regulate CDK activity and cell cycle progression: Summary	31
1.4 Cell Cycle Related Proteins in the Nervous System	31
a. CDKs and cdc2-related kinases expressed in nervous tissue	32
b. PSSLARE kinase or CDK5	34
c. Expression of cyclin proteins in neurons	38
d. The CDK inhibitors in neurons	42
e. Cell cycle related proteins in the nervous system: Summary	42
1.5 Goals and Approach of the Thesis	43
a. Goals	43
b. Approaches	45

Chapter 2

Materials and Methods

2.1 Introduction	51
2.2 A Mouse cdc2 Kinase Probe Made by Reverse Transcription and PCR	51
2.3 Low Stringency Screening of a Mouse Brain cDNA Library	52
2.4 Plasmid Subcloning	
a. pBluescript SK ⁺ plasmid subclones for sequencing and cDNA probes	53
b. Bacterial expression plasmid constructs	53
c. Eukaryotic expression plasmid constructs	55
2.5 Genomic DNA Analysis	57
a. Southern blot analysis	57
b. Detection of restriction fragment length polymorphisms	58
2.6 RNA Expression	58
a. Northern blot analysis	58
b. In situ hybridization	59
2.7 Protein Analysis	61

a. Production of bacterial fusion proteins	61
b. Antibody preparation	61
c. Antibody specificity	62
d. Immunoblotting	63
e. Indirect immunofluorescence	64
f. Kinase assays for endogenous PFTAIRE activity	64
g. Kinase assays with <i>in vitro</i> translated proteins	65
h. Kinase assays with PFTAIRE and D228N transfected cells	66
i. Kinase assays with bacterial fusion proteins	66
j. Two dimensional gel electrophoresis	67
2.8 Cell Culture and Transfections	68
a. Maintenance of cell lines	68
b. Transfections	68
c. Detection of transiently transfected cells by X-gal staining and indirect immunofluorescence	70

Chapter 3

A cDNA Encoding a Novel cdc2-Related Protein Kinase

and It's mRNA Expression in the Mouse Nervous System

3.1 Introduction	84
3.2 Cloning of a Novel cdc2-Related kinase from Mouse Brain	84
3.3 Physical Features of the Protein Kinase Encoded by a Novel cDNA	85
3.4 Genomic PFTAIRE	87
a. The PFTAIRE cDNA represents a single gene	87
b. PFTAIRE restriction fragment length polymorphisms and chromosomal localization of the mouse PFTAIRE gene, <i>Pftk1</i>	88
3.5 PFTAIRE mRNA Expression	90
a. PFTAIRE mRNA tissue distribution and developmental expression	90
b. In situ hybridization studies	92
3.6 Discussion	94
a. PFTAIRE is a new member of the cdc2 kinase family	94
b. The PFTAIRE gene and the chromosomal locations of	96

PFTAIRE, CDKs and other cdc2-related kinases	
c. Expression of PFTAIRE mRNA and cell cycle proteins	98
in the nervous system	
d. Conclusions	103

Chapter 4

Analysis of PFTAIRE Protein Expression and Kinase Activity

in the Mouse Nervous System

4.1 Introduction	115
4.2 Antibodies Specific for PFTAIRE	115
4.3 Characterization of PFTAIRE Protein Distribution Using the PFC5 Antibody	117
a. Immunoblot analysis of PFTAIRE distribution in mouse tissues	117
b. Localization of PFTAIRE in adult nervous tissue	118
4.4 PFTAIRE Kinase Activity	121
a. PFTAIRE kinase activity in adult mouse brain	121
b. Is PFTAIRE kinase activity immunoprecipitated from brain	124
due to PFTAIRE?	
4.5 What Proteins Does PFTAIRE Phosphorylate?	127
a. Are NF-H peptides substrates for PFTAIRE kinase activity?	128
b. What are the PFTAIRE-interacting proteins in brain homogenates?	129
4.6 PFTAIRE Is Phosphorylated by Proteins Present in the Brain	130
a. Phosphorylation of the GST-PFTAIRE fusion protein	130
b. Immunoprecipitated PFTAIRE is phosphorylated in kinase reactions:	131
two dimensional gel electrophoresis analysis of PFTAIRE kinase reactions	
4.7 Discussion	133
a. The PFC5 antibody recognizes a protein in the adult mouse	133
nervous system encoded by the PFTAIRE cDNA	
b. The kinase activity of PFTAIRE	136
c. What are the proteins co-immunoprecipitated with PFTAIRE?	138
d. Does phosphorylation regulate PFTAIRE kinase activity?	142
e. Conclusions	144

Chapter 5

Transfection of the PFTAIRE cDNA in Cell Lines

5.1	Introduction	159
5.2	Identifying Cell Lines That Express PFTAIRE	160
5.3	Generating Permanent Cell Lines That Overexpress the PFTAIRE cDNA	161
5.4	Using the pHermes-L/PF Amplicon construct to Produce PFTAIRE	162
	Herpes Viruses for Expression of the PFTAIRE cDNA in Cell Lines	
5.5	Transient Transfection With PFTAIRE Plasmid Constructs	164
	a. Changes in cell morphology detected by indirect immunofluorescence	164
	b. Demonstrating PFTAIRE kinase activity in transfected cells	167
5.6	Discussion	169
	a. Cell lines that constitutively overexpress PFTAIRE	169
	could not be derived	
	b. Does transient overexpression of PFTAIRE in proliferating cells	171
	lead to G ₁ arrest or cell death?	
	c. How might PFTAIRE overexpression in proliferating cells	174
	result in cell death?	

Chapter 6

General Discussion

6.1	The Homology Between PFTAIRE and Other CDKs	185
6.2	Developmentally Regulated Expression of PFTAIRE	187
	in the Nervous System	
	a. Neurofilament protein phosphorylation during neuronal development	187
	b. Developmental regulation of the expression of PFTAIRE and	189
	related kinases	
	c. Functional implications of the developmentally regulated expression	193
	of PFTAIRE and future directions	
	d. Conclusions	196
6.3	PFTAIRE Kinase Activity	196
	a. Substrates of PFTAIRE kinase and their potential function	196
	b. Functional implications of the characterization of PFTAIRE kinase	202
	activity and future directions	

	c. Conclusions	204
6.4	Consequences of ectopic overexpression of PFTAIRE in cell lines	204
	a. Consequences of ectopic expression of PFTAIRE in cell lines	204
	b. Functional implications of ectopic expression of PFTAIRE and future directions	207
6.5	Conclusions	208
	<u>References</u>	210

List of Tables

Table 1.1	Summary of vertebrate cdc2 kinase family
Table 2.1	Oligonucleotides used for cloning and subcloning
Table 3.1	Consensus motifs found in the PFTAIRE sequence
Table 5.1	Summary of transfection experiments

List of Figures

1.1	Schematic diagram depicting the four phases of the eukaryotic cell cycle	46
1.2	The posttranslational modifications of active and inactive p34 ^{cdc2} kinase	47
1.3	A schematic representation of the eukaryotic cell cycle "engine"	48
1.4	Developmentally regulated expression of mammalian cdc2 and CDK5 mRNA and histone H1 kinase activities	49
1.5	Locations of potential phosphorylation sites in neurofilament subunits	50
2.1	Positions of oligonucleotides derived from the mouse cdc2 kinase cDNA sequence	72
2.2	PFTAIRE-pBluescript SK ⁺ , the 2.5 kb cDNA in pBluescript SK ⁺	73
2.3	H312, H334, H323 and H345, <i>Hind</i> III fragments derived from the 2.5 kb cDNA and subclones in pBluescript SK ⁺	74
2.4	Positions of oligonucleotides derived from the PFTAIRE cDNA sequence	75
2.5	PF290ED-pBluescript SK ⁺ , a 5' 290 bp <i>Eco</i> RI- <i>Dra</i> I fragment from the 2.5 kb cDNA subcloned in pBluescript SK ⁺	76
2.6	GST-PFTAIRE bacterial fusion protein expression plasmids	77
2.7	pcDNA1-PFTAIRE eukaryotic expression plasmids	78
2.8	pcDNA3-PFTAIRE eukaryotic expression plasmids	79
2.9	pHermes-L and pHermes-L/PFTAIRE expression vectors	80
2.10	Location of PFTAIRE peptides used to raise antibodies	81
2.11	Scheme for <i>in vitro</i> binding assay	82
2.12	Scheme for kinase assay with GST-PFTAIRE and GST-D228N	83
3.1	Nucleotide and deduced amino acid sequence for murine PFTAIRE	105
3.2	Alignment of deduced amino acid sequences of PFTAIRE with p34 ^{cdc2} and cdc2-related kinases	106
3.3	The PFTAIRE gene represents a single gene	107
3.4	PFTAIRE restriction fragment length polymorphisms	108
3.5	Location of the PFTAIRE gene(<i>Pftk1</i>) on mouse chromosome 5	109

3.6	Northern blot analysis of PFTAIRE mRNA expression in adult mouse	110
3.7	The PFTAIRE cDNA represents a single mRNA transcript	111
3.8	Northern blot analysis of PFTAIRE mRNA expression during development	112
3.9	In situ localization of PFTAIRE, cdc2 and NF-L mRNA in adult mouse brain	113
3.10	Expression of PFTAIRE mRNA in adult mouse spinal cord and cerebellum	114
4.1	Reactivity and specificity of anti-PFTAIRE polyclonal antibodies	146
4.2	Protein expression of PFTAIRE in adult mouse tissues	147
4.3	Specificity of the PFC5 antibody for the 52 kDa PFTAIRE protein detected in mouse nervous tissue	148
4.4	Indirect immunofluorescence detection of PFTAIRE in transfected cells with the PFC5 antibody	149
4.5	Indirect immunofluorescence labelling of mouse spinal cord with antibodies recognizing PFTAIRE and hypophosphorylated NF-H	150
4.6	Indirect immunofluorescence labelling of mouse cerebellum with antibodies recognizing PFTAIRE and hypophosphorylated NF-H	151
4.7	Indirect immunofluorescence labelling of mouse sciatic nerve with antibodies recognizing PFTAIRE and hyperphosphorylated NF-H	152
4.8	PFTAIRE kinase activity in brain immunoprecipitates	153
4.9	Phosphorylation of 58-60 kDa proteins from brain following co-precipitation with <i>in vitro</i> translated PFTAIRE and D228N	154
4.10	Transfection of 293 cells with PFTAIRE and the PFTAIRE mutant D228N	155
4.11	Kinase assays with synthetic peptide substrates derived from mouse NF-H	156
4.12	Phosphorylation of GST-PFTAIRE and GST-D228N by proteins in a brain lysate	157
4.13	Two dimensional gel electrophoresis analysis of a PFTAIRE kinase assay sample	158
5.1	Immunoblot analysis of PFTAIRE expression in cell lines	178
5.2	Vero cells transfected with the pHermes-L and pHermes-L/PF plasmids	179
5.3	COS-7 and NIH 3T3 cells transfected with the pHermes-L and pHermes-L/PF plasmids	180

5.4	Indirect immunofluorescence labelling of NIH 3T3 and Vero cells transfected transiently with the PFTAIRE plasmid, pc3PF	181
5.5	Anti-PFTAIRE and anti-lamin A/C labelling of COS-7 cells transfected transiently with the PFTAIRE cDNA	182
5.6	Anti-PFTAIRE and anti-lamin A/C labelling of Vero cells transfected transiently with the PFTAIRE cDNA	183
5.7	Immunoblot analysis of cells transfected with pcDNA3 and pc3PF ~~off~~ plasmids	184
6.1	Sequence alignment of murine PFTAIRE Drosophila PFTAIRE	209

Abbreviations

bp	base pair
BSA	bovine serum albumin
CAK	CDK activating kinase
CDK	cyclin-dependent kinase
CDKI	cyclin-dependent kinase inhibitor
cM	centiMorgan
C-terminal	carboxy-terminal
dNTPs	2'-deoxynucleoside 5'-triphosphates
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β -amino-ethyl ether) N, N, N', N'-tetraacetic acid
ERK	extracellular signal regulated kinase
G418	geneticin
GFAP	glial fibrillary acidic protein
GSK-3	glycogen synthase kinase-3
GST	glutathione S-transferase
H ₃ PO ₄	phosphoric acid
HBBS	Hepes buffered balanced salt solution
IPTG	isopropylthio- β -galactoside
kb	kilobase
kDa	kiloDalton
MAP kinase	mitogen activated protein kinase
NaF	sodium fluoride
Na ₃ VO ₄	sodium orthovanadate
N-terminal	amino-terminal
NGF	nerve growth factor
NF-L	neurofilament light protein
NF-M	neurofilament medium protein
NF-H	neurofilament heavy protein
NLS	nuclear localization signal
PCR	polymerase chain reaction
PKA	protein kinase A (cAMP-dependent protein kinase)
PKC	protein kinase C
PMSF	phenylmethylsulfonylfluoride
SDS	sodium dodecyl sulfate
TE	tris-EDTA
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside

CHAPTER 1
INTRODUCTION

1.1 Protein phosphorylation and the eukaryotic cell cycle

a. Protein kinases

The posttranslational modification of cellular proteins by protein kinases and protein phosphatases is one of the most widely used and important mechanisms for transducing external signals across the cell membrane to the nucleus, to control crucial functions related to cell growth and differentiation. In eukaryotes, protein kinases form a large superfamily of homologous genes and proteins, and presently approximately 400 amino acid sequences for eukaryotic protein kinases are known (Hanks and Hunter, 1995). Within the superfamily of protein kinases there are two main classes: serine/threonine protein kinases and tyrosine protein kinases. There are also some protein kinases which phosphorylate serine/threonine and tyrosine residues and these are referred to as dual specificity protein kinases (eg. MAP kinase kinases). All of these proteins are related due to the homology of their catalytic domains, which consist of a core of approximately 250-300 amino acids. Within the catalytic domain there are 12 subdomains which are regions that are uninterrupted by large amino acid inserts and characterized by the conservation of specific amino acid residues. The 12 subdomains are highly conserved among the many different serine/threonine and tyrosine protein kinases in all species, from yeast to mammals, and considered essential for protein kinase function (Hanks and Hunter, 1995). The activities of protein kinases are generally regulated by binding of ligands to extracellular receptors (tyrosine kinases) or regulatory domains (serine/threonine kinases), association with regulatory subunits (serine/threonine kinases), and by phosphorylation and dephosphorylation of specific residues. Protein kinases catalyze the transfer of the γ -phosphate of Mg^{2+} or Mn^{2+} -ATP (or GTP) to the hydroxyl group of an acceptor serine, threonine or tyrosine residue in the substrate protein. The crystal structures of several protein kinases such as cAMP-dependent protein kinase (PKA), MAP kinase (ERK 2), cyclin-dependent kinase 2 (CDK2) and the insulin receptor protein kinase have provided a basis for understanding the functions of specific subdomains and conserved residues in the phosphorylation reaction, as well as the differences in specificity for the phosphoacceptor residue (Knighton et al., 1991; Zhang et al., 1994a; De Bondt et al., 1993; Hubbard et al., 1994).

b. The cell cycle is regulated by protein kinases

The cell cycle is the set of events that regulates duplication of the cell and in its simplest form consists of replication of chromosomal DNA (S phase) and segregation of replicated chromosomes to produce genetically identical daughter cells (mitosis). In many eukaryotic cells

the cell cycle is considered to consist of four phases (Figure 1.1). DNA replication occurs in S phase and segregation of replicated chromosomes and cell division (mitosis) occur in M phase. The two gap phases that occur in the cell cycle, G₁ and G₂, provide time for the cell to monitor its environment, cell size and DNA content. The G₂ gap phase separates the completion of DNA synthesis from the initiation of mitosis (M), and during this period cells monitor their size and DNA quality and content to ensure that DNA replication is complete before cell division begins. The G₁ gap phase separates the previous cell division (M phase) from the initiation of DNA synthesis in S phase (Hartwell et al., 1974). During G₁ newly divided cells are responsive to external signals from growth factors and they monitor their environment and size before passing the restriction point, the process that commits a cell to DNA replication and cell division without further need for mitogenic stimulation (Pardee, 1989). Cells in G₁ that do not pass the restriction point, either remain in a quiescent state for extended periods (up to years) or undergo terminal differentiation and permanent withdrawal from the cell cycle. Quiescent cells and terminally differentiated cells are referred to as being in G₀. Following mitogenic stimulation quiescent cells re-enter G₁ and progress through the cell cycle, however, terminally differentiated post-mitotic cells, such as skeletal and cardiac myocytes and neurons, do not re-enter the cell cycle in response to such stimuli.

The major components of the eukaryotic cell cycle are the cell cycle “engine” and the downstream events that occur in response to changes in the activity of the engine (Murray, 1992). The cell cycle engine consists of protein complexes that are activated and inactivated in an ordered sequence. Reactions involving these proteins control the occurrence of downstream events, such as DNA replication, chromosome condensation, spindle assembly and nuclear envelope breakdown before mitosis; chromosome segregation, spindle disassembly, nuclear envelope formation and cytokinesis after mitosis (Murray, 1992; Graña and Reddy, 1995).

Key components of the cell cycle engine are protein kinases that were first identified by genetic approaches in mutant yeast strains and by physiological and biochemical approaches in developmental studies using frog oocytes. The protein kinase activities are subject to external control by signals from growth factors or hormones and to internal control by interactions with a variety of positive and negative regulatory proteins. The kinases and proteins regulating their activities are the components of regulatory pathways within the cell that are required for controlling

the order and timing of cell cycle transitions. These pathways are referred to as cell cycle checkpoints and they ensure that critical events such as DNA replication and chromosome segregation are correctly completed before a cell progresses to the next phase of the cell cycle. The checkpoints respond to irregularities such as DNA damage by arresting cell cycle progression to allow time for repair. The loss or deregulation of a component of a cell cycle checkpoint frequently results in genomic instability, uncontrolled cell proliferation (transformation) or aborted cell cycles and cell death (Nigg, 1995; Elledge, 1996).

c. A cytoplasmic factor, maturation promoting factor (MPF), triggers key meiotic and mitotic events in frog oocytes and diverse eukaryotic species

Studies investigating the control of cell fate by cytoplasmic factors have provided a basis for our current understanding of how the eukaryotic cell cycle is regulated. This section reviews developmental biology studies that provided initial evidence for the involvement of protein phosphorylation in cell cycle progression. Cell fusion experiments by Rao and Johnson (1970) demonstrated that mitotic HeLa cells could induce features of mitosis, such as nuclear envelope breakdown and chromosome condensation, when fused to other HeLa cells in any stage of the cell cycle. In 1971 Masui and Markert demonstrated that injection of cytoplasm from M phase arrested frog oocytes was able to induce germinal vesicle breakdown (nuclear envelope breakdown) during meiotic metaphase and maturation of G2 arrested oocytes, even in the absence of protein synthesis. The factor present in the cytoplasm of M-phase arrested amphibian oocytes was referred to as maturation promoting factor (MPF). A similar activity, promoting nuclear membrane breakdown and chromosome condensation, was also identified in mouse oocyte cytoplasm (Balakier and Czolowska, 1977).

Experiments by Maller et al. (1977) demonstrated that during meiotic maturation of amphibian oocytes, germinal vesicle breakdown was preceded by a burst in protein phosphorylation and injection of MPF also stimulated an immediate increase in protein phosphorylation. Specific proteins, phosphorylated during M phase in frog oocyte cytoplasmic extracts and in intact eggs, were also phosphorylated following the addition of partially purified MPF to G₁/S phase extracts. This indicated that protein kinase activities were associated with MPF and may be involved in regulating the nuclear changes that occur during meiotic metaphase (Maller and Smith, 1985; Lohka et al., 1987). In addition to the MPF activity that promoted meiotic metaphase in oocytes, another maturation promoting factor demonstrating a peak of activity

(promoting nuclear membrane breakdown and chromosome condensation) at the G₂-M transition was also identified in the early cleavage stages of amphibian embryos (Wasserman and Smith, 1978). This data suggested that factors regulating nuclear envelope breakdown and chromosome condensation during meiosis and mitosis may be similar or identical.

Mitotic extracts prepared from mammalian (HeLa) cells were also shown to induce germinal vesicle breakdown and chromosome condensation when injected into G₂-arrested *Xenopus* oocytes. Low levels of mammalian MPF activity increased gradually throughout G₂ and showed a peak of activity in extracts prepared from mitotic cells (Sunkara et al., 1979). Mitosis in mammalian cells is characterized by the appearance of newly phosphorylated and hyperphosphorylated proteins, similar to the burst of protein phosphorylation that occurs prior to metaphase during meiosis in amphibian oocytes. Among the many proteins phosphorylated during mitosis, the phosphorylation of proteins such as lamins (Ottaviano and Gerace, 1985), histone H1 (Gurley et al., 1975; Matsukawa et al., 1985), vimentin (Chou et al., 1989) and non-muscle caldesmon (Yamashiro et al., 1990) is thought to lead to dramatic structural changes that occur during mitosis, such as disassembly of the nuclear envelope, condensation of chromosomes, reorganization of the cytoskeleton and formation of the mitotic spindle apparatus. The demonstration of a mammalian MPF activity that peaked at mitosis and triggered the same meiotic events in frog oocytes as amphibian MPF (Sunkara et al., 1979) and the association of protein phosphorylation with meiotic and mitotic cell divisions in diverse eukaryotic species suggested that a universal control mechanism may exist for regulating the changes that occur during all cell divisions.

d. Identification of genes that regulate the yeast cell cycle

As developmental biologists investigated cytoplasmic factors that controlled cell division, yeast genetists were identifying regulatory genes that were required for normal cell cycle progression. Temperature sensitive mutant strains of budding yeast (*S. cerevisiae*) and fission yeast (*S. pombe*) were used to identify genes that regulate the yeast cell cycle. The *cdc* (cell division cycle) mutants showed delayed mitotic onset leading to either cell cycle arrest or cell division at an increased size (Hartwell et al., 1973; Hartwell et al., 1974; Reed, 1980; Nurse, 1975; Nurse and Thuriaux, 1980; Nurse and Bissett, 1981) and *wee* mutants advanced to mitosis prematurely and divided at a reduced cell size (Nurse and Thuriaux, 1980; Nurse, 1975). Analysis of the *cdc* mutants revealed that in fission yeast the *cdc2* gene was required for the onset of

mitosis and for commitment to cell division in G₁ (Nurse and Thuriaux, 1980; Nurse and Bisset, 1981). Analysis of budding yeast *cdc* mutants demonstrated that the *cdc28* gene was similarly required for the G₁ commitment to cell division (Reed, 1980) and for mitosis (Piggot et al., 1982; Beach et al., 1982). *Cdc28* mutants were conditionally arrested at Start (restriction point in mammalian cells) and morphologically resembled cells responding to mating pheromone (differentiated phenotype) (Reed, 1980). The *cdc28* gene was shown to complement the G₁ and mitotic functions of the *cdc2* gene in *S. pombe cdc2* mutants, suggesting that these genes performed similar or identical functions (Beach et al., 1982). A requirement for the *cdc2* and *cdc28* genes for cell cycle progression was demonstrated and both genes were considered positive control elements of the cell cycle in two distantly related species of yeast. These findings suggested that the molecular mechanisms controlling the G₁ commitment to cell division and initiation of mitosis may be highly conserved among divergent eukaryotic species (Beach et al., 1982).

The predicted amino acid sequences of *cdc28* and *cdc2* showed homology to the ATP binding region of protein kinases such as bovine cAMP-dependent protein kinase and viral oncogenes like *src* and *v-mos* (Lőrincz and Reed, 1984; Hindley and Phear, 1984). The predicted amino acid sequences of the *cdc2* and *cdc28* shared approximately 62% amino acid identity and contained threonine and tyrosine phosphorylation acceptor sites, which suggested that these protein kinases may be regulated by other protein kinases (Hindley and Phear, 1984). Sequence data and the demonstration that *cdc2* and *cdc28* were functionally homologous regulators of the G₁ commitment to cell division and initiation of mitosis (Beach et al., 1982) indicated that these genes encoded protein kinases with similar functions. These findings also suggested that phosphorylation of substrate proteins by these protein kinases may be important for regulating events that occur at the two major transition points of the eukaryotic cell cycle, when cells commit to cell division in G₁ (G₁-S boundary) and at the onset of mitosis (G₂-M boundary). A human homologue of *cdc2* and *cdc28* was cloned based on its ability to complement *S. pombe cdc2* function in temperature-sensitive *cdc2* mutants and *cdc2* deletion mutants (Lee and Nurse, 1987).

Antibodies raised against *cdc2* amino acid sequences were used to immunoprecipitate and detect on immunoblots a 34 kDa protein in yeast and human cell lysates (Simanis and Nurse,

1986; Lee and Nurse, 1987; Draetta et al., 1987). Characterization of the p34^{cdc2} protein showed that during the cell cycle there was little change in the abundance of p34^{cdc2}, but its phosphorylation state varied (Draetta and Beach, 1988). p34^{cdc2} immunoprecipitated from yeast and human cell lysates phosphorylated histone H1 and casein in *in vitro* kinase assays, demonstrating that kinase activity was associated with p34^{cdc2} (Reed et al., 1985; Simanis and Nurse, 1986; Draetta et al., 1987; Draetta and Beach, 1988). A 40 kDa protein was associated with yeast p36^{cdc28} (Reed et al., 1985) and human p34^{cdc2} co-immunoprecipitated with a 62 kDa protein that appeared to be an endogenous substrate. Although variations in p34^{cdc2} protein levels during the cell cycle were not observed, a peak of activity occurred in extracts prepared from cells entering mitosis (Simanis and Nurse, 1986; Draetta and Beach, 1988). At the completion of cell division p34^{cdc2} kinase was inactive (Simanis and Nurse, 1986; Draetta and Beach, 1988) and in human cells the loss of protein kinase activity coincided with dissociation of the p34^{cdc2}-62 kDa protein complex (Draetta and Beach, 1988). These data demonstrated that: 1) p34^{cdc2} was a protein kinase, 2) p34^{cdc2} activity may be required for cells to initiate mitosis and 3) inactivation of p34^{cdc2} may be a signal for cells to exit from mitosis. The similarities in the data obtained from biochemical characterization of yeast and mammalian p34^{cdc2} homologues indicated p34^{cdc2} kinase and the molecular mechanisms underlying cell cycle control may be conserved among diverse eukaryotic species.

e. Identification of the protein components of MPF

Injection of MPF into oocytes of various species resulted in an immediate increase in protein phosphorylation (Maller et al., 1977; Maller and Smith 1985; Lohka et al., 1987) and MPF activity was lost during purification in the absence of protein phosphatase inhibitors (Lohka et al., 1988). These observations suggested that this factor either was a protein kinase, an activator of a protein kinase or part of a complex that contained a protein kinase. Maturation promoting factor and p34^{cdc2} kinase were identified in a wide range of eukaryotic species and both were considered potentially key regulators of cell division. The purification of MPF from oocytes revealed that the factor consisted primarily of two proteins of approximately 32-34 kDa and 45 kDa (Lohka et al., 1988). The presence of a 32-34 kDa protein in purified MPF suggested that p34^{cdc2} and the 32-34 kDa component of MPF may be similar or the same protein. In addition,

immunoprecipitated p34^{cdc2} and purified MPF both phosphorylated histone H1 kinase in *in vitro* kinase assays (Simanis and Nurse, 1986; Draetta et al., 1987; Lohka et al., 1988) and both showed similar activation and deactivation kinetics during the cell cycle (Simanis and Nurse, 1986; Draetta and Beach, 1988; Cyert and Kirshner, 1988).

A 34 kDa protein in purified MPF obtained from frog and starfish oocytes was detected specifically on immunoblots with antibodies raised against p34^{cdc2} and this protein was not detected following pre-adsorption of the antibodies with p34^{cdc2} peptides (Gautier et al., 1988; Arion et al., 1988; Labbé et al., 1988). The p34^{cdc2} antibody also co-immunoprecipitated the 32 and 45 kDa protein components of MPF as a protein complex that phosphorylated the 45 kDa subunit and histone H1 in *in vitro* kinase assays (Gautier et al., 1988). This data provided strong evidence that p34^{cdc2} was the protein kinase activity associated with MPF.

Further characterization of the proteins associated with p34^{cdc2} determined that the 45 kDa subunit from amphibian and starfish oocyte MPF, the 40 kDa protein associated with *S. cerevisiae cdc28*, and the 62 kDa protein that co-immunoprecipitated with p34^{cdc2} from HeLa cell lysates were cyclin proteins (Labbé et al., 1989a; Draetta et al., 1989). Cyclin was originally identified in sea urchin and starfish oocytes as a protein that is synthesized and accumulated during the cell cycle and then abruptly degraded at the completion of metaphase, as cells enter anaphase (Evans et al., 1983; Standart et al., 1987). Purification of starfish oocyte MPF demonstrated that MPF was a complex of one molecule of p34^{cdc2} and one molecule of cyclin B (Labbé et al., 1989a). Human p34^{cdc2} formed complexes with either cyclin A or cyclin B (Draetta et al., 1989). Thus the kinase activity of p34^{cdc2} in eukaryotic species from yeast to humans appeared to be regulated by association with cyclin proteins (Draetta et al., 1989; Labbé et al., 1989a) and by reversible phosphorylation at specific times during the cell cycle (Draetta and Beach, 1988; Labbé et al., 1989b).

1.2 A family of protein kinases related to p34^{cdc2} in higher eukaryotes

a. Several cdc2-related kinases are identified in mammals

The *cdc2* and *cdc28* genes in yeast were first identified as genes required for the G₁/S and G₂/M transitions of the cell cycle (Nurse, 1975; Nurse and Thuriaux, 1980; Nurse and Bissett,

1981; Piggot et al., 1982; Hartwell et al., 1973; Hartwell et al., 1974; Reed, 1980). Since the gene product of *cdc2*, p34^{cdc2}, was identified as the protein kinase catalytic subunit of MPF (Gautier et al., 1988; Arion et al., 1988; Labbé et al., 1988) homologues of the *cdc2* gene have been identified in most eukaryotic species. p34^{cdc2} and p36^{cdc28} are important regulators of the G₁-S and G₂-M transitions in *S. pombe* and *S. cerevisiae*, respectively. In yeast these protein kinases are activated at different times during the cell cycle upon association with a variety of G₁ cyclin proteins or cyclin B (mitotic cyclin) (Nash et al., 1988; Hadwiger et al., 1989). Experiments examining the kinase activity of p34^{cdc2} in multicellular eukaryotes indicated that p34^{cdc2}-cyclin B activity was associated predominantly with the G₂-M transition (Draetta and Beach, 1988; Labbé et al., 1989b). These observations suggested that in higher eukaryotes another protein (possibly related to p34^{cdc2}) may be involved in the G₁-S transition. The identification of *Xenopus* and *Drosophila* homologues of *cdc2* provided the first indication that other *cdc2*-related proteins existed in higher eukaryotes (Dunphy et al., 1988; Paris et al., 1991; Milarski et al., 1991; Lehner and O'Farrell, 1990). A 32 kDa protein that cross-reacted with anti-*cdc2* antibodies was identified in *Xenopus* oocyte protein extracts (Dunphy et al., 1988). When the *Xenopus cdc2* cDNA homologue was cloned two related cDNAs were identified; a *Xenopus cdc2* homologue which shared 87% homology with human *cdc2* and a *cdc2*-related gene referred to as Eg-1 which shared only 66% homology with the *Xenopus cdc2* cDNA (Paris et al., 1991; Milarski et al., 1991). Eg-1-specific antibodies immunoprecipitated a protein that phosphorylated histone H1 *in vitro*, similar to p34^{cdc2}. Unlike p34^{cdc2} kinase the activity of the Eg-1 protein remained relatively constant throughout the cell cycle, and did not increase dramatically at mitosis. Immunodepletion of Eg-1 from oocyte extracts did not affect initiation of mitosis but DNA replication was inhibited in the absence of Eg-1 protein (Fang and Newport, 1991). These experiments were the first to demonstrate that in multicellular eukaryotes, progression through the two major transition points of the cell cycle in higher eukaryotes, G₁-S and G₂-M, required the activity of more than one *cdc2* kinase.

Since the identification of p34^{cdc2} and Eg-1, large families of protein kinases related to p34^{cdc2} kinase and cyclin proteins have been identified in several vertebrate species, using a variety of cDNA cloning strategies and protein purification. The amino acid sequences predicted

from cDNA clones related to the *cdc2* gene represent the catalytic domains of putative serine/threonine protein kinases. p34^{cdc2} and several of the *cdc2*-related cDNA clones encode proteins that contain approximately 300 amino acids but some of the encoded proteins also have extended amino- and carboxy-terminal regions relative to p34^{cdc2}. All of the sequences contain the eleven highly conserved subdomains characteristic of serine/threonine protein kinase catalytic domains. All of the protein kinases related to *cdc2* share a significant amount of nucleotide and amino acid homology with *cdc2* (approximately 45-65% identical or similar amino acids), particularly within the 250-300 amino acid core corresponding to *cdc2*. Based on sequence similarities, the *cdc2* kinase family is phylogenetically classified with mitogen-activated protein kinases (MAP kinases), stress-activated protein kinases, glycogen synthase kinase-3 (GSK-3) and Jun kinase (Hanks and Hunter, 1995). Collectively, these families of kinases are referred to as proline-directed protein kinases because the serine/threonine residues phosphorylated by these kinases in substrate proteins are immediately preceded or followed by proline residues. Interestingly, many of the other proline-directed protein kinases related to the *cdc2*-kinase family regulate cellular responses to various external stimuli and are part of signal transduction pathways that regulate cell proliferation and differentiation, probably acting upstream of the CDKs and *cdc2*-related kinases. An amino acid motif, the PSTAIRE sequence (single letter amino acid code), located at positions 45-51 of the human p34^{cdc2} sequence (Lee and Nurse, 1987) is a unique feature conserved in all protein kinases in the *cdc2* family. This motif is an identifying feature of *cdc2*-related kinases, but for the majority of these kinases amino acid substitutions occur at various positions within the motif except at the Arg (R) and Glu (E) residues, which are strictly conserved.

The protein kinases related to *cdc2* are separated into two classes: 1) the cyclin-dependent kinases (CDKs), which have been shown to associate with cyclin regulatory subunits (Meyerson et al., 1991) and 2) the *cdc2*-related kinases, for which no interactions with cyclin proteins have been demonstrated. The CDKs are numbered in order of their discovery, starting with p34^{cdc2} as CDK1, Eg-1 as CDK2, up to CDK8 presently. The nomenclature for the *cdc2*-related kinases is based on the amino acid sequence corresponding to the PSTAIRE sequence of p34^{cdc2}. Thus a protein kinase related to p34^{cdc2} that has not been shown to associate with cyclin proteins is named by the amino acid sequence in this region (e.g., PCTAIRE, PITALRE),

which frequently diverges from the PSTAIRE sequence found in p34^{cdc2}, CDK2 and CDK3.

Several members of the *cdc2* kinase family were identified by amplifying human sequences by the polymerase chain reaction (PCR) with degenerate oligonucleotides corresponding to the conserved regions of *cdc2*. Novel sequences were subsequently cloned by hybridizing the PCR fragments to human cDNA libraries (Meyerson et al, 1991; Meyerson et al. 1992). A total of seven novel sequences related to *cdc2* were identified by this cloning strategy. The hybridization screening also yielded additional related cDNA clones that had been isolated previously including *cdc2*, CDK2, PSK-J3 (Hanks, 1987) and p58^{GTA} (Bunnell, 1990). The PSK-J3 cDNA was subsequently shown to interact with D-type cyclins and renamed CDK4 (Matsushime et al., 1992). p58^{GTA} is now referred to as PITALRE (Graña et al., 1994a). Since the cloning of the seven novel human *cdc2*-related genes by Meyerson et al. (1991; 1992) homologues of some of these genes have been cloned in other vertebrate and invertebrate species (Hellmich et al., 1992; Lew et al., 1992b; Okuda et al., 1992; Sauer et al., 1996). In addition, other previously unidentified genes related to *cdc2* have been isolated from various species (Graña et al., 1994b; Brambilla and Draetta, 1994; Fisher and Morgan, 1994; Mäkelä et al., 1994; Tassan et al., 1995). Thus fifteen *cdc2*-related genes have been identified in various vertebrate and invertebrate species. Among the *cdc2*-related genes that have been identified in vertebrate species, six encode proteins that associate with cyclins and are considered cyclin-dependent kinases (e.g., CDK2, CDK4, CDK5, CDK6, CDK7 and CDK8). Although CDK3 has never been shown to associate with cyclin proteins (Pines, 1993), it was classified as a cyclin-dependent kinase based on its ability to complement *cdc28* function in *S. cerevisiae cdc28* temperature-sensitive mutants (Meyerson et al., 1992). The seven remaining members of the family are considered *cdc2*-related kinases since no cyclin partners have been identified for these putative protein kinases to date (reviewed in Pines, 1993; Hunter and Pines, 1994; Nigg, 1995). A summary of the vertebrate *cdc2*-related kinases, CDKs and the regulatory proteins with which they are known to interact is presented in Table 1.1.

Yeast homologues have not been identified for the majority of the protein kinases listed in Table 1.1. Presently only PITALRE and CDK7 have been identified in yeast and higher eukaryotes (Bunnell et al., 1990; Buck et al., 1995; Damagnez et al., 1995). The PHO85 cyclin dependent kinase and the cyclins that regulate its activity (PHO80, PCL1 and PCL2) form CDK-

cyclin complexes that have been identified only in yeast (*S. cerevisiae*) and not in higher eukaryotes (reviewed in Nigg, 1995). Only two of the CDKs that have been identified in higher eukaryotes, CDK2 and CDK3, complemented the *cdc28* function in *S. cerevisiae cdc28* temperature-sensitive mutants (Meyerson et al., 1992), suggesting that both are involved in cell cycle regulation. Expression of dominant negative CDK2 and CDK3 mutants in mammalian cells has provided additional evidence to suggest that both of these protein kinases are required for cell cycle progression (van den Heuvel and Harlow, 1993). Although most of the protein kinases in the *cdc2* family do not substitute *cdc2* or *cdc28* functions in mutant strains of yeast, at least six CDKs may be involved in some aspect of mammalian cell cycle regulation; including progression through G₁ and the G₁-S transition, initiation of DNA replication, regulation of p34^{cdc2} and other CDKs, regulation of RNA polymerase activity and mitosis (reviewed in Pines, 1993; Hunter and Pines, 1994; Nigg, 1995). Cdc2-related kinases such as PITSLRE, PITALRE and PISSLRE may also be involved in cell cycle regulation (Bunnell et al., 1990; Graña et al., 1994b; Li et al., 1995). There is also evidence indicating that at least one member of the *cdc2* kinase family, CDK5, may function only in postmitotic neurons in the nervous system (Tsai et al., 1993; Oshima et al., 1996). Thus many of the activities that are regulated by p34^{cdc2} (or p36^{cdc28}) and multiple cyclin partners in yeast are regulated by multiple CDKs and cyclins in higher eukaryotes. The requirement for several different CDKs and cyclins to regulate cell cycle in multicellular organisms may reflect the greater degree of complexity in signalling pathways required for cell growth, division, development and differentiation compared to the pathways utilized by unicellular organisms such as yeast.

b. The conserved features of CDKs and *cdc2*-related kinases

All of the CDKs and *cdc2*-related kinases contain a central core of approximately 300 amino acids showing high homology to p34^{cdc2}. In addition to the conserved kinase subdomains located within this core, all CDKs and *cdc2*-related kinases have phosphoacceptor residues equivalent to Thr14, Tyr15 and Thr161 (Thr167) of human (yeast) p34^{cdc2} and a PSTAIRE-like motif, which are required for regulation of kinase activity (Gould and Nurse, 1989; Gould et al., 1991; Norbury et al., 1991; Krek and Nigg, 1991b; Ducommun et al., 1991).

Conserved phosphorylation sites

The abundance of p34^{cdc2} does not change dramatically during the cell cycle. However,

p34^{cdc2} kinase activity fluctuates in all eukaryotic cell cycles and peak activity occurs as cells initiate mitosis. Experiments examining the variations in p34^{cdc2} activity during the cell cycle demonstrated that changes in kinase activity were associated with changes in the phosphorylation state of p34^{cdc2} (Simanis and Nurse, 1986; Draetta and Beach, 1988). Threonine 14, tyrosine 15 and threonine 161, are the three major phosphorylation sites that have been identified in all homologues of p34^{cdc2}. In most other CDKs and cdc2-related kinases the amino acids located in the corresponding positions are either identical (Tyr15) or may have serine substitutions (Thr14 and Thr161). The reversible phosphorylation of each of these residues ensures that p34^{cdc2} kinase is activated at the appropriate time in the cell cycle. The conservation of these residues in other CDKs and cdc2-related kinases suggests that similar mechanisms may be used to regulate the kinase activities of these proteins.

The first indications that p34^{cdc2} activity may be regulated by tyrosine phosphorylation came when p34^{cdc2} was identified as a major phosphotyrosine-containing protein in HeLa cells (Draetta et al., 1988). The demonstration that tyrosine dephosphorylation coincided with p34^{cdc2} activation and entry into mitosis led to the hypothesis that tyrosine phosphorylation of p34^{cdc2} inhibited its activity until a cell is ready to divide (Morla et al., 1989; Dunphy et al., 1989; reviewed in Atherton-Fessler et al., 1993). Tyrosine 15, which is located in the ATP-binding region of p34^{cdc2}, was shown to be the site of tyrosine phosphorylation (Gould and Nurse, 1989; Krek and Nigg, 1989). Mutation of Tyr15 to a non-phosphorylatable phenylalanine residue confirmed that tyrosine phosphorylation inhibited the kinase activity of yeast p34^{cdc2} and prevented cells from entering mitosis prematurely (Gould and Nurse, 1989). In higher eukaryotes Thr14 was also shown to be phosphorylated (Krek and Nigg, 1991a). Mutation of Thr14 and Tyr15 demonstrated that phosphorylation of both residues is required for inhibition of p34^{cdc2} kinase activity and prevention of premature mitosis (Norbury et al., 1991; Krek and Nigg, 1991b). The location of Thr14 and Tyr15 in the ATP-binding region suggests that phosphorylation of these residues could inhibit kinase activity by interfering with ATP binding. The solution of the crystal structure of inactive human CDK2 has demonstrated that only the hydroxyl group of Thr14 is close enough to the γ -phosphate of ATP to directly disrupt the conformation of the ATP phosphates and interfere with ATP binding. The CDK2 crystal structure data showed that the

hydroxyl group of Tyr15 is located at a greater distance from the ATP than Thr14, suggesting that phosphorylation of Tyr15 would not affect ATP binding (DeBondt et al., 1993; reviewed in Pines, 1993). The more recent solution of the crystal structure of the partially activated human CDK2-cyclin A complex has provided a clearer understanding of how phosphorylation of Thr14 and Tyr15 may inhibit p34^{cdc2} or CDK2 activity. The crystal structure of the CDK2-cyclin A complex has indicated that cyclin binding induces conformational changes in CDK2 that move Thr14 and Tyr15 further into the catalytic cleft in the CDK2-cyclin A complex. Thus the phosphorylation of these residues may hinder phosphate transfer from ATP to a bound substrate through steric hindrance or electrostatic repulsion (Jeffrey et al., 1995).

In contrast to the inhibitory effects that phosphorylation of Thr14 and Tyr15 have on p34^{cdc2} kinase activity phosphorylation of Thr167 of *S. pombe* p34^{cdc2} (Thr161 of human p34^{cdc2}) positively regulates p34^{cdc2}. Gould et al. (1991) demonstrated that phosphorylation of Thr167 is essential for p34^{cdc2} activation at mitosis and for cell division to occur. Mutating Thr167 to a serine residue did not affect the activation of p34^{cdc2}, but overexpression of p34^{cdc2} mutants with Glu or Ala substitutions at position 167 demonstrated that the non-phosphorylatable p34^{cdc2} mutant proteins were unable to rescue *cdc2* temperature-sensitive mutants that arrest at the G₁-S and G₂-M transitions. These observations suggested that the non-phosphorylatable p34^{cdc2} mutants were inactive. In addition, the p34^{cdc2} mutant proteins were associated with less p56^{cdc13} (cyclin B) when immunoprecipitated and lacked histone H1 kinase activity (Gould et al., 1991). In higher eukaryotes phosphorylation of the equivalent threonine residue (Thr161 in human p34^{cdc2}) is required for p34^{cdc2} activation (Ducommun et al., 1991; Solomon et al., 1992). In agreement with the observation that less cyclin associated with non-phosphorylated forms of yeast p34^{cdc2} (Gould et al., 1991), Ducommun et al. (1991) demonstrated that phosphorylation of Thr161 may also be a prerequisite for cyclin binding to human p34^{cdc2}. However, others have shown in *in vitro* binding experiments that cyclins bind to p34^{cdc2} in the absence of Thr161 phosphorylation (Solomon et al., 1992; Desai et al., 1992). Dephosphorylation of Thr161 is required for inactivation of p34^{cdc2} at the end of metaphase (Lorca et al., 1992). The crystal structure of inactive CDK2 provided some insight into the

mechanism by which phosphorylation of equivalent site in CDK2, Thr160, may lead to kinase activation. The crystal structure revealed that Thr160 is located in a large loop of protein referred to as the 'T loop' (residues 152-170) which almost completely blocks the core of the protein substrate binding site. The T loop prevents substrates from interacting with the CDK2-Mg²⁺-ATP complex (DeBondt et al., 1993). Based on this data it was hypothesized that phosphorylation of Thr160 may promote an interaction between this residue and basic residues in the carboxy-terminal lobe of CDK2 to effectively move the T loop away from the protein substrate binding site and allow substrates to bind to the kinase (DeBondt et al., 1993; Pines, 1993). The crystal structure of the partially active CDK2-cyclin A complex has provided a clearer picture of how phosphorylation of Thr160 promotes complete activation of CDK2. The binding of cyclin A to one side of the CDK2 catalytic cleft induces a conformational change that directs the T loop away from the entrance of the catalytic cleft, which relieves steric hindrance at the entrance to the catalytic cleft and enable substrates to bind. These changes expose Thr160, making it available for phosphorylation, which contributes to CDK2 activation (Jeffrey et al., 1995; reviewed in Johnson et al., 1996). Although observations by Gould et al. (1991) and Ducommun et al. (1991) indicated that phosphorylation of Thr167/Thr161 may be a prerequisite for cyclin binding to p34^{cdc2}, the crystal structure of the partially active CDK2-cyclin A has demonstrated that prior to cyclin A binding, Thr160 is buried in the catalytic cleft of the CDK2 monomer and partial activation via Thr160 phosphorylation can only occur after cyclin binding has made this residue more accessible for phosphorylation. Thus it has been suggested that phosphorylation of the threonine residue may help stabilize certain CDK-cyclin complexes (Pines, 1995). Phosphorylation of a residue corresponding to Thr161 of p34^{cdc2} may be a general mechanism of activation for kinases, because other protein kinases such as cAMP-dependent protein kinase A, Src and insulin receptor tyrosine kinases require autophosphorylation of threonine or tyrosine residues located in the equivalent position for activation (Pines, 1993). It is now known that phosphorylation of the equivalent site in CDK4 (Thr172) is also required for its activation, but not for its association with cyclin D1 (Kato, 1994).

The three phosphoacceptor residues corresponding to Thr14, Tyr15 and Thr161 of p34^{cdc2} are conserved in all other CDKs and cdc2-related kinases. In addition to p34^{cdc2} two other members of the CDK family, CDK2 and CDK4, are regulated by phosphorylation of all or

some of these sites. It is possible that the phosphorylation reactions that regulate p34^{cdc2} activity are general mechanisms for regulating the activities of several CDKs and cdc2-related kinases. A schematic diagram depicting the phosphorylation states of active and inactive p34^{cdc2} is presented in Figure 1.2 and the protein kinases and phosphatases involved in regulating p34^{cdc2} activity are discussed in Section 1.3e.

The PSTAIRE region

The PSTAIRE motif is a cluster of amino acids that is conserved in the amino acid sequences of all p34^{cdc2} homologues, CDK2 and CDK3. Variations of this motif are found in all CDKs and cdc2-related kinases where amino acid substitutions may occur at all positions except those corresponding to Arg50 and Glu51 of human p34^{cdc2}. In alanine scanning mutagenesis experiments designed to determine which conserved residues in the p34^{cdc2} kinase sequence were required for p34^{cdc2} to interact with cyclin A, Ducommun et al. (1991) demonstrated that Arg50 and Glu51 of the PSTAIRE motif and several highly conserved residues flanking kinase subdomains I and II were required for cyclin A binding to p34^{cdc2}. In agreement with this data, the crystal structure of the CDK2-cyclin A complex has revealed that the PSTAIRE region of CDK2 and the highly conserved cyclin box region of cyclin A are critical components of the CDK2-cyclin A interface (Jeffrey et al., 1995). Although the exact sequence in the PSTAIRE region may be important for determining the specificity of which cyclin binds to which CDK (Pines, 1995), the strict conservation of Arg50 and Glu51 in all CDKs and cdc2-related kinases suggests that these residues are of more general importance for CDK-cyclin interactions.

1.3 The regulation of CDK activity by other proteins and how key components of the cell cycle engine may regulate cell cycle progression

In order to ensure the proper timing and coordination of cell cycle events, the activities of CDKs are subject to many levels of regulation. The cellular levels of CDKs remain in constant excess throughout the cell cycle and regulation of the kinase activities occurs primarily through association with other proteins and post-translational modifications (Morgan, 1995). In general, all CDK activities are regulated positively by forming complexes with cyclin proteins. G₁ CDK-cyclin complexes are regulated negatively by association with at least two families of CDK inhibitory

proteins (CDKs). The reversible phosphorylation of phosphoacceptor amino acids corresponding to Thr14, Tyr15 and Thr161 of human p34^{cdc2} by protein kinases, such as Wee1, Myt1 and CAK (CDK7/cyclin H), and the CDC25 phosphatases provides an additional level of regulation for the activities of some CDKs. A review of the current understanding of how other proteins regulate CDK activity will be presented in this section.

a. Cyclin proteins are positive regulators of CDKs

Cyclin was first identified as a protein that showed oscillations in cellular levels following fertilization of sea urchin eggs and during cell cycles of developing embryos (Evans et al., 1983). Variations in cyclin levels during the cell cycle were shown to be periodic. The fluctuations were attributed to the periodic destruction of cyclin which coincided with cell division (Evans et al., 1983). Although Evans et al. (1983) noted similarities in the cyclic fluctuations of MPF activity described by Wasserman and Smith (1978) and cyclin, the role of cyclin in cell cycle regulation was not evident until MPF was purified and its components were identified as p34^{cdc2} and cyclin B (Labbé et al., 1989a; Draetta et al., 1989). Since cyclin B was first described by Evans et al. (1983) other cyclins have been identified that are present at relatively constant levels throughout the cell cycle but these proteins are considered cyclins because of structural and functional similarities (Sherr, 1994). It is now known that cyclin binding is an essential step in the activation of CDKs (Solomon et al., 1990; Jeffrey et al., 1995) and that two highly conserved regions, located within the cyclin box, are required for interactions with and activation of CDKs (Lees and Harlow, 1993; Jeffrey et al., 1995). Cyclin proteins are thought to regulate CDK activity in two ways. The synthesis and periodic destruction of cyclin proteins ensures that their CDK partners will be active at the appropriate time in the cell cycle (Nigg, 1995). In addition, cyclins may also help to target CDKs to specific substrates (Peeper et al., 1993).

In yeast and in multicellular eukaryotes cyclins are classified as G₁ cyclins or mitotic cyclins, depending on when they function in the cell cycle. In yeast (*S. cerevisiae*) twelve cyclin proteins have been identified. The G₁ cyclins CLN1-3 associate with p36^{cdc28} (reviewed in Nigg, 1995) and PCL1 and PCL2 associate with PHO85 (Measday et al., 1994; Espinoza et al., 1994). The CLB1-6 cyclins regulate p36^{cdc28} activity during mitosis and S phase (reviewed in Nigg, 1995). The CDK PHO85 is also regulated by the cyclin protein PHO80 and this CDK-cyclin complex is involved in transcriptional control and regulation of phosphate metabolism (Kaffman et al., 1994).

The cyclin family in vertebrates also consists of twelve proteins. Some of these cyclins are known to associate with only a single CDK, such as cyclin B1, B2, C and H, while others interact with and may regulate the function of more than one CDK, such as cyclin A, D1, D2, D3 and E. The CDK partners for cyclins F and G have not yet been determined. Cyclins D1, D2, D3 and E are the G₁ cyclins. Cyclin A is required at two points in the cell cycle, in S phase and during M phase in addition to other mitotic cyclins; cyclins B1, B2. Cyclin H is expressed throughout the cell cycle and may be active during multiple phases. The functions of cyclins C, F and G have not yet been determined (reviewed in Nigg, 1995). A summary of the cyclin proteins identified to date is presented in Table 1.1. The remainder of this literature review will focus on the regulatory roles of cyclins in vertebrates.

b. The G₁ CDK-cyclin complexes, restriction point and the G₁-S transition

Key regulators of G₁ progression in mammalian cells include cyclins D1, D2 and D3 which form complexes with CDK4 or CDK6 and cyclin E which associates with CDK2 later in G₁ (reviewed in Sherr, 1994). Cyclin D1 and cyclin E were identified in a search for mammalian cDNAs that could rescue *S. cerevisiae* strains lacking G₁ cyclins (Xiong et al., 1991; Koff et al., 1991; Lew et al., 1991). Cyclins D2 and D3 were identified as genes that were upregulated in murine macrophage cell lines in response to colony stimulating factor 1 treatment (Matsushime et al., 1991).

The D-type cyclins show some cell lineage specificity and many cell types express cyclin D3 and D1 or D2 (Sherr, 1993). It is unclear whether the D-type cyclins are functionally redundant in response to different signals. However, the lineage-specific expression patterns of the D-type cyclins and their CDK partners suggest that different complexes may respond differently to distinct signals during G₁ (Sherr, 1993; Sherr, 1994). The expression of D-type cyclins is growth factor inducible and is part of the delayed early response to mitogens (Sherr, 1994). These proteins are synthesized while mitogens are present, show little variation in expression during the cell cycle, but undergo rapid degradation upon withdrawal of growth factors, regardless of the phase of the cell cycle (Matsushime et al., 1991; review in Sherr, 1994; Hunter and Pines, 1994). Although microinjection of cyclin D1 antibodies into normal fibroblasts during G₁ and the degradation of D-type cyclins in response to mitogen withdrawal prevents entry into S phase, neither treatment interferes with cell cycle progression at other times, even at the G₁-S phase

transition (Baldin et al., 1993; Matsushime et al., 1991). In addition, overexpression of D-type cyclins in cultured fibroblasts shortens the duration of G₁ and accelerates entry into S phase (Quelle et al., 1993). These observations suggested that D-type cyclins may function partly as growth factor sensors and provided further evidence for their role as regulators of G₁ progression (Sherr, 1994; Hunter and Pines, 1994).

The accumulation and increasing activity of CDK4-or CDK6-cyclin D complexes during G₁ in response to mitogenic signals may regulate progression through the restriction point of the cell cycle (Matsushime et al., 1992; Matsushime et al., 1994; Meyerson and Harlow, 1994; Sherr, 1994; Hunter and Pines, 1994). When CDK4- and CDK6-cyclin D complexes are formed *in vitro* using proteins expressed from baculovirus vectors in insect cells or by immunoprecipitation of endogenous proteins from cell lines, these complexes preferentially phosphorylated the retinoblastoma tumor suppressor protein (pRB). CDK4-cyclin D complexes phosphorylate pRB *in vitro* on many of the sites that are phosphorylated *in vivo* in late G₁. Unlike other CDK-cyclin complexes, CDK4- and CDK6- cyclin D complexes do not phosphorylate histone H1 (Matsushime et al., 1992; Kato et al., 1993; Meyerson and Harlow, 1994). Direct interactions between D-type cyclins and pRB have been demonstrated *in vitro* and when endogenous proteins were immunoprecipitated from cells (Kato et al., 1993; Ewen et al., 1993; Dowdy et al., 1993). All of this data has led to the hypothesis that D-type cyclins may target CDK4 and CDK6 to pRB during G₁ (reviewed in Sherr, 1993; Sherr, 1994; Hunter and Pines, 1994). In normal proliferating cells the phosphorylation of pRB near the G₁ restriction point by CDK4- and CDK6-cyclin D complexes is thought to promote progression through the restriction point and entry into S phase by inactivating the growth suppressing functions of pRB (Sherr, 1994). The hypophosphorylated and active (growth suppressing) form of pRB is bound to the transcription factor E2F, thereby negatively regulating the transcriptional activity of E2F that is important for the G₁-S transition. When pRB is phosphorylated, E2F is released from pRB (Kato et al., 1993). Disruption of pRB-E2F complexes enables E2F to activate transcription of genes required for entry into S phase and DNA transcription, such as *c-myc*, cyclin D, cyclin E, dihydrofolate reductase, thymidine kinase and DNA polymerase α (reviewed in Nevins, 1992; Field et al., 1996). Thus the CDK4- and CDK6-cyclin D complexes that form during G₁ in response to mitogenic stimulation are the first CDK complexes to be activated during the cell cycle (Meyerson and Harlow, 1994) and may be

responsible for driving cells through the restriction point. Once cells have passed the restriction point and are committed to DNA replication and cell division, they are no longer dependent on growth factors or environmental signals for regulation of the cell cycle .

The activation of CDK2 by association with cyclin E is thought to be required for the G₁-S phase transition. Cyclin E is synthesized periodically during the cell cycle, and is most abundant in late G₁ cells. CDK2-cyclin E complexes are assembled and active later in G₁ than the CDK4-CDK6-cyclin D complexes (Draetta, 1994). The assembly of cyclin E with CDK2 results in maximal CDK2-cyclin E kinase activity at the G₁-S transition and this activity declines as cells progress through S, G₂ and mitosis (Lew et al., 1991; Koff et al., 1992; Dulic et al., 1992). These observations and the ability of mammalian cyclin E to rescue G₁ cyclin-deficient *S. cerevisiae* (Koff et al., 1991; Lew et al., 1991) suggested that cyclin E may be required for triggering the G₁-S transition. The constitutive overexpression of cyclin E in cultured fibroblasts shortens the duration of G₁, accelerates entrance into S phase, and correlates with decreased cell size and decreased serum requirements at the G₁-S transition. This data indicated that the level of cyclin E (and CDK2-cyclin E complexes) regulated the rate of progression through the G₁ phase of the cell cycle (Ohtsubo and Roberts, 1993). Additional evidence for the requirement for CDK2-cyclin E activity at the G₁-S transition came from experiments demonstrating: 1) microinjection of CDK2-specific antibodies could prevent cultured fibroblasts from entering S phase and 2) depletion of either CDK2 or cyclin E from lysates prepared from late G₁ fibroblasts eliminated the majority of the histone H1 kinase activity normally associated with the CDK2 or cyclin E immune complexes (Tsai et al., 1993).

The target substrate proteins of the CDK2-cyclin E complexes may include pRB, the RB-related protein p107 and proteins involved in the initiation of DNA replication (reviewed in Weinberg, 1995; Sherr, 1996). Hatakeyama et al. (1994) demonstrated that when pRB was ectopically expressed in *S. cerevisiae*, phosphorylation of pRB was similar to the phosphorylation that occurs in mammalian cells and required the expression of the yeast G₁ cyclins, CLN3 and CLN1 or CLN2. In *S. cerevisiae* lacking CLN3 or CLN1/CLN2 function, pRB was phosphorylated following expression of either mammalian cyclin D1 or cyclin E (Hatakeyama et al., 1994). These results indicated that pRB may be a substrate for multiple CDK-cyclin complexes in mammalian

cells, including CDK4- and CDK6-cyclin D and CDK2-cyclin E. It has been hypothesized that in mammalian cells the CDK4-, CDK6-cyclin D complexes may initially phosphorylate pRB and that the CDK2-cyclin E complexes may maintain the hyperphosphorylated state of pRB. Alternatively, phosphorylation of pRB by CDK4-, CDK6-cyclin D may make pRB a more attractive substrate for further modification by CDK2-cyclin E (Weinberg, 1995). Direct evidence in support of either hypothesis is presently lacking. It has also been suggested that the CDK2-cyclin E complex may promote DNA synthesis by phosphorylating proteins that are part of the DNA replication machinery (Sherr, 1996). Once cells enter S phase the CDK2-cyclin E complex is inactivated by the ubiquitin-mediated proteolysis of cyclin E (Won and Reed, 1996) and CDK2 associates with cyclin A to regulate progression through S phase.

c. CDK2-cyclin A and S phase

Cyclin A is first detected in mammalian cells at the onset of S phase (Pines and Hunter, 1990; Girard, et al., 1991; Pagano et al., 1992) and its expression coincides with the timing of CDK2 activation (Pagano et al., 1992; Rosenblatt et al., 1992). The association of CDK2 with cyclin A was demonstrated by immunoprecipitation of the two proteins from cell extracts with CDK2 and cyclin A specific antibodies (Tsai et al., 1991; Pagano et al., 1992; Rosenblatt et al., 1992; Tsai et al., 1993). The CDK2-cyclin A complexes immunoprecipitated from S phase cell extracts phosphorylated histone H1. This activity was not detected in CDK2-cyclin A immune complexes formed from extracts that were immunodepleted with either cyclin A or CDK2-specific antibodies prior to immunoprecipitation (Pagano et al., 1992, Rosenblatt et al., 1992). Experiments in which microinjection of CDK2 or cyclin A-specific antibodies or antisense plasmids resulted in inhibition of DNA replication and progression through S phase have demonstrated the requirement for CDK2 and cyclin A during S phase (Fang and Newport, 1991; Girard et al., 1991; Pagano et al., 1992; Tsai et al., 1993). The co-localization of CDK2 and cyclin A with the DNA replication and repair factor, proliferating cell nuclear antigen (PCNA), at subnuclear foci of DNA replication in cultured cells also suggests that CDK2-cyclin A may be involved in regulating DNA replication (Cardoso et al., 1993).

Although a role for CDK2-cyclin A in regulating DNA replication and S phase progression has been implicated from experiments described above, the events triggered by CDK2-cyclin A activity that are required for initiation of DNA replication have not yet been determined. CDK2-cyclin A accumulates during S phase in complexes with E2F and pRB or E2F and the RB-related

protein, p107 (Devoto et al., 1992). CDK2-cyclin A has been shown to phosphorylate p107 *in vitro* and it has been suggested that a p107-CDK2-cyclin A complex may modulate the activity of E2F (Peeper et al., 1993). Presently, it is unclear how these complexes may regulate S phase progression but it has been suggested that CDK2-cyclin E in association with E2F and p107 at the G₁-S transition may stimulate E2F-mediated gene transcription while CDK2-cyclin A-E2F-p107 complexes may inhibit their transcription later in S phase (Hunter and Pines, 1994). The regulation of DNA replication has been best studied in *S.cerevisiae*. One model to emerge from these studies suggests that an S phase CDK-cyclin complex may be responsible for phosphorylating MCM proteins, which are essential for DNA replication and have been identified in a wide range of eukaryotic species. The MCM proteins associate with chromatin and may contribute to putting replication origins into an initiation-competent state. During S phase MCM proteins are phosphorylated and cyclic changes in phosphorylation are correlated with changes in MCM affinity for chromatin (reviewed in Wuarin and Nurse, 1996; Stillman, 1996; Chong et al., 1996). The MCM proteins bind to chromatin in late mitosis and remain bound until they are gradually removed as S phase progresses (reviewed in Stillman, 1996). Whether the S phase CDK2-cyclin A kinase activity regulates DNA replication by phosphorylating the MCM proteins remains to be determined.

d. p34^{cdc2} kinase-cyclin B and the G₂-M transition

It is now well established that p34^{cdc2} kinase and B-type cyclins are the components of the cytoplasmic factor, maturation promoting factor, which is capable of inducing nuclear membrane breakdown and chromosome condensation in G₂ arrested cells. As the first CDK-cyclin complex identified, p34^{cdc2}-cyclin B is the best characterized CDK-cyclin complex, in terms of how its activity is regulated and what events it may be directly involved in as cells undergo mitosis. The activation of p34^{cdc2} is a multistep process that initially requires binding to cyclin B (Solomon et al., 1990). Although mammalian cyclin B is synthesized throughout the cell cycle, there is a large increase in the level of cyclin B in the G₂ phase of the cell cycle (Pines and Hunter, 1989). Cyclin B and p34^{cdc2} begin to form complexes late in S phase that accumulate during G₂, and the association of the two proteins induces phosphorylation of three regulatory amino acids, Thr14 and Tyr15 and Thr161 (Norbury et al., 1990; Solomon et al., 1990; Ducommun et al.,

1991). While p34^{cdc2} is phosphorylated on Thr14 and Tyr15 the p34^{cdc2}-cyclin B complexes remain inactive throughout G₂ and are only activated at the G₂-M transition following dephosphorylation of these residues (Norbury et al., 1991; Krek and Nigg, 1991a, 1991b; reviewed in Norbury and Nurse, 1992; King et al., 1994). The phosphorylation of Thr161 positively regulates p34^{cdc2}, is required for full activation of p34^{cdc2}, and this site remains phosphorylated during mitosis (Gould et al., 1991; Solomon et al., 1992; Krek and Nigg, 1991b). The inactivation of p34^{cdc2}-cyclin B complexes at the end of mitosis and entry into anaphase may depend on the dephosphorylation of Thr161 (Gould et al., 1991; Lorca et al., 1992) and the degradation of cyclin B (Lorca et al., 1992) by ubiquitin-mediated proteolysis (reviewed in King et al., 1996).

The kinase activity of p34^{cdc2}-cyclin B complexes is implicated in the mitotic hyperphosphorylation of several target substrate proteins that contain phosphorylation sites in the context of the consensus for phosphorylation by p34^{cdc2}, (S/T)-P-X-K/R, where X is a polar amino acid (Moreno and Nurse, 1990). The substrates of p34^{cdc2}-cyclin B include several structural proteins such as nuclear lamins, vimentin, caldesmon, myosin regulatory light chain, histone H1 and pp60^{c-src}. Most of these proteins are phosphorylated on the same sites *in vitro* by p34^{cdc2}-cyclin B as they are *in vivo* during mitosis (reviewed in Moreno and Nurse, 1990; Nigg, 1993). Thus the phosphorylation of many of these proteins by p34^{cdc2} contributes to the major structural reorganization of cells during mitosis.

e. The protein kinases and phosphatases that regulate CDK activity

In section 1.2b I described the three regulatory phosphorylation sites of mammalian p34^{cdc2} (Thr14, Tyr 15 and Thr161) which are conserved in all p34^{cdc2} homologues throughout evolution and in all CDKs and cdc2-related kinases. This suggests that the proteins regulating p34^{cdc2} activity via reversible phosphorylation of these residues may also be evolutionarily and functionally conserved, possibly regulating the activities of many different members of the CDK family. In this section I will review the literature describing: 1) the protein kinases Myt1 and Wee1 which are responsible for inhibiting p34^{cdc2}-cyclin B activity through phosphorylation of Thr14 and Tyr15, 2) the CDK-activating kinase known as CAK (or p40^{MO15} or CDK7-cyclin H) that

phosphorylates Thr161 and 3) the protein phosphatase CDC25 which dephosphorylates Tyr15 to activate p34^{cdc2}-cyclin B at the onset of mitosis.

Wee1 and Myt1 kinases are negative regulators of p34^{cdc2} activity

Wee1 was first identified in yeast genetic studies as the gene mutated in *S. pombe* “wee” mutants that initiated mitosis and cell division prematurely, at half the size of wild type cells. These observations indicated that the wee phenotype, resulting from mutations in the *wee1* gene, was due to the inactivation of an inhibitor protein that normally delayed onset of mitosis until cells had grown to a certain size (Nurse, 1975; Nurse and Thuriaux, 1980). When the *S. pombe wee1* gene was cloned, it was found to encode a putative serine/threonine protein kinase that exerted its inhibitory effects on mitosis in a dose-dependent manner. Overexpression of the *wee1* gene resulted in a prolonged G₂ phase and cells overexpressing *wee1* divided at a much larger size than wild type cells (Russell and Nurse, 1987). Wee1 is directly responsible for the phosphorylation of p34^{cdc2} kinase on Tyr15, and inhibiting the histone H1 kinase activity of yeast and human p34^{cdc2} (Parker et al., 1992; Parker and Piwnica-Worms, 1992; McGowan and Russell, 1993). The phosphorylation of p34^{cdc2} by Wee1 remains relatively constant during the cell cycle while p34^{cdc2} is inactive. During mitosis p34^{cdc2} is activated following dephosphorylation of Tyr15 (and Thr14) and Wee1 is inactive as the p34^{cdc2} Tyr15 kinase (McGowan and Russell, 1995).

Wee1 from *S. pombe* was shown to be a dual-specificity kinase, phosphorylating serine and tyrosine residues (Parker et al., 1992). Based on the conservation of structure and function of many cell cycle regulatory proteins through evolution it was predicted that phosphorylation of Thr14 and Tyr15 of vertebrate p34^{cdc2} may be accomplished by the human Wee1 homologue (Lindberg et al., 1992). However, human Wee1 is not a dual-specificity kinase and does not phosphorylate Thr14 of p34^{cdc2} (Parker and Piwnica-Worms, 1992; McGowan and Russell, 1993). A cDNA cloned from *Xenopus laevis* encodes a dual-specificity protein kinase named Myt1 that was shown to phosphorylate Thr14 and Tyr15 of p34^{cdc2}. Myt1 is most homologous to the Wee1 kinases. Myt1 is the predominant p34^{cdc2} Thr14-specific kinase in *Xenopus* egg extracts and its activity is regulated similar to the Tyr15-specific activity of Wee1 during the cell

cycle (Mueller et al., 1995). Thus in higher eukaryotes the inhibition of p34^{cdc2} kinase activity prior to the onset of mitosis appears to be regulated by two related protein kinases. Although conserved or identical residues are located in positions equivalent to Thr14 and Tyr15 of p34^{cdc2} in all CDKs and cdc2-related kinases that have been identified, it is presently unclear whether phosphorylation of these residues by Myt1 and Wee1 (or other unidentified protein kinases) regulate the activities of other members of the cdc2 kinase family. Preliminary data has suggested that a Wee1-like protein exists in the brain, which may regulate the nervous system-specific kinase activity of CDK5 (Lew et al., 1995). Further studies are required to determine whether the Thr14- and Tyr15-equivalent sites are used to regulate other CDK activities in a manner similar to p34^{cdc2}.

Cdc25 phosphatase activates p34^{cdc2} at mitosis

Like *wee1*, *cdc25* was first described in *S. pombe* as a gene that functioned in the regulation of cell cycle by coordinating cell growth with mitosis. In the absence of *cdc25*, *S. pombe cdc25* mutants were unable to initiate mitosis, suggesting that *cdc25* was a positive regulator of mitosis (Nurse and Thuriaux, 1980). Increases in *cdc25* expression resulted in corresponding decreases in cell size at mitosis (i.e., cells initiated mitosis prematurely), demonstrating that the level of *cdc25* expression was rate-limiting for initiation of mitosis (Russell and Nurse, 1986). In addition, the *cdc25* gene function was shown to counteract the mitosis inhibiting function of the *wee1* gene in *S. pombe* (Russell and Nurse, 1986). The levels of *cdc25* mRNA and protein expression are regulated during the cell cycle, increasing through interphase and peaking at mitosis (Moreno et al., 1990). All of this data indicated that in *S. pombe* the accumulation of *cdc25* to a critical level leads to the onset of mitosis.

The nucleotide sequences of *S. pombe cdc25* and *cdc25* homologues from budding yeast, *Drosophila* and humans revealed that *cdc25* was evolutionarily conserved and the carboxy-terminal segment of the predicted proteins was the most highly conserved region. This region was later shown to be the functional domain of the *cdc25* protein (reviewed in Atherton-Fessler et al., 1993). Indirect evidence suggested that *cdc25* possessed tyrosine phosphatase activity, and that it might induce entry into mitosis by dephosphorylation of Tyr15 of p34^{cdc2}. The *S. pombe cdc25* mutants that were unable to initiate mitosis accumulated p34^{cdc2} in a tyrosine phosphorylated form. Expression of a non-phosphorylatable Tyr15Phe mutant of p34^{cdc2}

eliminated the requirement for *cdc25* gene function for cells to enter mitosis (Gould and Nurse, 1989). More direct evidence for the tyrosine phosphatase function of *cdc25* was provided by experiments demonstrating that when bacterially expressed *cdc25* proteins were added to oocyte extracts or purified p34^{cdc2}-cyclin B, *cdc25* induced rapid tyrosine dephosphorylation and activation of p34^{cdc2} histone H1 kinase activity (Kumagai and Dunphy, 1991; Strausfeld et al., 1991). Human *cdc25* is also capable of dephosphorylating Thr14 and activating p34^{cdc2} and p33^{cdk2} *in vitro* (Sebastian et al., 1993), indicating that it is a dual-specificity phosphatase.

In mammals, three *cdc25* homologues have been identified; CDC25A, B and C (reviewed in Atherton-Fessler et al., 1993). Of the three mammalian CDC25 proteins, only CDC25C is expressed predominantly in G₂ (Sadhu et al., 1990) and functions as an activator of p34^{cdc2} and mitosis. CDC25A mRNA and protein expression peak in late G₁ just prior to onset of S phase (Jinno et al., 1994) and the peak of CDC25B expression is closer to the G₁-S boundary (Kakizuka et al., 1992). In addition, microinjection of CDC25A antibodies into cultured cells caused most cells to arrest in G₁, indicating that CDC25A is not a mitotic inducer but that it is required for cells to enter S phase. This data suggests that CDC25A may regulate the kinase activity of a G₁ CDK, such as CDK4 or CDK2, which have been shown to be dephosphorylated *in vitro* by a human *cdc25* protein (Sebastian et al., 1993). Other data suggest that CDC25A and CDC25B are potential human oncogenes. When normal mouse embryo fibroblasts were co-transfected with each of the three CDC25 proteins and oncogenic versions of p53 and H-RAS, only the CDC25A and CDC25B co-transfectants formed transformed foci. In fibroblasts lacking pRB, transfection of CDC25A alone caused transformation. In addition, both CDC25A and CDC25B are overexpressed in some primary breast carcinomas (Galaktionov et al., 1995). It has recently been demonstrated that the transcription of CDC25A is controlled by *c-myc*, which regulates normal cell growth, oncogenesis and apoptosis (Galaktionov et al., 1996), but the physiological substrates of CDC25A and CDC25B remain to be determined in order to understand how these proteins may function as oncogenes.

The characterization of Wee1 and *cdc25* has demonstrated that the activation of p34^{cdc2} and initiation of mitosis depends largely on the opposing regulatory actions of these two proteins. Phosphorylation of Tyr15 by Wee1 (and of Thr14 in vertebrate species by Myt1) inhibits

premature activation of p34^{cdc2} and mitosis, possibly by interfering with ATP binding, until cells grow to the appropriate size for cell division. The abrupt dephosphorylation of these regulatory sites by cdc25 fully activates p34^{cdc2} and promotes mitosis.

The CDK activating kinase (CAK or CDK7/cyclin H)

Phosphorylation of Thr167 of *S. pombe* p34^{cdc2} was shown to be essential for p34^{cdc2} activation at mitosis and for cell division to occur (Gould et al., 1991). Although the equivalent site in several other protein kinases requires autophosphorylation for activation (Hanks et al., 1988), highly purified p34^{cdc2}-cyclin B does not self-phosphorylate (Labbé et al., 1991). The protein kinase responsible for phosphorylating the equivalent of Thr167 in mammalian p34^{cdc2} (Thr161), CDK2 (Thr160), CDK4 (Thr172) and CDK6 (Thr177) was initially purified from *Xenopus* and starfish oocyte extracts as a complex of two proteins, p40^{MO15} and p37. Phosphorylation of the threonine residue by this CDK-activating kinase (CAK) was shown to activate the histone H1 kinase activities of p34^{cdc2} and CDK2 and the pRB kinase activities of CDK4 and CDK6 (Fesquet et al., 1993; Poon et al., 1993; Solomon et al., 1993; Kato et al. 1994; Aprelikova et al., 1995). Further characterization of CAK demonstrated that CAK expression and activity was constant throughout the cell cycle (Tassan et al., 1994), possibly reflecting its ability to regulate the activities of multiple CDKs. The p37 component of the mammalian homologue of CAK was identified as cyclin H, following purification of the mammalian CAK (Fisher and Morgan, 1994) and by using a mammalian MO15 homologue for a yeast two-hybrid screen (Mäkelä et al., 1994). Reconstitution of cyclin H with the mammalian catalytic MO15 subunit, *in vitro*, showed that MO15 was a CDK (referred to as CDK7), which was capable of phosphorylating Thr160 or Thr161 of CDK2- or p34^{cdc2}-cyclin complexes and promoting their histone H1 kinase activities (Fisher and Morgan, 1994; Mäkelä et al., 1994). Like other CDKs, CDK7 contains a threonine at the position equivalent to Thr161 and also requires phosphorylation of this residue for full activity (Fisher and Morgan, 1994).

In addition to its function as a CDK activating kinase, CAK was also shown to be essential component of a basal transcription-DNA repair factor, TFIIH. In this protein complex the kinase activity of CAK phosphorylates the C-terminal domain of the large subunit of RNA polymerase II. It has been suggested that CAK may exist in two forms, TFIIH-bound and unbound forms (Roy et

al., 1994). The purification and cloning of CAK (Cak1p or Civ1) from *S. cerevisiae* has indicated that in yeast, the CDK activating and RNA polymerase II C-terminal domain kinase functions are performed by distinct proteins. The yeast CAK, referred to as Cak1p or Civ1, is not a homologue of mammalian CDK7 and is active as a monomer. Cak1p (Civ1) does not have RNA polymerase II C-terminal domain kinase activity (Kaldis et al., 1996; Thuret et al., 1996). The CDK7-cyclin H homologues in budding yeast are Kin28p and Ccl1p, respectively. Kin28p and Ccl1p are subunits of TFIIH and phosphorylate the C-terminal domain of RNA polymerase II, but do not have CAK activity (reviewed in Kaldis et al., 1996; Thuret et al., 1996). Based on these findings, it has been suggested that another CAK, similar to *S. cerevisiae* Cak1p (Civ1), may exist in higher eukaryotes (Kaldis et al., 1996).

f. The CDK inhibitor proteins (CDKIs)

Another major mechanism for regulating CDK activity during the cell cycle involves the association of CDKs with CDK inhibitor proteins (CDKIs) that bind to and inactivate CDK-cyclin complexes. Although four of these proteins (FAR1, p40^{Sic1}, PHO81 and rum1) have been identified in yeast (reviewed in Nigg, 1995; Morgan, 1995), in this section I will limit the review of inhibitor proteins to those that have been identified in mammals. These proteins are separated into two groups and include: 1) p21 (also referred to as Cip1, Waf1, Sdi1, Cap20) and p27 (also known as Kip1), which are related proteins that interact preferentially with CDK2- and CDK4-cyclin complexes and 2) the INK4 family (including p16^{INK4a}, p15^{INK4b}, p18^{INK4c} and p19^{INK4d}), which are specific inhibitors of CDK4- and CDK6-cyclin D complexes.

The p21 and p27 CDK inhibitor proteins

p 2 1

The first CDKI protein identified was p21. In normal cells, CDKs exist predominantly in complexes that also contain cyclin, proliferating cell nuclear antigen (PCNA) and the p21 protein (Xiong et al., 1992; Xiong et al., 1993a; Gu et al., 1993). The majority of studies examining the activities and regulation of CDKs have been done using transformed cells, such as HeLa cells, and initially p21 escaped notice because in transformed cells PCNA and p21 are dissociated from the CDK2- and CDK4-cyclin complexes that they are part of in normal cells (Xiong et al., 1993a). When normal fibroblasts were transformed with DNA tumor viruses, CDK-cyclin complexes did not contain p21 and PCNA but acquired new subunits, p16 or p19. This indicated that in normal cells the subunit composition of CDK-cyclin complexes was more complicated than originally thought

and that p21 may be an additional regulatory subunit. These observations also suggested that a transformed phenotype was related to alterations in the CDK-cyclin-PCNA-p21 subunit composition (Xiong et al., 1993a).

In vitro reconstitution experiments demonstrated that p21, PCNA, CDK2, CDK4 and cyclins expressed in a baculovirus system formed the same quaternary complexes *in vitro* that were formed *in vivo* in normal fibroblasts (Xiong et al., 1993b). Addition of p21 to CDK2-cyclin A or CDK4-cyclin D complexes resulted in abrupt inhibition of these kinase activities towards histone H1 and pRB substrates when p21 reached stoichiometric levels (Xiong et al., 1993b). Similar results were obtained by Gu et al. (1993) using p21 purified from mouse fibroblasts. It has been suggested that variations in the stoichiometry of the CDK-cyclin-PCNA-p21 quaternary complex determine whether the kinase is active or inactive. One molecule of p21 in a complex permits kinase activity, but two or more molecules of p21 are inhibitory (Zhang et al., 1994b).

The p53-deficient cells derived from Li-Fraumeni patients do not carry any known DNA tumor viruses, but they undergo spontaneous immortalization in culture. The CDK-cyclin complexes immunoprecipitated from these cells lack p21 and PCNA subunits, and do not contain p16 or p19 subunits either (Xiong et al., 1993a). Cells lacking p53, from mice homozygously null for p53, show a 50-fold reduction in p21 mRNA levels compared to normal cells (Xiong et al., 1993b). p53 is a known tumor suppressor protein (Donehower and Bradley, 1993) and mice lacking p53 are susceptible to spontaneous tumor formation (Donehower et al., 1992). In addition, mutations in the p53 gene are associated with a wide range of human cancers (reviewed in Donehower and Bradley, 1993). p53 null mice develop fully, which suggested that p53 may not be required for normal progression through the mitotic cell cycle (Donehower et al., 1992). However, p53 is required for regulation of cell cycle progression. p53 is a cell cycle checkpoint factor that is required for arresting the cell cycle in G₁ when DNA is damaged by radiation, chemicals or viral infection (reviewed in Cox and Lane, 1995). There is strong evidence to suggest that p53 mediates cell cycle arrest by functioning as a transcription factor that regulates expression of the CDKI protein, p21. In addition to the alterations in p21 mRNA expression in cells lacking p53 observed by Xiong et al. (1993a and 1993b) others have demonstrated that p21 expression is induced a p53-dependent manner (El-Diery et al., 1993) and that the CDK-cyclin inhibitory activity of p21 was induced by γ -irradiation of human cells manner (Dulic et al., 1994). When embryonic fibroblasts from p21 null mice, wild-type mice and p53 null mice were exposed to

γ -irradiation, the number of p21 null cells that arrested in G₁ was intermediate between wild-type and the p53 null cells, suggesting that p21 inhibitory activity may be redundant. In addition, the p21 null mice do not show a tendency toward spontaneous early tumorigenesis, unlike p53 null mice, which also suggests that p21 CDK inhibitory activity at the G₁ checkpoint may be partially redundant (Deng et al., 1995). All of this data strongly supports the hypothesis that p21 may act downstream of p53 to partially mediate cell cycle arrest induced by DNA damage through inhibition of CDK2- and CDK4-cyclin kinase activities during G₁. Induction of p21 has also been correlated with exit from cell cycle or terminal differentiation of skeletal muscle in a p53-independent but MyoD-dependent pathway (Parker et al., 1995; Halevy et al., 1995). However, because the targeted disruption of the p21 gene did not affect the development of mice it is possible that the role of p21 in terminal differentiation may also be redundant or that its activity may be involved in maintenance of the differentiated state (Deng et al., 1995).

In addition to mediating cell cycle arrest in G₁, p21 may also inhibit DNA replication by blocking the activity of CDK2-cyclin E complexes (Jackson et al., 1995) or through direct interactions with PCNA (Waga et al., 1994; Jackson et al., 1995).

p 27

The CDK inhibitory protein, p27 (or Kip1) was identified by yeast two-hybrid screening in search for proteins that interacted with CDK4-cyclin D1 complexes (Toyoshima and Hunter, 1994) and by protein purification from extracts of cells made quiescent by TGF- β treatment (Polyak et al., 1994). Cloning of p27 revealed that p27 showed homology to p21 and that amino acid similarity was predominantly in N-terminal regions of these proteins. Although p27 associates predominantly with CDK4-cyclin D1 complexes, p27 activity inhibited the kinase activities of several CDK-cyclin complexes *in vitro*, including CDK4-cyclin D1, CDK2-cyclin A, CDK2-cyclin E and p34^{cdc2}-cyclin B (Toyoshima and Hunter, 1994; Polyak et al., 1994). The N-terminal region of p27, which shares the most homology with p21, is essential for the inhibition of CDK2-cyclin A kinase activity (Polyak et al., 1994). This finding suggests that the N-terminal region is the functional domain of p27 and p21. It has been suggested that the activity of p27 may mediate quiescence induced by TGF- β treatment of fibroblasts in culture and the presence of inactive CDK2-cyclin E complexes in these cells may be due to association with p27 (Koff et al., 1993). When p27 was added to extracts of proliferating cells, cyclin E-associated CDK2 was not

phosphorylated on Thr160 and these complexes were inactive, suggesting that the inhibitor protein may act by preventing Thr160 phosphorylation (Polyak et al., 1994). Although neither p27 nor p21 interact directly with CAK (the Thr160 kinase), both proteins appeared to inhibit phosphorylation of CDK2 at Thr160 by rendering CDK2 inaccessible to CAK (Aprelikova et al., 1995). The recent solution of the crystal structure of p27 bound to CDK2-cyclin A revealed that p27 binding to CDK2 induces conformational changes that alter the the shape of the catalytic cleft. p27 fills the catalytic cleft and mimics ATP. In addition, the occupation of the catalytic cleft by p27 may produce steric hindrance that interferes with the binding of CAK to the T loop, thereby inhibiting phosphorylation of CDK2 at Thr160 (Russo et al., 1996).

Overexpression of p27 correlates with cell cycle arrest in G₁ and failure to enter S phase (Polyak et al., 1994; Toyoshima et al., 1994). Targeted disruption of the p27 gene resulted in mice with increased body size, enlargement of multiple organs and spontaneous pituitary tumors (Nakayama et al., 1996; Fero et al., 1996; Kiyokawa et al., 1996). The presence of spontaneous pituitary tumors in p27 null mice is similar to the phenotype observed in pRB null mice (Jacks et al., 1992; Lee et al., 1992), suggesting that p27 and pRB may be components of the same pathway involving CDK4-cyclin D1. Surprisingly, cultures of T cells derived from p27 null mice displayed cell cycle arrest in response to TGF- β treatment, indicating that p27 was not essential for mediating anti-proliferative signals via TGF- β (Nakayama et al., 1996). However, the enlargement of multiple organs in these mice suggests that p27 is partially involved in the control of cell proliferation.

INK4 CDK inhibitors

The CDK inhibitor p16^{INK4a} was first identified in CDK4-cyclin D1 immune complexes from cells transformed by DNA tumor viruses, that lacked the p21 and PCNA components of this complex found in normal cells (Xiong et al., 1993a). Recombinant p16 binds specifically to CDK4 or CDK6, but not to other CDKs or cyclins and its association with CDK4- and CDK6-cyclin D complexes inhibits their pRB kinase activity, *in vitro* (Serrano et al., 1993). The biochemical characterization of p16 has suggested that it is a negative regulator of the proliferation of normal cells. It has been hypothesized that the function of p16 in normal cells may be to inhibit CDK4 activity once pRB has been inactivated by phosphorylation near the G₁ restriction point (Serrano et al., 1993; Sherr and Roberts, 1995). Overexpression of p16 in normal cells prevents

proliferation, by arresting cells in G₁, and deletions or mutations in the p16 gene occur in several types of primary tumors. This data indicates that p16 may function as a tumor suppressor (reviewed in Sherr and Roberts, 1995).

The three additional genes related to the p16^{INK4a} gene encode p15^{INK4b}, p18^{INK4c} and p19^{INK4d}, which are also specific inhibitors of CDK4- and CDK6-cyclin D kinase activities. The expression of p15^{INK4b} was induced in cultures of human keratinocytes following TGF- β treatment, suggesting that p15 may mediate the cell cycle arrest induced by TGF- β by inhibiting the CDK4 and CDK6 kinases (Hannon and Beach, 1994). It is presently unclear whether the four related INK4 proteins perform distinct biological functions, since each inhibits CDK4-or CDK6-cyclin D activity *in vitro*. It has been suggested that one protein may function primarily as an inhibitor of CDK4 or CDK6 activity when normal cycling cells exit G₁, while another member of the family may normally be expressed at low levels but be induced in response to DNA damage, anti-mitogenic signals, or during terminal differentiation (Sherr and Roberts, 1996). The biological function of each of these proteins remains to be determined.

g. The proteins that regulate CDK activity and cell cycle progression:

Summary

Within the past several years our understanding of how the eukaryotic cell cycle is regulated has advanced at a rapid pace. Although many of the key components of the cell cycle engine and different levels of regulation are conserved from yeast to mammals, the complexity of mammalian organisms obviously means that many more complicated pathways are required for the development of an organism. Much of what has been learned about the cell cycle in recent years is related to understanding the regulation of CDKs and relative to this, considerably less is known about the actual substrate proteins phosphorylated by CDKs in mammals. Figure 1.3 summarizes the current understanding of how and when the proteins that I have described in this section may function to regulate mammalian cell cycle progression.

1.4 Cell cycle related proteins in the nervous system

Significant progress has been made toward understanding what factors control eukaryotic cell cycle progression in proliferating cells and how deregulation of specific proteins may contribute to uncontrolled proliferation (oncogenesis) or cell death. Comparatively little is known

about whether these proteins or related proteins influence withdrawal from the cell cycle to help establish and possibly maintain the terminally differentiated and postmitotic state of cells such as neurons.

Although all of the CDKs and *cdc2*-related kinases that have been identified in mammals to date show widespread expression in mitotic tissues, some of these kinases have also been identified in postmitotic nervous tissue (Meyerson et al., 1992; Hellmich et al., 1992; Lew et al., 1992a; Okuda et al., 1992; Tsai et al., 1993; Graña et al., 1994a; Graña et al., 1994b; Brambilla et al., 1994). The expression of putative *cdc2*-related kinases in postmitotic tissue suggests that their functions may not be restricted to the proliferative state and that some of these kinases may regulate other processes pertinent to the differentiated state (Meyerson et al., 1992). Thus the CDKs, *cdc2*-related kinases and other regulators of the cell cycle that are expressed in mitotic cells and in postmitotic neurons, may perform different functions in different types of cells. In addition, novel proteins related to the cell cycle regulatory proteins may be expressed predominantly or exclusively in the nervous system to perform specialized functions, unrelated to regulation of the cell cycle. In this section I will review literature describing the expression of CDKs, *cdc2*-related kinases, cyclins, CDKIs and tumor suppressor proteins in the nervous system and what is currently known about the activities or functions of these proteins in postmitotic neurons.

a. CDKs and *cdc2*-related kinases expressed in nervous tissue

The first study to examine the expression of *cdc2* in the developing rat nervous system, established that *cdc2* mRNA and protein were highly expressed in proliferating neuronal precursor cells, and that a dramatic downregulation of expression correlated with periods of neuronal differentiation in the cortex (E15-E20) and in the cerebellum (P9-P22) (Hayes et al., 1991). The very low level of p34^{*cdc2*} expression detected in nervous tissue at the end of neurogenesis is maintained in adult animals (Hayes et al., 1991) in discrete populations of postmitotic neurons and in some proliferating precursor cells (Okano et al., 1993). The downregulation of p34^{*cdc2*} expression during neurogenesis raised the question of whether other *cdc2*-related kinases may be upregulated at this time, to regulate the switch from a proliferative to terminally differentiated state. The reduction in *cdc2* mRNA and protein expression observed in rat brain and cerebellum by Hayes et al. (1991) has also been demonstrated in mouse spinal cord (Zhao et al., 1995) and occurs in cultures of rat superior

cervical ganglia and PC12 cells following treatment with nerve growth factor (NGF), a neuronal differentiation and survival factor (Freeman et al., 1994; Yan and Ziff, 1995).

The low level of p34^{cdc2} expression maintained by certain populations of terminally differentiated neurons raised the question of whether p34^{cdc2} phosphorylated components of the neuronal cytoskeleton, since many of cytoskeletal rearrangements that occur during mitosis are the result of direct phosphorylation of cytoskeletal proteins by p34^{cdc2} (Hayes et al., 1991; reviewed in Nigg, 1993). A study by Hisanaga et al. (1991) demonstrated that p34^{cdc2} prepared from starfish oocytes could phosphorylate purified, alkaline phosphatase-treated (dephosphorylated) neurofilament proteins *in vitro*. Phosphorylation of the neurofilament-heavy protein (NF-H) by p34^{cdc2}, *in vitro*, produced the same anomalous electrophoretic mobility of NF-H on SDS-polyacrylamide gels that occurs when it is posttranslationally phosphorylated *in vivo*. Peptides derived from the carboxy-terminal domain of NF-H, containing multiple repeats of the amino acid sequence KSP (single letter code), were used as substrates in *in vitro* kinase assays with p34^{cdc2}. Only the peptide containing KSP motifs similar to the consensus for phosphorylation by p34^{cdc2} (S/T-P-X-K/R) was phosphorylated (Hisanaga et al., 1991). The low level of p34^{cdc2} in adult nervous tissue suggested that other protein kinases related to p34^{cdc2} may be more abundant in postmitotic neurons to phosphorylate neurofilament proteins *in vivo*.

The identification of several cDNAs encoding a family of cdc2-related protein kinases in mammals by Meyerson et al. (1992) revealed that the majority of these putative kinases are expressed in a wide variety of mitotic tissues. Some of these kinases, such as PSSLARE (CDK5), PCTAIRE-1, PCTAIRE-2, PCTAIRE-3, PLSTIRE (CDK6) and KKIALRE, are expressed in mitotic tissues and in the brain. The genes encoding these kinases did not complement the function of the *cdc28* gene that was lacking in *cdc28* temperature sensitive mutants (Meyerson et al., 1992). The mRNA transcripts of other cdc2-related kinases including isoforms of PITSLRE, PISSLRE, and PITALRE have also been detected in brain and a variety of other tissues (Xiang et al., 1994; Graña et al., 1994b; Brambilla and Draetta, 1994; Graña et al., 1994a). Although many of the cdc2-related kinases that have been identified are expressed in brain, none are expressed exclusively in postmitotic nervous tissue. Only one of the cdc2-related kinases, PSSLARE or CDK5, has been characterized extensively by molecular and biochemical approaches in order to determine

its biological function in postmitotic neurons.

b. PSSLARE kinase or CDK5

Human PSSLARE kinase is expressed abundantly in several different tissues, but is also enriched in the brain (Meyerson et al. 1992). Rat, bovine and mouse homologues of PSSLARE have been identified and all show similar mRNA enrichment in nervous tissue (Hellmich et al., 1992; Lew et al., 1992b; Ino et al., 1994). When PSSLARE was immunoprecipitated from a normal human fibroblast cell line (WI38) it co-immunoprecipitated with cyclins D1 and D3 and PCNA (Xiong et al., 1992), and because of its association with cyclins PSSLARE was renamed CDK5. Although CDK5 associates with D-type cyclins no protein kinase activity has been found associated with this complex and expression of a dominant negative CDK5 mutant in human osteosarcoma cells did not reveal an essential role for CDK5 in cell cycle regulation (van den Heuvel and Harlow, 1993).

The CDK5 proteins from bovine and rat nervous tissue co-purified with proteins having molecular weights of 25 kDa and 62 kDa, which did not correspond to any known cyclin proteins expressed in postmitotic nervous tissue (Lew et al., 1992a; Shetty et al., 1993). CDK5, purified from bovine brain, phosphorylated histone H1 *in vitro*, in a cyclin-independent manner. The histone H1 kinase activity of CDK5 was comparable to that of p34^{cdc2}-cyclin B purified from HeLa cells (Lew et al., 1992a). Although CDK5 is expressed in several tissues and cell lines, the histone H1 kinase activity associated with CDK5 has only been detected in CDK5 immunoprecipitated or purified from nervous tissue (Lew et al., 1992a; Shetty et al., 1993; Tsai et al., 1993; Tsai et al., 1994).

The abundant expression of CDK5 mRNA in nervous tissue in neurons that contain high levels of neurofilament proteins (NF-M and NF-H), such as dorsal root ganglion neurons (Hellmich et al., 1992), suggested that CDK5 may be the cdc2-related protein kinase that phosphorylates *in vivo* the KSP motifs located in the carboxy-terminal domain of neurofilament proteins. Independent studies demonstrated that bovine and rat CDK5, purified from nervous tissue, phosphorylated alkaline phosphatase-treated NF-M and NF-H and peptides derived from the carboxy-terminal domain of NF-H in *in vitro* kinase assays (Lew et al., 1992b; Shetty et al., 1993). Trypsin digestion of bovine NF-M and NF-H, following phosphorylation *in vitro* by bovine CDK5 or p34^{cdc2} purified from HeLa cells, demonstrated that both kinases phosphorylated neurofilament

proteins on identical sites (Lew et al., 1992b). The phosphorylation of the bovine neurofilament proteins by CDK5 does not produce the same electrophoretic mobility shift of NF-H observed by Hisanaga et al. (1991), following phosphorylation by p34^{cdc2}, unless the incubation time for the kinase reaction is extended to over 4 hours (Hisanaga et al., 1993). With extended incubation of kinase reactions, phosphorylation of dephosphorylated NF-H by CDK5 and p34^{cdc2} resulted in the incorporation of similar amounts of phosphate by NF-H (Hisanaga et al., 1993). The prolonged incubation time required for phosphorylation of bovine NF-H by CDK5 compared to p34^{cdc2} suggests that 1) CDK5 may not readily phosphorylate as many sites on completely dephosphorylated NF-H *in vitro* as p34^{cdc2} does or 2) NF-H may need to be partially phosphorylated on other sites (possibly by another kinase, *in vivo*?) before CDK5 can phosphorylate all of the sites phosphorylated by p34^{cdc2}. Only a slight shift in the electrophoretic mobility of rat NF-H on SDS-polyacrylamide gels occurs following phosphorylation *in vitro* by purified rat CDK5, even after kinase reactions were incubated for 6 hours (Shetty et al., 1993). CDK5 only phosphorylated an NF-H peptide containing the KSP motifs KSPXK and KSPEK (where X is usually an uncharged amino acid residue, such as Ala, Gly or Val), which are the same KSP motifs preferentially phosphorylated by p34^{cdc2} (Hisanaga et al., 1991; Shetty et al., 1993; Hisanaga et al., 1993). The discrepancy in the effects of phosphorylation by p34^{cdc2} and CDK5 on bovine and rat NF-H electrophoretic mobility may be due to differences in the numbers of KSPXK and KSPEK motifs available for phosphorylation, which vary among different species (Shetty et al., 1993). In species such as rat and mouse, there are considerably fewer NF-H KSP motifs that are similar to the consensus for phosphorylation by p34^{cdc2} (8 in mouse NF-H; Julien et al., 1988) compared to KSP repeats that occur in the context of K-S/T-P-X-E or K-S/T-PX-X (42 in mouse NF-H; Julien et al., 1988). NF-H peptides containing KSP sequences in the context of K-S/T-P-X-E or K-S/T-P-X-X were not substrates for p34^{cdc2} or CDK5 (Hisanaga et al., 1991; Hisanaga et al., 1993; Shetty et al., 1993). This observation indicates that other protein kinases phosphorylate these sites, possibly other cdc2-related kinases or proline-directed kinases. Extracellular signal-regulated kinases (ERKs) and glycogen synthase kinase-3 (GSK-3), which are proline-directed protein kinases like CDKs have been shown to phosphorylate NF-H *in vitro* (Guan et al., 1991; Roder and Ingram, 1991). Similar to neurofilament proteins, the microtubule-

associated protein tau also contains multiple phosphoacceptor serine or threonine residues in the context of the consensus for phosphorylation by p34^{cdc2}. CDK5 and p34^{cdc2} phosphorylate identical sites on tau *in vitro*, and some of these sites are phosphorylated in paired helical filaments obtained from Alzheimer's disease brains (Paudel et al., 1993; Baumann et al., 1993). In addition, phosphorylation of NF-H by p34^{cdc2} and CDK5 alters interactions between neurofilaments and microtubules (Hisanaga et al., 1991; Miyasaka et al., 1993), which suggests that CDK5 activity could be important for the organization or maintenance of the neuronal cytoskeleton in mature axons. Thus the biochemical characterization of CDK5 has indicated that CDK5 may function in the nervous system as a kinase that phosphorylates proteins of the neuronal cytoskeleton.

Characterization of CDK5 expression and activity patterns during development demonstrated that CDK5 expression in the nervous system begins as early as 12 days gestation (E12) in mice and gradually increases throughout embryonic and postnatal development (Tsai et al., 1993; Ino et al., 1994). Increases in histone H1 kinase activity associated with CDK5 during development are consistent with increases in CDK5 expression. The maximum levels of p34^{cdc2} expression and kinase activity in embryonic brain occur at approximately E11-12 in mice, when CDK5 expression and kinase activity are at minimum levels. During neurogenesis the increase in CDK5 expression and kinase activity to maximum levels by E17 coincides with the downregulation of p34^{cdc2} expression and kinase activity to minimum levels during this period (Tsai et al., 1993). This data is summarized in Figure 1.4. In mice moderate expression of CDK5 mRNA was detected throughout the neural tube at E14 (Ino et al., 1994), and CDK5 protein was detected only in axonal pathways in non-proliferating zones (Tsai et al., 1993). At all stages of embryonic development examined and in adults CDK5 immunoreactivity was restricted to axonal pathways, particularly in fasciculated axons. CDK5 was not detected in immature axons during axonal outgrowth (Tsai et al., 1993). Although the biochemical characterization of CDK5 has demonstrated that CDK5 phosphorylates NF-H *in vitro*, the expression and phosphorylation of NF-H is associated with axonal maturation. The NF-L and NF-M subunits of neurofilaments have been detected in developing neurons as early as E12 (rat). Expression of NF-H begins by E15, but remains low throughout embryonic development relative to NF-L and NF-M expression. NF-H expression continues to increase for several weeks during postnatal development. Axons

contain highly phosphorylated NF-M by E13, but phosphorylated forms of NF-H were not detected prior to E19 (Carden et al., 1987). CDK5 is expressed and active in embryos by E11-12 (Tsai et al., 1993), prior to detection of phosphorylated NF-M and NF-H. This observation suggests that during embryonic development CDK5 may phosphorylate protein substrates other than NF-H. A recent publication by Nikolic et al. (1996) demonstrated that in primary cortical neuronal cultures, CDK5 and its regulatory component p35 (see below) are distributed throughout postmitotic neurons; in cell bodies, nuclei, neurites and growth cones. This distribution of CDK5 in cultured neurons differs from the exclusively axonal localization in embryonic and adult mouse nervous tissue, described previously (Tsai et al., 1993). The localization of CDK5 in the terminals of growing neurites was similar to the distribution of actin. In addition, CDK5 kinase activity was required for the neurite outgrowth in the primary neuronal cultures. These findings suggest that CDK5 activity may be required for axonal outgrowth *in vivo* during neuronal differentiation (Nikolic, et al., 1996), but do not exclude the possibility that this kinase activity may also be required in mature animals to phosphorylate components of the neuronal cytoskeleton, such as NF-H or tau.

The purification of CDK5 from nervous tissue demonstrated that CDK5 co-purified with other proteins, including a 25 kDa and a 62 kDa protein (Lew et al., 1992a; Shetty et al., 1993). The molecular and biochemical characterization of each of these proteins has shown that they are the gene products of cDNAs encoding a 35 kDa protein (p35) and a 67.5 kDa protein (p67) (Tsai et al., 1994; Lew et al., 1994; Shetty et al., 1995). The 25 kDa protein that co-purified with CDK5 is the truncated form of the larger p35 precursor protein, and is the predominant form of the protein in extracts of nervous tissue (Lew et al., 1994). Based on sequence homology searches neither protein shares homology with cyclin proteins (Lew et al., 1994; Tsai et al., 1994; Shetty et al., 1995). p67 is identical to the synaptic vesicle fusion protein, Munc-18, previously identified by Hata et al. (1993). Expression of p35 and p67 is restricted to nervous tissue where each is localized exclusively in neurons (Tsai et al., 1994; Shetty et al., 1995). Each protein positively regulates CDK5 histone H1 kinase activity *in vitro* (Tsai et al., 1994; Lew et al., 1994; Shetty et al., 1995) and association with p25 (p35) is essential for the histone H1 kinase activity of CDK5 (Tsai et al., 1994; Lew et al., 1994). These data indicate that both proteins are regulators of neuronal CDK5 kinase activity. The lack of CDK5 kinase activity in mitotic tissues and proliferating cell lines that express the kinase is likely due to the absence of these regulatory proteins in non-neuronal

cells. Although the regulatory phosphorylation sites of p34^{cdc2} are conserved in CDK5 (T14, Y15, S158 of human CDK5) it has not been established whether phosphorylation of these sites is also required to regulate CDK5 activity. Wee1-like and CAK-like activities have been detected in bovine brain extract but it is presently unclear whether these kinases perform similar regulatory functions in the nervous system and whether CDK5 is a substrate for either kinase (Lew et al., 1995).

The targeted disruption of the CDK5 gene in embryonic stem cells has been used to generate CDK5 null mice. The elimination of the CDK5 gene resulted in a high rate of embryo lethality, and 64% of CDK5 null mice died *in utero* after E16.5. Analysis of E18.5 and newborn CDK5 null mice revealed that morphological abnormalities were restricted to nervous tissue. These mice displayed abnormal development of nervous tissue, which lacked the normal stratification of the cerebral and cerebellar cortices. Neuronal degeneration occurred in populations of CNS neurons, but not in dorsal root ganglia. In addition, accumulations of phosphorylated and non-phosphorylated neurofilaments were detected in neuronal cell bodies in the CDK5 null mice, suggesting defects in axonal transport. The initial analysis of the CDK5 null mice has indicated that CDK5 is important for normal brain development and differentiation (Oshima et al., 1996). The abnormal development of the CNS in CDK5 null mice is consistent with the hypothesis that CDK5 activity may be required at early stages of neuronal differentiation, possibly for axonal outgrowth (Nikolic et al., 1996). The neurodegeneration and accumulation of phosphorylated neurofilaments in neuronal cell bodies that occurs in the CDK5 null mice resembles the pathological changes characteristic of a variety of neurodegenerative disorders, including amyotrophic lateral sclerosis, motor neuron disease and Lewy body disease. These findings suggest that loss of CDK5 may trigger aberrant activity by other kinases that phosphorylate neurofilaments in pathological conditions (Oshima et al., 1996) and the CDK5 null mice will provide a useful model for studying this hypothesis.

c. Expression of cyclin proteins in neurons

The expression of G₁ and mitotic cyclins in the nervous system has not been well studied. In the CNS expression of cyclin A and cyclin B appears to be restricted to neuronal precursor cells. The downregulation of both cyclins coincides with the downregulation of *cdc2* mRNA and protein expression as precursor cells undergo terminal differentiation (Hayes et al., 1991; Zhao et al., 1995). When the mRNA expression of cell cycle related proteins was examined in cultures of rat

superior cervical ganglia (SCG) neurons following NGF-induced differentiation there was a downregulation of cyclin A mRNA during differentiation, but cyclin B mRNA expression was unchanged (Freeman et al., 1994). In similar experiments with PC12 cells (rat pheochromocytoma), NGF-induced differentiation had no effect on the expression of cyclin A protein, but cyclin B declined to undetectable levels after eight days of NGF treatment (Yan and Ziff, 1995). Differentiated PC12 cells are considered to have a sympathetic neuron-like phenotype, so it is surprising that the changes observed in cyclin A and cyclin B expression were different in SCG culture and PC12 cells.

Changes in the expression of G₁ cyclins during neuronal differentiation have generated more interest than the downregulation of mitotic cyclins as cells become postmitotic. However, there are several inconsistencies in the changes reported, which may reflect differences in the tissues or cells examined. Recently, Miyajima et al. (1995) demonstrated that the mRNA levels of cyclin E do not change significantly throughout development in the mouse brain. The cyclin E protein was detected in brain and spinal cord extracts as two isoforms of 51 kDa and 56 kDa that showed developmentally regulated expression. Prior to neurogenesis (E13-15), only the 56 kDa cyclin E isoform was detected. Between E17 and P10 expression of this isoform was extremely low, and expression of the 51 kDa cyclin E isoform was elevated. By postnatal day 15 and in adult animals only the 56 kDa isoform was expressed (Miyajima et al., 1995). Immunoprecipitation of cyclin E from extracts prepared from embryonic and adult brain extracts demonstrated that CDK2 co-immunoprecipitated with cyclin E from embryonic brain extracts (E12) but not from adult extracts. CDK5 was co-immunoprecipitated with cyclin E from adult brain extracts but not from embryonic brain extracts (E15), which suggests that CDK5 and cyclin E may only be associated in postmitotic neurons (Miyajima et al., 1995). Although these findings suggest that p35 and p67 may not be the only proteins to regulate CDK5 activity in the mature nervous system, it has not been determined whether the CDK5-cyclin E complex is an active kinase complex. In contrast to this data, Yan and Ziff (1995) demonstrated that only one form of cyclin E protein was detected in proliferating and NGF-differentiated PC12 cells and that differentiation did not affect the level of cyclin E protein in these cells.

Spatial and temporal variations in the expression of D-type cyclins occur in the CNS. In mouse spinal cord expression of cyclin D1 and cyclin D2 mRNA was not detected beyond E13.5. However, during the period of expression, cyclin D1 mRNA was detected in proliferating cells in

the ventricular zone and in the marginal zone, which contained differentiated neurons.

Expression of cyclin D2 was restricted to cells in the marginal zone (Zhao et al., 1995). Cyclin D1 and D2 expression has also been demonstrated in the rat brain. During embryonic development (E16) cyclin D2 mRNA and protein were abundantly expressed and then downregulated to undetectable levels in postnatal and adult animals. Little variation in the abundance of cyclin D1 mRNA occurred between embryonic, postnatal and adult animals, but the levels of cyclin D1 protein in the brain showed a progressive increase during development (Tamaru et al., 1994). A histone H1 kinase activity is associated with cyclin D1 immunoprecipitated from cytosolic and nuclear protein preparations of two week old rat hippocampi, but the identity of the protein kinase has not been determined (Tamaru et al., 1993).

In mouse brain a cDNA was identified that encodes a form of cyclin D2, referred to as MN20, which differs from the more widely expressed cyclin D2 only in the 3' untranslated region. MN20/D2 cyclin is expressed predominantly in the nervous system in the cerebral cortex and cerebellum and its restricted tissue distribution was detected only with probes derived from the the unique 3' untranslated region of the MN20 cDNA (Ross and Risken, 1994). The expression of MN20/D2 cyclin mRNA in the CNS is restricted to specific populations of immature postmitotic neurons, in which it is transiently expressed. The transient expression of MN20 in recently differentiated neurons suggests a possible role for this form of D2 cyclin in preventing further cell divisions from occurring, but the actual function of MN20 in these cells remains to be determined (Ross and Risken, 1994; Ross et al., 1996).

All three D-type cyclin mRNA transcripts (D1, D2, D3) were detected in differentiated SCG cultures, although cyclin D1 expression was relatively low (Freeman et al., 1994). NGF-induced differentiation of PC12 cells resulted in a dramatic upregulation of cyclin D1 protein, but expression of cyclin D2 protein declined after several days of NGF treatment (Yan and Ziff, 1995; Tamaru et al., 1994). Cyclin D1 expression was not induced in proliferating PC12 cells treated with mitogenic factors such as epidermal growth factor (EGF) or insulin, suggesting that upregulation of cyclin D1 expression is associated with PC12 cell differentiation (Tamaru et al., 1994; Yan and Ziff, 1995). These findings are consistent with the demonstrated increases in cyclin D1 expression in the brain during development (Tamaru et al., 1994), but disagree with the downregulation observed by Zhao et al. (1995) during neurogenesis in the spinal cord.

The upregulation of cyclin D1 during neuronal differentiation and its continued

expression in postmitotic neurons is surprising, because there is evidence suggesting that in many proliferating cells cyclin D1 mediates progression through the restriction point by activating the pRB kinase activity of CDK4 and CDK6 (Hunter and Pines, 1994; See also Chapter 1 Section 1.3b). Experiments by Yan and Ziff (1995) demonstrated that during PC12 cell differentiation: 1) cyclin D1 was upregulated 2) p34^{cdc2}-, CDK2- and CDK4-cyclin D1 complexes were increased relative to undifferentiated cells 3) the kinase activities of CDK-cyclin D1 complexes were decreased relative to undifferentiated PC12 cells. The induction of cyclin D1 in NGF-differentiated cells also correlated with increased expression of the CDK inhibitor p21 and association of p21 with CDK-cyclin D1 complexes. Although this data suggested that upregulation of cyclin D1 may lead to differentiation, transient overexpression of cyclin D1 alone was insufficient for differentiation to occur. However, cyclin D1 overexpression did cause a dramatic decrease in PCNA expression (Yan and Ziff, 1995). These results suggest that multiple factors, acting through a cyclin D1 pathway may mediate differentiation induced by NGF in PC12 cells. The upregulation of cyclin D1 during NGF-induced differentiation may mediate repression of PCNA, thereby inhibiting DNA replication and entry into S phase. The upregulation of p21 that occurs during differentiation may lead to inhibition of CDK-cyclin D1 kinase activity during G₁, which would prevent phosphorylation of pRB and inactivation of its growth suppressive activity. In addition, p21 may interact directly with PCNA to inhibit DNA replication (Waga et al., 1994; Jackson et al., 1995).

Additional studies examining cyclin D1 function in postmitotic cells have demonstrated that induction of cyclin D1 accompanies programmed cell death (Freeman et al., 1994; Kranenberg et al., 1996). Withdrawal of NGF from SCG cultures results in almost complete neuronal cell death within 48 hours (Martin et al., 1988) and following NGF withdrawal the mRNA levels of several cell cycle related proteins, including cyclins B, C, D3, and E, CDK4 and CDK5, pRB, PCNA, p53 and *c-myc* show decreased mRNA expression. Cyclin D1 mRNA was selectively upregulated and reached a peak of expression between 15 and 20 hours following NGF withdrawal (Freeman et al., 1994). Further analysis of the involvement of cyclin D1 in apoptotic cell death has demonstrated that transient overexpression of cyclin D1 induced apoptosis in proliferating cell lines, and in cultures of postmitotic neurons neuronal apoptosis was accompanied by a dramatic elevation in endogenous cyclin D1 protein (Kranenberg et al., 1996). Increases in the amounts of CDK4-cyclin D1 complexes and CDK4-cyclin D1 pRB kinase activity

were observed during neuronal apoptosis, even though the level of CDK4 protein was not elevated. When cultures of neuronal precursor cells were transfected transiently with either cyclin D1 or lacZ expression plasmids and induced to differentiate, withdrawal of serum resulted in a higher percentage of apoptotic cells in the cyclin D1-transfected cultures. When the CDK inhibitor p16^{INK4a} was co-transfected with a reporter plasmid in a similar experiment, a higher percentage of reporter activity was retained in these cultures than in cultures that were co-transfected with vector and reporter plasmids. All of these data indicate that upregulation of cyclin D1 and increased CDK4-cyclin D1 kinase activity are required for neuronal apoptosis (Kranenberg et al., 1996). The analysis of cyclin D1 expression during neuronal apoptosis has provided strong evidence in support of the hypothesis that neuronal apoptosis may result from an aborted attempt by postmitotic neurons to re-enter the cell cycle (Freeman et al., 1994; Kranenberg et al., 1996).

d. The CDK inhibitors in neurons

As proteins that inhibit the activities of CDKs during the G₁ phase of the cell cycle, one might predict that the CDK inhibitor proteins would be expressed or upregulated in cells that no longer divide and have become terminally differentiated, such as neurons. Analysis of CDKI expression in the nervous system has not been extensive. Expression of p21 has been demonstrated in adult brain but not in embryonic brain (Parker et al., 1995) and Yan and Ziff (1995) have demonstrated increased expression of p21 in differentiated PC12 cells. p16^{INK4a} mRNA is also expressed in adult brain and not in embryonic brain. Expression of p16^{INK4a} mRNA is also induced upon differentiation of the teratocarcinoma cell line NT2 into postmitotic neurons (Lois et al., 1995). These observations and data demonstrating that transfected p16^{INK4a} may prevent apoptosis induced by increased CDK4-cyclin D1 activity in apoptotic neuronal cultures (Kranenberg et al., 1996) indicate that CDKI proteins like p21 and p16^{INK4a} may be involved in maintaining the terminally differentiated state of neurons, by inhibiting the kinase activities of G₁ CDK-cyclin complexes in neurons.

e. Cell cycle related proteins in the nervous system: Summary

The analysis of CDKs, cyclins and CDKIs in the nervous system has been limited to date. These studies have demonstrated that proteins such as CDK5 and the nervous system-specific proteins that regulate its activity (p35, p67) are active only in the nervous system, where they perform functions that may be required only in postmitotic neurons. The downregulation of

p34^{cdc2}, CDK2, cyclin A and cyclin B expression and activity correlates with proliferating neuronal precursor cells becoming postmitotic neurons. In addition the increased expression of CDKI proteins, such as p21 and p16^{INK4a} in postmitotic neurons may contribute to maintaining the terminally differentiated state. Although proteins that are involved in regulating cell cycle progression, such as cyclin D1 and CDK4, are also expressed and active in postmitotic neurons, the aberrant upregulation of cyclin D1 in postmitotic neurons can contribute to apoptotic cell death. The cyclin D1-mediated apoptosis that occurs in neurons may be the result of the inappropriate activation of a pathway that is normally used by proliferating cells for cell cycle progression from G₁-S phase.

1.5. Goals and approach of the thesis

a. Goals

An intriguing observation for many investigators of neurofilament protein phosphorylation was the demonstration by Hisanaga et al. (1991) that *in vitro* phosphorylation of purified, dephosphorylated NF-H by p34^{cdc2} kinase produced the same anomalous electrophoretic mobility of NF-H on SDS-polyacrylamide gels that occurs when this protein is posttranslationally modified *in vivo*. This was the first demonstration of a protein kinase that produced this effect on NF-H electrophoretic mobility, following phosphorylation *in vitro*. Various regions of the three neurofilament proteins contain potential sites for phosphorylation by a variety of protein kinases, including cAMP-dependent protein kinase, casein kinase I, protein kinase C, Ca/CAM kinase, MAP-2 (ERK) kinase, and p34^{cdc2} kinase (reviewed in Pant and Veeranna, 1995 and summarized in Figure 1.5). Many of these protein kinases, as well as other less well characterized kinases, co-purify with neurofilament preparations. The carboxy-terminal tail regions of NF-M and NF-H contains numerous KSP (lysine-serine-proline) repeats, which vary in number (~40-50 repeats per molecule of NF-H) depending on the species. The majority of KSP repeats are phosphorylated on axonal NF-M and NF-H *in vivo*, making these proteins among the most highly phosphorylated proteins known (Julien and Mushynski, 1983; Geisler et al., 1987; Lee et al., 1988). The highly phosphorylated carboxy-terminal tail regions of NF-M and NF-H form sidearm projections at the periphery of the assembled neurofilament (Julien and Mushynski, 1983; Hisanaga and Hirokawa, 1988), which may mediate spacing and interactions between filaments

(Hirokawa et al., 1984; reviewed in Julien and Grosveld, 1991). A subset of these repeats occur in a sequence motif similar to the consensus for phosphorylation by p34^{cdc2} (X-S/T-P-X-K/R). Purified p34^{cdc2} preferentially phosphorylated a peptide derived from NF-H containing KSP motifs that were similar to this consensus sequence (Hisanaga et al., 1991). The downregulation of cdc2 kinase mRNA and protein during neurogenesis and the low level of cdc2 expression in the adult nervous system (Hayes et al. 1991; Okano et al., 1993; Zhao et al., 1995) indicated that p34^{cdc2} kinase may not be the kinase responsible for phosphorylation of the KSP sites in neurofilament proteins *in vivo*. This raised questions about whether other kinases related to p34^{cdc2} kinase may exist in the nervous system to mediate this function and other specialized functions acquired by terminally differentiated neurons.

The majority of phosphorylated epitopes on neurofilaments are located in axons, but in certain pathological conditions, such as motor neuron disease, amyotrophic lateral sclerosis, Lewy body disease and intoxication by neurotoxic substances such as aluminum or β , β -iminodipropionitrile, neurofilaments are hyperphosphorylated and concentrated in neuronal cell bodies (reviewed in Julien, 1995; Pant and Veeranna, 1995). In general, neurofilament phosphorylation may be important for: 1) increasing axonal caliber by increasing neurofilament spacing, and slowing axonal transport 2) increasing neurofilament stability during axonal growth and 3) conferring resistance to proteolysis (reviewed in Pant and Veeranna, 1995). The identification of protein kinases involved in the phosphorylation of the KSP repeats of NF-M and NF-H may provide answers to questions such as: 1) how are these proteins phosphorylated? 2) what is the role of neurofilament phosphorylation in neuronal function? and 3) why and how do neurofilament proteins become aberrantly phosphorylated in certain pathological conditions?

Thus the goals of this thesis were to identify and characterize a novel protein kinase cDNA related to the cell division cycle kinase p34^{cdc2}, which may function as a neurofilament kinase in the adult mammalian nervous system. During the course of this work, CDK5 was identified and found to be enriched in the brain. Extensive molecular and biochemical characterization of various mammalian CDK5 homologues has indicated that CDK5: 1) may function exclusively in postmitotic neurons 2) phosphorylates some of the KSP repeats of NF-H *in vitro*, and 3) may be important for the establishment or organization of the neuronal cytoskeleton during development. Thus the characterization of CDK5 has provided limited insight into the function of this kinase in

the nervous system. The characterization of other related proteins in the nervous system may reveal that similar to the requirement for the coordinated activities of several CDKs to regulate cell cycle progression, a subset of the cdc2 kinase family may be pertinent to changes that affect the formation and functioning of the mature nervous system, such as maintaining the terminally differentiated state, loss of regenerative capacity or cell death.

b. Approaches

Because I sought to identify a cDNA encoding a novel cdc2-related protein kinase, the work presented in this thesis began by using a mouse cdc2 kinase cDNA fragment to screen a cDNA library prepared from postnatal mouse brain, using low stringency hybridization conditions. The purified cDNA clone was the fundamental tool for pursuing the characterization of the putative cdc2-related protein kinase encoded by this cDNA. The cdc2-related kinase cDNA was used to determine the chromosomal location of the corresponding gene in mice. The cDNA was also used to identify a mRNA transcript and analyze its temporal and spatial expression patterns by Northern blotting and *in situ* hybridization. To study the protein encoded by this cDNA, I have raised polyclonal antibodies against regions of the protein that do not show homology to other known proteins. One of these antibodies was used for analysis of the protein distribution, subcellular localization and characterization of the kinase activity associated with this protein to begin to address questions about the regulation and function of this novel protein in the adult mammalian nervous system.

To further address the function of this protein kinase, I have used the cDNA to construct a transgene that has been used to generate mice that overexpress this gene in the nervous system. My colleague Dr. Qinzhang Zhu, a research associate in Dr. Jean-Pierre Julien's laboratory, cloned a partial genomic PFTAIRE clone and generated a targeting vector for homologous recombination in embryonic stem cells. Presently in Dr. Julien's laboratory there are strains of transgenic mice that overexpress this cDNA and mice that are nullizygous for this gene. The mice generated by both approaches bear no gross morphological phenotypes, but the analysis of these mice may provide further insights into the function of this protein that were not obtained from the molecular and biochemical characterization that is presented in this thesis.

Table 1.1 Summary of vertebrate cdc2 kinase family

Cyclin-dependent kinases (cdks)	References	Associated cyclins	References
cdk1 (p34 ^{cdc2})	Lee and Nurse, 1987; Spurr et al., 1990	cyclin A, B1, B2, B3	Giordano et al., 1989; Pagano et al., 1992; Pines and Hunter, 1989
cdk2 (Eg-1)	Paris et al., 1991; Millarski et al., 1991; Meyerson et al., 1992	cyclin A, D1, E	Pines and Hunter, 1990; Pagano et al., 1992; Devoto et al., 1992; Xiong et al., 1992; Koff et al., 1992
cdk3	Meyerson et al., 1992	?	Pines 1993
cdk4 (PSK-J3)	Hanks, 1987; Matsushime, 1992; Meyerson et al., 1992	cyclin D1, D2, D3	Xiong et al., 1992; Ewen et al., 1993
cdk5 (PSSALRE)	Meyerson et al., 1992; Hellmich et al., 1992; Lew et al., 1992b	cyclin D1, D3, E, p35	Xiong et al., 1992; Miyajima et al., 1995; Tsal et al., 1994; Lew et al., 1994
cdk6 (PLSTIRE)	Meyerson et al., 1992	cyclin D1, D2, D3	Meyerson and Harlow, 1994
cdk7 (p40 ^{MO15})	Poon et al., 1993; Fesquet et al., 1993; Solomon et al., 1993; Fisher and Morgan, 1994; Mäkelä et al., 1994	cyclin H	Fisher and Morgan, 1994; Mäkelä et al., 1994
cdk 8	Tassan et al., 1995	cyclin C	Tassan et al., 1995
cdc2-related kinases			
PCTAIRE-1	Meyerson et al., 1992; Okuda et al., 1992		
PCTAIRE-2	Meyerson et al., 1992		
PCTAIRE-3	Meyerson et al., 1992; Okuda et al., 1992		
KKIALRE	Meyerson et al., 1992		
PITSLRE (p58 ^{GTA})	Bunnell et al., 1990; Meyerson et al., 1992; Xiang et al., 1994		
PISSLRE	Graña et al., 1994b; Brambilla and Draetta, 1994		
PITALRE	Graña et al., 1994a		

Figure 1.1 Schematic diagram depicting the four phases of the eukaryotic cell cycle.

G₁ is the gap phase separating the previous mitosis (M phase) and DNA synthesis (S phase).

During G₁ daughter cells are responsive to mitogenic signals from growth factors and they monitor their environment and size before passing the restriction point. Passing the restriction point commits a cell to DNA replication and progression through the cell cycle without further need for growth factor stimulation. Cells that do not pass the restriction point either become quiescent or terminally differentiated, entering a phase referred to as G₀. Cells with damaged and unrepaired DNA may undergo apoptosis prior to the restriction point, preventing replication of damaged DNA and the risk of transformation. G₂ is the gap phase that separates the completion of DNA synthesis in S phase from initiation of mitosis. During G₂ cells monitor their size, DNA quality and content to ensure that DNA replication is complete and correct before cell division begins.

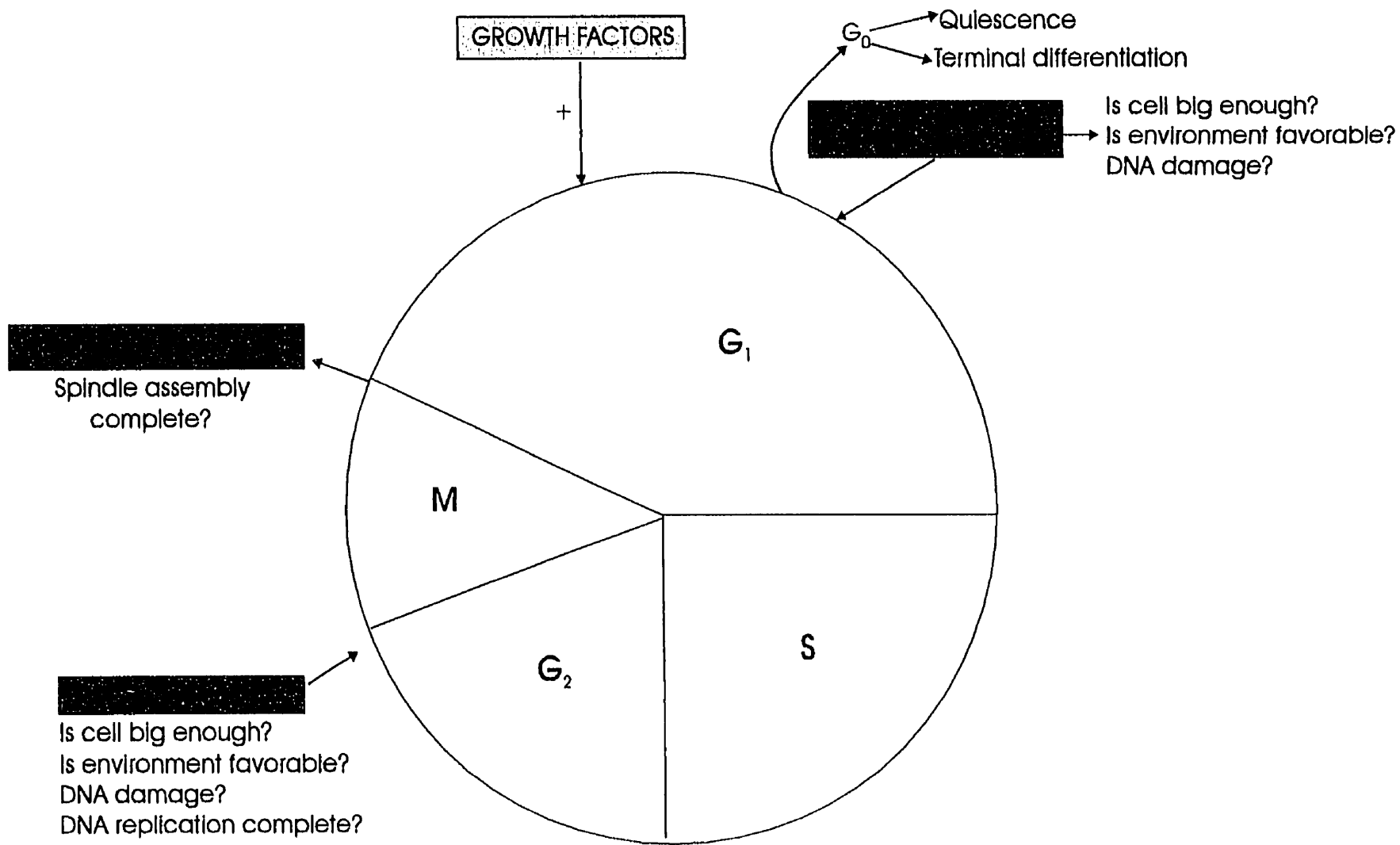
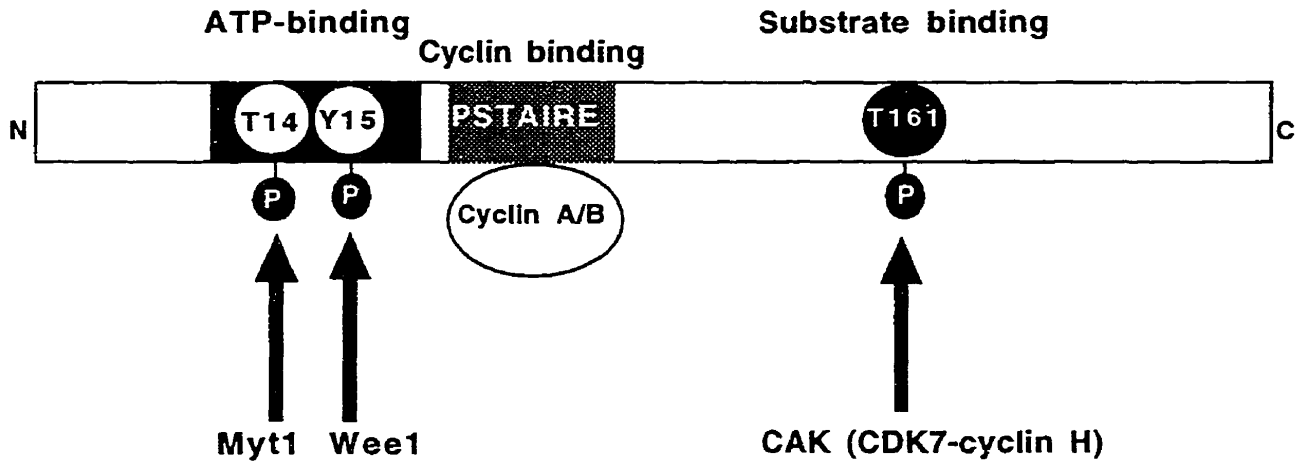


Figure 1.2 The posttranslational modifications of active and inactive p34^{cdc2} kinase.

The formation of p34^{cdc2}-cyclin B complexes late in S phase induces inhibitory phosphorylation of p34^{cdc2} on T14 and Y15 by Myt1 and Wee1 kinases, and phosphorylation of T161, by the CDK-activating kinase CAK (CDK7-Cyclin H) which is a positive regulator of p34^{cdc2} kinase activity. The p34^{cdc2}-cyclin B complex is phosphorylated on these three residues throughout G₂. Dephosphorylation of T14 and Y15 by the dual-specificity phosphatase CDC25C results in the abrupt activation of p34^{cdc2}-cyclin B at the onset of mitosis.

INACTIVE p34^{cdc2} (G₂ phase)



ACTIVE p34^{cdc2} (M phase)

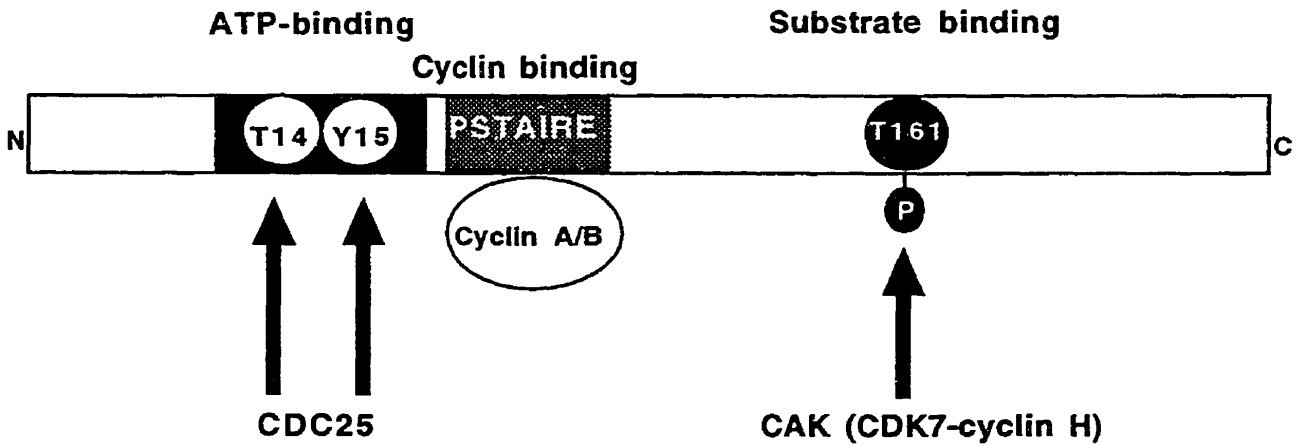
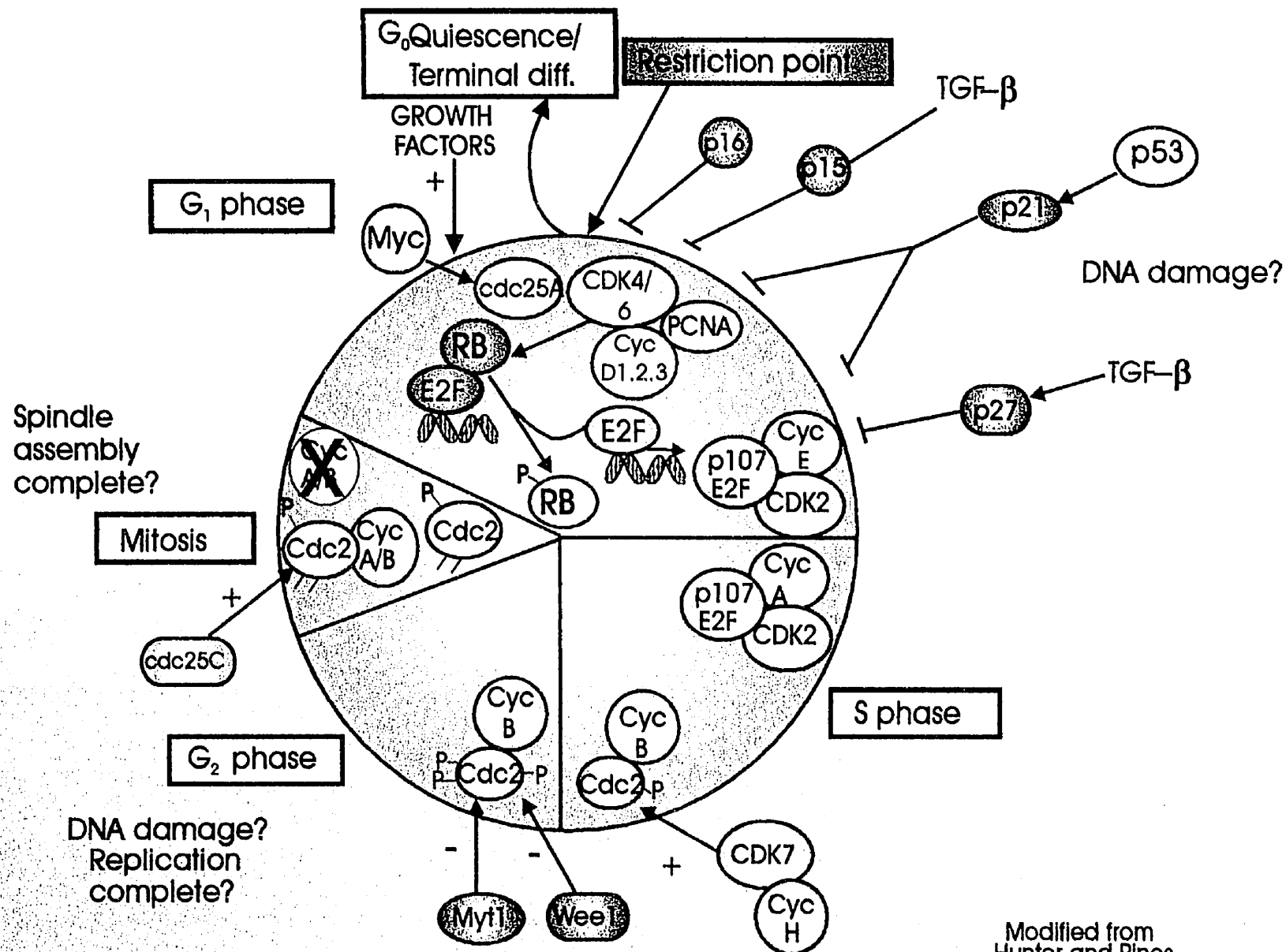


Figure 1.3 A schematic representation of the eukaryotic cell cycle “engine”.

In this diagram proteins depicted in red are negative regulators of cell cycle progression and proteins shown in green are positive regulators. During G₁ expression of D-type cyclins is inducible by mitogens and CDK4- or CDK6-cyclin D complexes accumulate. The commitment to DNA synthesis and cell division at the restriction point, involves the activation of CDK4/6-cyclin D kinases which may drive the cell through the restriction point by phosphorylating and inactivating the growth suppressive activity of pRB. Phosphorylation of pRB disrupts its association with the transcription factor E2F, allowing E2F to activate transcription of genes required for DNA replication. The CDK4/6-cyclinD complexes are subject to negative regulation by the CDKI proteins of the INK4 family (p15, p16, p18, p19) and p21^{CIP1}, which can halt progression through G₁ by inhibiting the activities of CDK4/6-cyclinD complexes. At the G₁ checkpoint the cell monitors itself for DNA damage. In response to DNA damage the tumor suppressor/transcription factor p53 induces expression of p21^{CIP1}, which can inhibit the kinase activities of CDK4/6-cyclinD complexes. Late in G₁ CDK2-cyclin E complexes associate with and may phosphorylate pRB and the RB-related protein p107 to promote progression through the G₁-S phase transition by modulating E2F activity. The CDK2-cyclin E complexes are also subject to negative regulation by the CDKI protein p27. CDK2-cyclin A activity in S phase is required for DNA replication and progression through S phase and the effects of this kinase activity may be mediated through phosphorylation of p107 and regulation of E2F transcriptional activity. Late in S phase and in G₂, inactive p34^{cdc2}-cyclin B complexes that are phosphorylated on T14, Y15 and T161 accumulate in an inactive state. At the G₂ checkpoint the cell monitors its size, DNA quality and DNA content before progressing to mitosis. The dephosphorylation of T14 and Y15 by CDC25C results in the activation of p34^{cdc2}-cyclin B and the onset of mitosis. Phosphorylation of several structural proteins and histone proteins by p34^{cdc2} may be responsible for the major structural changes and chromosome condensation that are characteristic of mitosis.



Modified from
Hunter and Pines
1994 Cell 79: 573-582

Figure 1.4 Developmentally regulated expression of mammalian cdc2 and CDK5 mRNA and histone H1 kinase activities.

Prior to neurogenesis cdc2 mRNA and protein are abundantly expressed in neuronal precursor cells. Downregulation of cdc2 occurs during neurogenesis (E16-20, rodent cortex) and cdc2 expression and kinase activity in postnatal and adult brain remains very low. In the rodent cortex expression of CDK5 mRNA, protein and kinase activity begin at approximately E11-12 and progressively increase throughout development .

Representation of cdc2 expression and activity is from Hayes et al., 1991 and Tsai et al.,1993.

Representation of CDK5 expression and activity is from Tsai et al., 1993 and Ino et al., 1994.

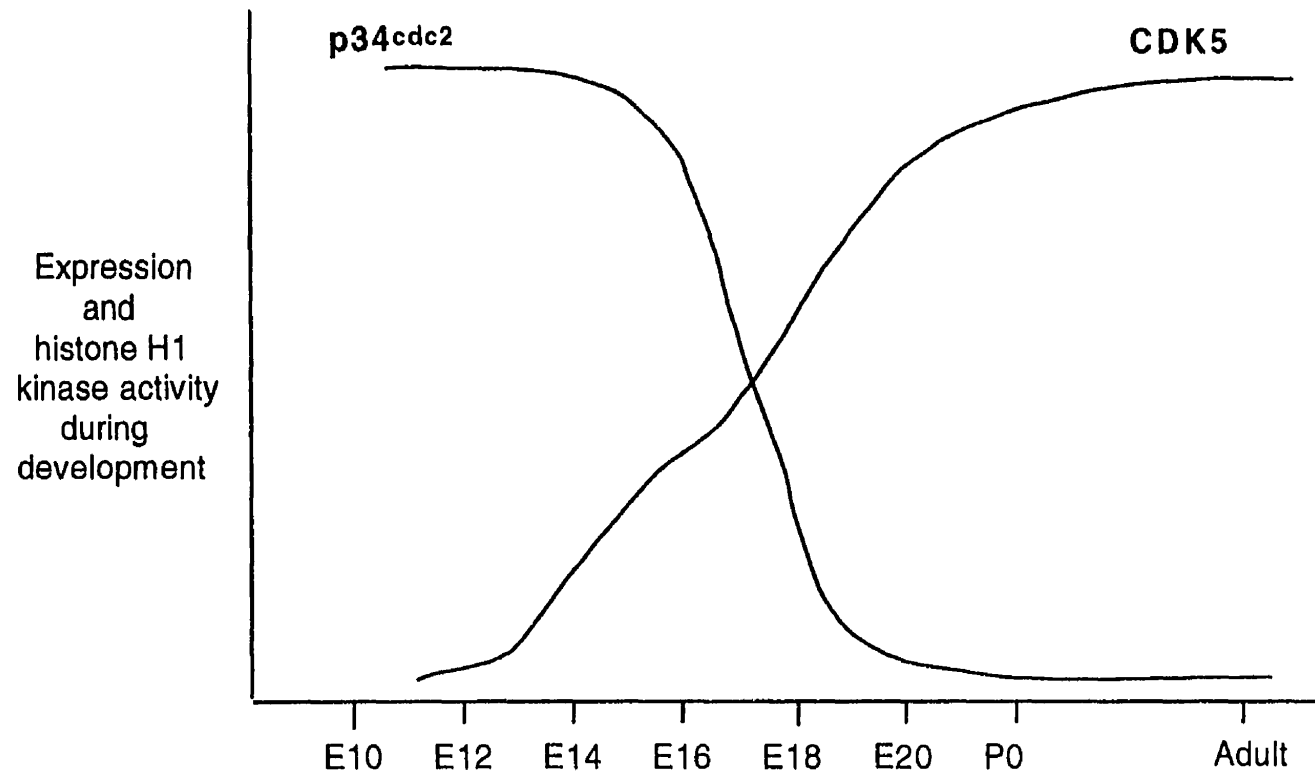
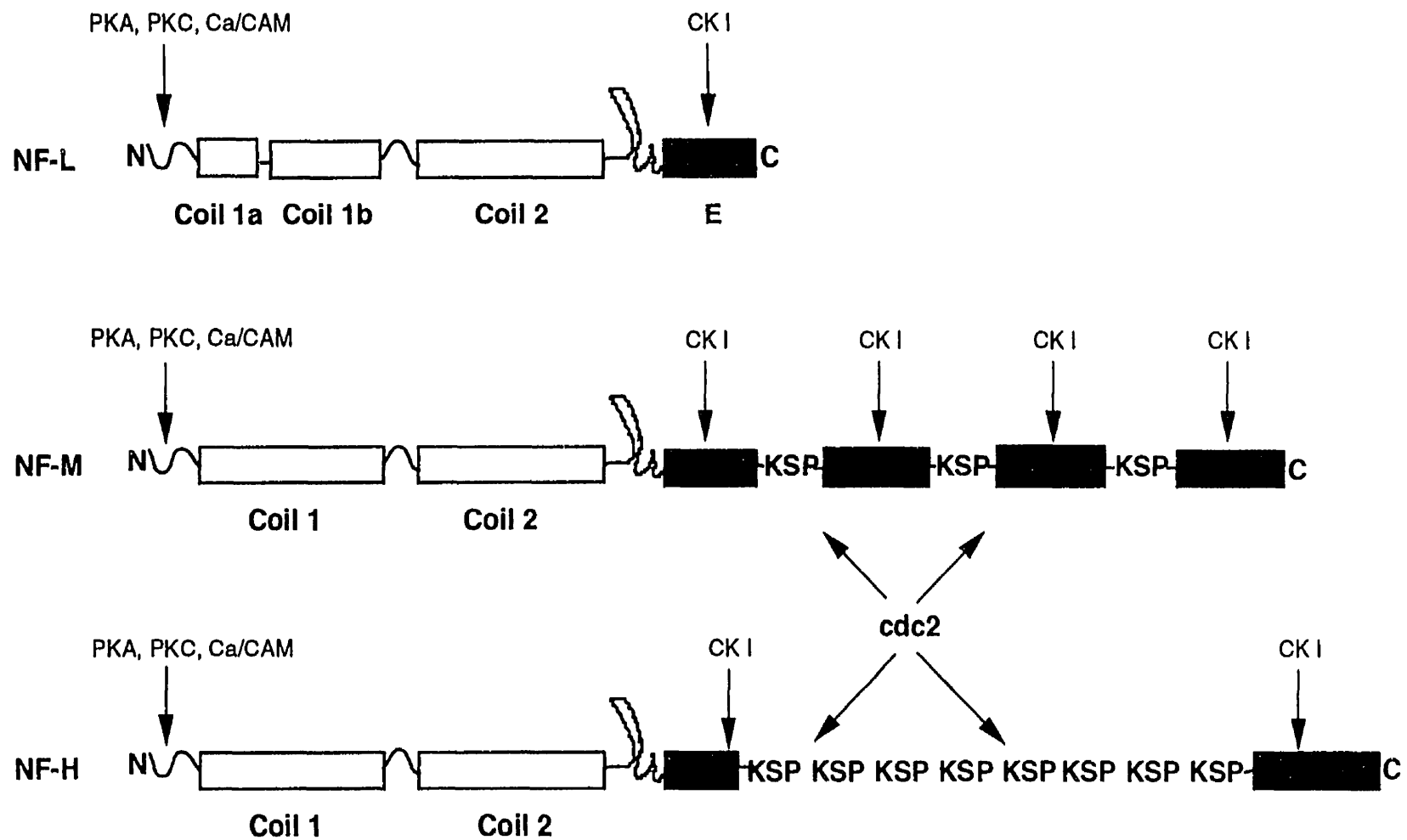


Figure 1.5 Locations of potential phosphorylation sites in the neurofilament subunits.

All three neurofilament proteins, NF-L (68 kDa), NF-M (95 kDa) and NF-H (115 kDa) contain α -helical rod domains (Coil 1a, 1b, and 2) separated by non-helical spacers or linkers and E segments which are rich in glutamic acid (solid boxes). NF-M and NF-H contain several repeats of lysine-serine-proline (KSP) in their carboxy-terminal tail domains. Based on the identification of consensus sequences, potential sites of phosphorylation occur in the amino-terminal regions of all three subunits (cAMP dependent protein kinase, protein kinase C and Ca^{2+} /CAM kinase). The consensus sequence for phosphorylation by casein kinase I occurs in the glutamic acid rich regions of all three neurofilament proteins and multiple p34^{cdc2} consensus sequences are located within the KSP repeats in the carboxy-terminal tail regions of NF-M and NF-H.



Adapted from:
 Pant and Veeranna 1995 Biochem. Cell Biol. 73: 575-592

CHAPTER 2
MATERIALS and METHODS

2.1 Introduction

This project involved the characterization of a new gene and many of the reagents or "tools" required to complete this work needed to be generated from the cloned cDNA. The chapter on Materials and Methods details how these reagents were generated and provides the methodology for the experiments using these reagents, presented in this thesis. Several oligonucleotides that were synthesized for the purposes of cloning, DNA sequencing and plasmid subcloning are described in this chapter. Also described here are several plasmids that have been constructed with this new cDNA for the purposes of 1) producing cDNA probes for DNA and RNA blot analysis and in situ hybridization, 2) *in vitro* transcription and translation, 3) producing bacterial fusion proteins and 4) transfection experiments. The characterization of the protein kinase encoded by the PFTAIRE cDNA required the use of antibodies that were raised against peptide sequences predicted from the cloned cDNA and they are described in this chapter. These reagents will be useful for further characterization of the PFTAIRE kinase.

2.2 A mouse cdc2 kinase cDNA probe made by reverse transcription and PCR

The oligonucleotides cdc609 (CCGTCGTAACCTGTTGAGTAAC (5')) and cdc608 (CGTCTGTCCCTGTAGGTAGGTC (3')) spanning nucleotides derived from the mouse cdc2 kinase cDNA (Figure 2.1 and Table 2.1) were used for reverse transcription and the polymerase chain reaction (PCR) to generate a cDNA encoding mouse p34^{cdc2} kinase. For reverse transcription reactions, 2 µg of total RNA extracted from mouse spleen was combined with 1 x Vent polymerase buffer, containing 10 mM KCl, 20 mM Tris-HCl pH 8.8, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-100 (New England Biolabs), 1 unit RNAGuard RNAase inhibitor (Pharmacia), 500 µM dNTPs (Pharmacia), 2 µM of the 3' oligonucleotide and 10 units AMV reverse transcriptase (Pharmacia). Reactions were incubated at 37°C for 30', diluted 2.5-fold with 1 X Vent polymerase buffer and heated to 95°C for 5 min. prior to starting the PCR. For amplification of the cDNA by PCR 5' and 3' oligonucleotides (2 µM final concentration) and 1 unit of Vent polymerase were added to each reaction. Conditions for the PCR were 30 cycles of 94°C for 1 min., 60°C for 3 min. and 72°C for 5 min.

The 943 bp mouse cdc2 kinase PCR product, contained 23 bp of 5' untranslated sequence, 894 bp of coding sequence and 26 bp of 3' untranslated sequence. The PCR

product was purified, phosphorylated at the 5' ends (Sambrook et al., 1989) and inserted into the *Sma*I site of the pBluescript SK+ plasmid vector (Stratagene). Restriction digests with *Hind*III and *Pst*I produced fragments of the expected sizes; 580 bp for *Hind*III, 225 bp and 717 bp for *Pst*I. Partial sequence analysis of clones confirmed the identity of the PCR product as *cdc2*.

2.3 Low stringency screening of a mouse brain cDNA library

The mouse brain cDNA library, LGM 1, prepared from mRNA isolated from brains of 18 day old NIH-Swiss mice, was a gift from Dr. R.A. Lazzarini. The cDNA library was prepared in the λ gt11 bacteriophage cloning vector and propagated in the host *E. coli* bacterial strain Y1090.

Approximately 5×10^5 clones were plated to screen for nervous system-specific *cdc2*-related genes. Filter replicates of the plated clones were screened with the cDNA probe encoding the open reading frame of mouse *cdc2* kinase, labelled by random priming (Feinberg and Vogelstein, 1983) with [α - ^{32} P]dATP and purified on Sephadex G50 (Pharmacia). Hybridization was for 16 hours using low stringency conditions at 55°C in 5xSSC (750 mM NaCl, 85 mM sodium citrate, pH 7.0), 5xDenhardt's solution (0.1% Ficoll, 0.1% polyvinyl pyrrolidone, 0.1% bovine serum albumin), 5mM EDTA, 25mM sodium phosphate, 0.1% sodium pyrophosphate, 1% sodium dodecyl sulfate (SDS), 100 μ g/ml sonicated and denatured salmon sperm DNA and 500 ng/ml *Hinf*I digested λ DNA. Hybridized filters were washed in 2xSSC-0.1% SDS at room temperature for 10 min.; 2xSSC-0.1%SDS at 55°C twice for 15 min.; and 1xSSC-0.1%SDS at 55°C once for 10 min. Filters were exposed to film (Kodak X-OMat AR) with intensifying screens at -80°C overnight and then re-exposed for 96 hours.

A positive clone obtained in the primary screening was purified after two additional rounds of screening using the conditions described above. Lambda bacteriophage particles from isolated clones were eluted from agarose plugs in SM buffer (100 mM NaCl, 10 mM tris, pH 7.4, 10 mM MgSO₄) and DNA was prepared from the purified clones by propagation in the Y1090 host strain in liquid cultures (Sambrook et al., 1989). Lambda bacteriophage DNA from a purified clone was digested with *Eco*RI to release a 2.5 kb cDNA insert for subcloning into plasmid vectors.

2.4 Plasmid subcloning

a. pBluescript SK+ plasmid subclones for sequencing and cDNA probes

The 2.5 kb *EcoRI* cDNA insert isolated from the purified lambda clone was ligated to the pBluescript SK+ vector (Stratagene) that had been digested with *EcoRI* and dephosphorylated by treatment with calf intestinal alkaline phosphatase (Sambrook et al., 1989). The ligation reactions were used to transform the DH5 α strain of *E. coli* bacteria made competent for transformation with plasmid DNA (Chung et al., 1989). The DNA obtained from ampicillin-resistant plasmid subclones was used for restriction digest analysis (Refer to Figure 2.2 for plasmid map.). To sequence the 5' and 3' ends of the 2.5 kb cDNA clone, the Bluescript subclone was used as a DNA template with T7 and T3 oligonucleotide primers and the Sequenase DNA sequencing kit obtained from United States Biochemicals.

Restriction digestion of the 2.5 kb cDNA clone with *HindIII* yielded fragments of 524 bp, 650 bp, 482 bp and 740 bp which were all subcloned into pBluescript SK+. Plasmid maps of the *HindIII* subclones are shown in Figure 2.3. Each *HindIII* subclone was sequenced using T7 and T3 oligonucleotide primers. Additionally, subclones containing unidirectional deletions extending into the full length cDNA were generated for sequencing by exonuclease III/S1 nuclease digestion (Henikoff, 1984) using the Erase-a-Base System (Promega). Specific internal oligonucleotide primers were also made for sequencing some regions of the cDNA clone to obtain complete sequence from both strands of the cDNA. Sequence analysis was performed using the BLAST network service of the NCBI (U.S.A.), Intelligenetics programs, DNA Star and PROSITE programs.

The first 290 bp of the 2.5 kb cDNA is an *EcoRI-DraI* fragment which includes 118 bp of 5' untranslated sequence and 172 bp of amino-terminal coding sequence. This fragment was inserted into *EcoRI-SmaI* digested pBluescriptSK+ plasmid (Figure 2.5). This subclone, pPF290ED, was used to make cDNA probes for Southern blots, Northern blots and in situ hybridizations.

b. Bacterial expression plasmid constructs

In order to express PFTAIRE in bacteria, two plasmid constructs were prepared using the PFTAIRE cDNA and a glutathione S-transferase (GST) bacterial expression plasmid. The expression of proteins in bacteria as GST fusion proteins was first described by Smith and Johnson (Smith and Johnson, 1988) and is now a widely used technique. The expression of

fusion proteins in this plasmid system is inhibited by the presence of the *lac19* repressor which binds to the *lac* promoter controlling expression of the fusion protein. The addition of an inducer, isopropylthiogalatoside (IPTG), to growing cultures in mid-log phase displaces the repressor allowing expression of the fusion protein.

The two GST-PFTAIRE constructs encoded fusion proteins containing 1) the entire open reading frame of PFTAIRE and 2) an amino-terminal peptide of PFTAIRE spanning amino acids 1-68. Subcloning PFTAIRE cDNA fragments into the GST bacterial expression plasmid, pGEX1 λ T (Pharmacia) required that each fragment contain only coding sequence and that the sequences were subcloned in frame between the sequence encoding GST and a termination codon in the vector. To facilitate the ligation of the PFTAIRE cDNA fragments to the pGEX1 λ T plasmid, 5' and 3' PFTAIRE oligonucleotides were synthesized with *Eco*RI restriction sites so that PCR generated PFTAIRE cDNA fragments could be inserted at the *Eco*RI cloning site of the pGEX1 λ T plasmid vector. The sequences of oligonucleotides used for subcloning are presented in Table 2.1 and their locations in the PFTAIRE cDNA are shown in Figure 2.4. The PCR was performed using the following conditions: 10 ng of PFTAIRE-SK+ plasmid DNA template was combined with 1 x Vent polymerase buffer, containing 10 mM KCl, 20 mM Tris-HCl pH 8.8, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-100 (New England Biolabs), 500 μ M dNTPs, 2 μ M of each oligonucleotide, and 0.5 unit Vent polymerase (New England Biolabs) for 29 cycles of 94°C for 30 sec., 55°C for 3 min. and 72°C for 5 min.. The PCR products were digested with *Eco*RI and ligated to the *Eco*RI digested pGEX1 λ T plasmid vector. Ampicillin-resistant clones with inserts in the proper orientation were sequenced to determine that the PFTAIRE fragments had ligated in frame with the GST sequence and termination codon. Plasmid maps for the GST fusion protein constructs named PGPf19 (entire PFTAIRE coding sequence) and PGN5 (N-terminal PFTAIRE) are presented in Figure 2.6A and C.

A mutation made in PFTAIRE for expression of the inactive kinase by the pcDNA3 plasmid vector (described in Section 2.4c) was also subcloned into the pGEX1 λ T plasmid. The mutation lies within the 650 bp *Hind*III fragment of PFTAIRE. The PGPf19 plasmid was digested with *Hind*III to remove the 650 bp wild type *Hind*III fragment and the 650 bp *Hind*III fragment containing the D228N mutation (excised from the pc3D228N plasmid construct, see below) was ligated to the prepared PGPf19 plasmid. Restriction digests and DNA sequencing were used to identify a

clone, PGD228N, containing the mutated fragment in the correct orientation.

c.Eukaryotic expression plasmid constructs

pcDNA1 constructs

To over-express the PFTAIRE cDNA in a wide variety of mammalian cell lines, the cDNA was subcloned into plasmid vectors containing the human cytomegalovirus (CMV) promoter. For transient expression in cell lines, the entire 2.5 kb PFTAIRE cDNA was inserted into the pcDNA1 plasmid vector (Invitrogen) at a unique *EcoRI* cloning site following the CMV promoter. Plasmid maps of pcDNA1 clones containing the PFTAIRE cDNA in the sense (pcPF) and antisense (pcPF-AS) direction are shown in Figure 2.7.

pcDNA3 constructs: Wild type PFTAIRE and a PFTAIRE mutant, D228N

For the selection of stable transfectants and for transient expression in cell lines the entire 2.5 kb PFTAIRE cDNA was inserted into the pcDNA3 plasmid vector, which contains the neomycin-resistance selection marker expressed from the SV40 early promoter (Invitrogen). The PFTAIRE cDNA was ligated to pcDNA3 at the unique *EcoRI* cloning site following the CMV promoter. Plasmid maps of pc3PF and pc3PF-AS clones containing the PFTAIRE cDNA in the sense and antisense directions are shown in Figure 2.8.

A dominant negative mutation of PFTAIRE was generated to use as a "kinase inactive" control for kinase assays with *in vitro* translated proteins and for transient transfections. A point mutation was made using the Altered Sites II mutagenesis system (Promega) to change Asp²²⁸ to an asparagine residue. This residue is conserved in all protein kinases and its mutation in cAMP-dependent kinase, cdc2, CDK2, and CDK3 has produced "kinase inactive" mutants that lead to dominant negative inhibition of endogenous kinase activities when over-expressed (Taylor et al. , 1993; van den Heuvel and Harlow, 1993). To generate the point mutation in the Mg²⁺-ATP binding region of PFTAIRE, an oligonucleotide (PF2701A) derived from the PFTAIRE cDNA sequence changing guanosine 800 to adenosine was synthesized for the mutagenesis reaction. (Refer to Table 2.1 for PF2701A sequence and Figure 2.4 for the location of PF2701A in the PFTAIRE cDNA.) This mutation in the cDNA sequence results in the translation of an asparagine rather than aspartate at residue 228 of PFTAIRE. For the mutagenesis reaction the PFTAIRE cDNA was subcloned in the pALTER-1 plasmid vector, which contains genes for ampicillin and tetracycline resistance that have been inactivated and enables preparation of a single-stranded DNA template of the subcloned cDNA. The PFTAIRE oligonucleotide and an ampicillin repair

oligonucleotide were annealed simultaneously to single-stranded PFTAIRE-pALTER-1 template DNA to prime synthesis of the PFTAIRE mutant strand and restore ampicillin resistance to mutant clones. The mutant cDNA strand was synthesized with T4 DNA polymerase and ligated. The mutant PFTAIRE, named D228N, was excised from the pALTER-1 plasmid and inserted at the *EcoRI* site of the pcDNA3 plasmid. The map of the PFTAIRE mutant plasmid, pc3D228N, is shown in Figure 2.8

PFTAIRE and D228N open reading frame constructs

A low level of transfection efficiency of pc3PF and pc3D228N by various transfection methods in several cell lines, suggested the possibility that the 5' and 3' untranslated regions of PFTAIRE in these plasmid constructs may have an inhibitory effect on translation, resulting in what appeared to be low transfection efficiency. To eliminate the possibility of translational control by 5' and 3' untranslated sequences (Sachs, 1993), pcDNA3 plasmids were constructed that contained only the coding sequences of PFTAIRE and D228N. These plasmids were constructed using the PGPF19 and PGD228N plasmids, which contained only coding sequence. The pcDNA3 plasmid vector contains the polyadenylation signal from bovine growth hormone (see plasmid maps in Figure 2.8) so removal of the PFTAIRE polyadenylation signal was not expected to alter transcription of the subcloned cDNAs. The entire PFTAIRE or D228N coding sequence (1.272 kb) was excised from the pGEX1 λ T plasmid constructs and ligated to the pcDNA3 plasmid. The subclones containing only coding sequence are referred to as pc3PF $_{orf}$ and pc3D228N $_{orf}$.

pHermes-L/PF amplicon construct, a PFTAIRE-lacZ expression vector

The entire 2.5 kb PFTAIRE cDNA was subcloned into the HSV-1 (herpes simplex virus 1) derived vector, pHermes-L (constructed by Dr. M.E.Fotaki and J.Czemohorsky, gift of Dr. R.Dunn, McGill University, Centre for Research in Neuroscience). The pHermes-L vector contains plasmid DNA (pGem7Z) enabling propagation and selection in bacteria, an HSV-1 origin of DNA replication for replication in mammalian cells and an HSV-1 packaging signal. The pHermes-L vector also contains two promoters (CMV and SV40) in opposite orientations for controlling transcription of two different genes. The CMV promoter controls expression of the subcloned cDNA, while the SV40 promoter controls expression of the lacZ gene. The presence of the lacZ gene enables rapid screening of transfectants and determination of virus titers during the process of generating viral stocks, by staining fixed cells with the β -galactosidase substrate X-gal (Life

Technologies). When pHermes-L DNA is introduced into cultured Vero cells by transfection and transfected cells are infected with HSV-1 helper virus, the pHermes-L DNA is packaged within Herpes virions. Virions produced in the cells contain either helper virus DNA or pHermes-L DNA and are collected from the medium and lysed cells and stored as a virus stock. After transfection of new cultures with the pHermes-L vector the cells are infected with the virus stock collected from the first round of transfection-infection. Once virus stocks with a high ratio of pHermes-L DNA to helper virus DNA are obtained these stocks may be used for infecting mammalian cells in culture or *in vivo* (Breakefield and DeLuca, 1991).

The pHermes-L plasmid was modified for subcloning by inserting an oligonucleotide adaptor containing *Hind*III, *Sal*I and *Eco*RI restriction sites at the unique *Hind*III cloning site. The *Sal*I site was unique in the pHermes-L vector and used as the cloning site. *Xho*I adaptors were ligated to the 2.5 kb PFTAIRE cDNA fragment for insertion at the *Sal*I cloning site in pHermes-L. Restriction digest analysis with *Hind*III identified subclones containing the PFTAIRE cDNA. Orientation of the PFTAIRE insert was determined by sequence analysis. The map of the final construct, pHermes-L/PF, is shown in Figure 2.9.

Ultimately the production of virus stocks containing a high level of PFTAIRE cDNA relied on a high transfection efficiency of the pHermes-L constructs in the Vero cell line. Relative to the transfection efficiency obtained when pHermes-L was transfected in Vero cells (~20-50%), the efficiency obtained with pHermes-L/PF transfections was extremely low (<5%), precluding its use as a tool for producing virus stocks.

2.5 Genomic DNA analysis

a.Southern blot analysis

Genomic DNA was prepared from mouse tail biopsies after digesting the tissue in a buffer of 100 mM NaCl, 10 mM Tris pH 8.0, 25 mM EDTA, 0.5% SDS and 100 µg/ml proteinase K overnight at 50°C (Gross-Bellard et al., 1973). Genomic DNA (10 µg) was digested with several restriction endonucleases and fractionated by electrophoresis on 0.8% agarose gels, containing ethidium bromide, in a 1x Tris-borate (TBE) buffer (Sambrook et al., 1989). Southern blots were prepared by alkaline transfer of DNA from agarose gels to Hybond N⁺ (Amersham) nylon filter membranes with a 0.4 N NaOH solution (Sambrook et al., 1989). Filter membranes were pre-hybridized for 4-6 h at 65°C in a buffer containing 5xSSC (750 mM NaCl, 85 mM sodium citrate, pH

7.0), 5xDenhardt's solution (0.1% Ficoll, 0.1% polyvinyl pyrrolidone, 0.1% bovine serum albumin), 5 mM EDTA, 25 mM sodium phosphate, 0.1% sodium pyrophosphate, 1% sodium dodecyl sulfate (SDS), and 200 µg/ml denatured and sonicated salmon sperm DNA. cDNA probes (2.5 kb PFTAIR, 290 bp 5' PFTAIR *EcoRI-DraI* fragment and 740 bp *HindIII* fragment) were labelled by random priming with [α - ^{32} P]dATP, denatured and diluted in fresh buffer for hybridization overnight (16-18 hours) at 65°C. After hybridization the membranes were washed as follows: once for 5 min. at room temperature in 2xSSC-0.1% SDS, once for 10 min. at 60°C in 2x SSC-0.1% SDS, once for 15 min. at 60°C in 0.5x SSC-0.1% SDS and once for 10 min. at 60°C in 0.1x SSC-0.1% SDS. Membranes were exposed to Kodak XAR or Kodak Biomax MR film at -80°C with intensifying screens.

b. Detection of restriction fragment length polymorphisms

The 290 bp PFTAIR cDNA probe (*EcoRI-DraI* fragment) which shows little homology to other CDKs and *cdc2*-related kinases, was used for Southern blot analysis to identify PFTAIR restriction fragment length polymorphisms in genomic DNA from the mouse strains C57BL/6JEi and SPRET/Ei. Genomic DNA from each strain was digested with *MspI*, *PvuII*, *EcoRI* and *TaqI*. The 94 progeny of a BSS backcross panel ((C57BL/6JEi x SPRET/Ei) F_1 x SPRET/Ei), The Jackson Laboratory, Bar Harbor, ME) were screened for inheritance of PFTAIR *TaqI* polymorphic fragments by hybridizing Southern blots prepared from *TaqI* digested genomic DNA obtained from the animals with the 290 bp PFTAIR cDNA probe. The inheritance of the PFTAIR *TaqI* polymorphic fragments was compared with the inheritance of approximately 2300 markers that have been typed with the BSS backcross panel in order to determine the chromosomal location of the PFTAIR gene.

2.6 RNA expression

a. Northern blot analysis

For analysis of mRNA expression during development, embryos were removed from timed pregnant C3H mice on days 12.5 and 15.5 of gestation. All embryos and dissected brain tissue from postnatal pups and adults were frozen immediately in liquid nitrogen. For analysis of mRNA tissue distribution organs were dissected from adult mice and immediately frozen in liquid nitrogen. Total RNA was extracted from total embryos (gestation day 12.5), whole heads (gestation day 15.5) and dissected tissues following homogenization in a guanidinium

thiocyanate buffer (Chomczynski and Sacchi, 1987). RNA samples (20 µg) containing ethidium bromide were fractionated by electrophoresis on 1% agarose-formaldehyde gels (Sambrook et al., 1989). After electrophoresis gels were transferred to a Hybond N⁺ membrane (Amersham) using 10x SSC as the transfer solution. UV crosslinking was used to covalently bind RNA to the nylon membrane.

Northern blots were hybridized with PFTAIRE cDNA probes (2.5 kb PFTAIRE cDNA, the 5' 290 bp PFTAIRE fragment or a 740 bp *HindIII* PFTAIRE fragment from the 3' untranslated region) labelled with [α -³²P]dATP by random priming. Membranes were pre-hybridized for 2-6 hours at 42°C in 50% formamide, 1M NaCl, 50 mM Tris pH 7.5, 0.2% Ficoll, 0.2% polyvinyl pyrrolidone, 0.1% sodium pyrophosphate, 1% SDS and 200 µg/ml sonicated and denatured salmon sperm DNA. Denatured probes, diluted to 1-2 x 10⁶ cpm/ml, were added to pre-hybridization buffer for overnight (16 hours) hybridizations at 42°C. Blots were washed in 2xSSC-0.1% SDS at room temperature for 10 min., twice for 10 min. in 2xSSC-0.1% SDS at 65°C, once for 15 min. in 0.5xSSC-0.1% SDS at room temperature and once for 10 min. in 0.1xSSC-0.1% SDS at 65°C. Blots were exposed to film (Kodak, X-OMat AR or Biomax MR) with intensifying screens for 18 hours at -80°C. Some blots were stripped and re-hybridized with a mouse β -actin cDNA probe under the same conditions, to determine that equivalent amounts of total RNA were present in all lanes. Following hybridization with β -actin two additional washes in 0.1xSSC-0.1% SDS for 15 min. at 65°C were done and blots were exposed to film (Kodak, XRP) for 5-6h at -80°C.

b. In situ hybridization

Anesthetized adult C3H mice were perfused through the left ventricle with 4% phosphate-buffered paraformaldehyde. Tissues were dissected, post-fixed in 4% phosphate-buffered paraformaldehyde for 1-2 hours, placed in 15% sucrose overnight at 4°C and then frozen in OTC embedding medium (Miles) in -80°C isopentane. Sections (10 µm) were cut on a cryostat, thaw-mounted on to ProbeOn (Fisher) slides and stored at -80°C until hybridization. Prior to hybridization, slides were placed in 95% ethanol at -80°C, rehydrated in 80%, 70% and 50% ethanol at room temperature and rinsed in diethyl pyrocarbonate treated water. Sections were digested in 5 µg/ml proteinase K for 15 min. at 37°C, followed by rinses in 1xTE buffer and 2xSSC at room temperature. Control sections were treated with 50 µg/ml RNAase A for 1 hour at 37°C and rinsed in 2xSSC, prior to pre-hybridization. Sections were pre-hybridized in buffer

containing 50% formamide, 600 mM NaCl, 20 mM Tris pH 7.5, 1 mM EDTA, 0.2 % Ficoll, 0.2 % polyvinyl pyrrolidone, 0.1% BSA, 10 mM DTT, 500 µg/ml sonicated and denatured salmon sperm DNA and 50 µg/ml denatured yeast tRNA for 1 hour at 45°C. Hybridizations were performed with cDNA probes labelled by random priming with 50µCi each [$\alpha^{35}\text{S}$]-dATP and [$\alpha^{35}\text{S}$]-dCTP (1000 Ci/mmol; Amersham) per reaction (Ausabel et al., 1992) and purified with Sephadex G50 to remove unincorporated nucleotides. Hybridization buffer was the same as pre-hybridization buffer except that dextran sulfate was added to a final concentration of 10% (w/v), the concentration of salmon sperm DNA was reduced to 200 µg/ml and denatured probes were added at a dilution of approximately 2.5×10^6 cpm per slide. Hybridizations were performed overnight, for approximately 18 hours, under sealed coverslips in a humidified box at 45°C. Following hybridization, sections were washed over a period of 6 hours as follows: 2xSSC for 2 hours (4 changes) at room temperature ; 1xSSC for 2 hours (4 changes) at room temperature; 0.5xSSC for 1 hour (3 changes) at room temperature; 0.5xSSC for 1h (3 changes) at 45°C and in 0.1xSSC for 10 min. at room temperature. Slides were rinsed in 70% ethanol and exposed to Kodak XAR-5 film for 48 hours at room temperature. Slides were dipped in Kodak NTB2 emulsion and exposed for eight to ten days prior to developing. After developing, slides were counterstained with a dilute cresyl violet solution to reveal cell structure. Slides were dehydrated and mounted with Permount for observation using a Leitz microscope equipped with dark and bright field optics.

In situ hybridizations to examine PFTAIRE mRNA expression were performed using cDNA probes made from either the 5' 290 bp PFTAIRE fragment from the pPF290ED plasmid or a *HindIII* fragment spanning nucleotides 1-523 (H312PFTAIRE-SK+ plasmid, refer to Figure 2.3). The localization of neurofilament-L (NF-L) mRNA was detected using a 300 bp *PstI* fragment, from the mouse NF-L cDNA (P. Liesi et al., 1986). To verify the specificity of in situ hybridizations with PFTAIRE and NF-L cDNA probes, each probe was hybridized with sections from mouse liver or skeletal muscle, which do not express these mRNAs. As an additional control for specificity of in situ hybridizations a 700 bp *EcoRI* fragment from the rat prolactin cDNA was hybridized with sections of brain, cerebellum and spinal cord, which do not express prolactin.

2.7 Protein analysis

a. Production of bacterial fusion proteins

GST-PFTAIRE bacterial fusion proteins were prepared following the method of Smith and Cocoran (Smith and Johnson, 1988; Ausubel et al., 1992). Small volume cultures were grown overnight from single bacterial colonies in LB medium supplemented with 100 µg/ml ampicillin (LBA) at 37°C with vigorous shaking. Overnight cultures were diluted ten-fold in fresh LBA and grown to approximately mid-log phase (optical density of $A_{600}=0.6-0.8$) for 1.5-2 hours. To induced expression of fusion proteins IPTG was added to cultures at a final concentration of 100 µM, and cultures were incubated an additional 3-4 hours at 37°C. The cells were collected by centrifugation and pellets were resuspended in 9 ml ice cold phosphate-buffered saline (PBS) pH 7.4 (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4) per one liter of culture . The cell suspension was kept on ice and sonicated to lyse cells. The lysate volume was measured and a volume of 10% Triton-X100 equal to one-ninth the lysate volume was added to give a final concentration of 1% Triton-X100. Following brief mixing the lysate was centrifuged for 5 min. at 10,000 x g at 4°C to pellet insoluble material and intact cells.

For batch purification of fusion proteins, the supernatants were added to 0.5 ml aliquots of a 50% glutathione-Sepharose 4B (Pharmacia) slurry, pre-equilibrated with PBS. The lysate and glutathione-Sepharose were mixed slowly on an end-over-end rotating mixer for 15 min. at room temperature to bind the fusion proteins to the matrix. After collecting the Sepharose beads by brief centrifugation at 500 x g, supernatants were removed and the beads were washed three times with 50 ml ice cold PBS. The fusion proteins were eluted in 5-6 fractions by adding 0.25 ml of 50 mM Tris-HCl pH 8.0/10 mM reduced glutathione (Sigma) and gently mixing for 5 minutes. Eluants were analyzed by electrophoresis on 10% SDS-polyacrylamide gels stained with Coomassie Blue to 1) compare amounts of fusion protein in each elution with the amount of insoluble fusion protein in the bacterial pellet and 2) to confirm that the fusion proteins migrated at the expected molecular mass.

b. Antibody preparation

Two polyclonal antibodies were raised in rabbits against amino- and carboxy-terminal regions of PFTAIRE, which have little homology to other known CDKs or cdc2-related kinases. The amino-terminal antibody, PGN5, was raised against a bacterial fusion protein of residues 1-68 of PFTAIRE, fused in frame to glutathione S-transferase. A peptide derived from residues 409-

422 near the carboxy-terminus of PFTAIRE was synthesized with a cysteine residue added at the carboxy-terminus to facilitate conjugation to the carrier protein (keyhole limpet hemacyanin, KLH) and used to raise a second PFTAIRE antibody (see Figure 2.10). For the primary immunization rabbits (~2-2.5 kg female New Zealand white) received subcutaneous injections of fusion protein or peptide mixed with complete Freund's adjuvant. Rabbits were boosted three times after the initial immunization with fusion protein or peptide mixed with incomplete Freund's adjuvant. Ten days after the final immunization polyclonal antisera were processed from collected blood (Harlow and Lane, 1988).

c. Antibody specificity

The specificity of the anti-PFTAIRE antibodies was determined by immunoprecipitation experiments using *in vitro* -translated PFTAIRE. PFTAIRE sense-strand mRNA was transcribed *in vitro* using the 2.5 kb PFTAIRE-pBluescript SK+ plasmid as a DNA template. The transcription reaction, containing 1x transcription buffer (40 mM Tris pH 7.9, 6 mM MgCl₂, 10 mM DTT, 2 mM spermidine), 2 units RNAGuard RNAase inhibitor, 500 µM ATP, CTP, UTP, 50 µM GTP, 500 µM m⁷G(5')ppp(5')G RNA cap analog (New England Biolabs), 5 µg BSA, 5 µg linearized PFTAIRE cDNA template and 2 units T3 RNA polymerase (Pharmacia), was incubated for 1 hour at 37°C. The DNA template was digested at the end of the transcription reaction by the addition of RNAase-free DNAase1 for 15 min. at 37°C, followed by phenol-chloroform extractions and ethanol precipitation. The mRNA was translated *in vitro* for 1 hour at 30°C in a 50 µl reaction containing 1-2 µg mRNA (heated 10 min. at 67°C), 1 unit RNAGuard, 20 µM amino acid mix minus methionine (Promega), 35 µl nuclease-free rabbit reticulocyte lysate (Promega) and 40-50 µCi ³⁵S-methionine (translation-grade, 1000 Ci/mmol, New England Nuclear). For immunoprecipitations the translation product was diluted and combined with either the carboxy-terminal antibody (PFC5), amino-terminal antibody (PGN5) or the appropriate pre-immune serum and protein A-Sepharose (Pharmacia). For control immunoprecipitations, antibodies were pre-incubated with their corresponding peptide antigens prior to immunoprecipitating the PFTAIRE translation product.

To verify that both antibodies recognized the same protein, lysates of COS7 cells transfected transiently with the pcPF PFTAIRE expression plasmid were used for immunoprecipitation and western blotting. Cells were lysed in ice cold 150 mM NaCl, 50 mM Tris,

pH 7.5, 5 mM EDTA, pH 8, 0.5% Nonidet P-40, 5 mM DTT, 1 µg/ml leupeptin, aprotinin, pepstatin and 1 mM PMSF. Lysed cells were kept on ice for 10 min., centrifuged at 12,000 x g in an Eppendorf microfuge and the protein concentrations of supernatants were determined by the Bradford assay. The cytosolic protein fractions (100 µg protein) were used for immunoprecipitations with pre-immune serum, the carboxy-terminal PFTAIRE antibody (PFC5) or the amino-terminal PFTAIRE antibody (PGN5). Proteins in immunoprecipitated samples were separated by electrophoresis on a 10% SDS-polyacrylamide gel, electrophoretically transferred to nitrocellulose membranes and immunoblotted with either PGN5 (1:350) or PFC5 (1:2500) as described in the next section.

d. Immunoblotting

For analysis of expression of PFTAIRE protein, tissues were dissected from adult C3H mice and frozen immediately in liquid nitrogen. Tissues were homogenized in ice cold buffer containing 150 mM NaCl, 50 mM Tris pH 8.0 and 1% Nonidet P-40 with either a mechanical Teflon tissue grinder or a Polytron tissue grinder. Homogenates were kept on ice for 10 min., centrifuged for 15 min. at 12,000 x g in an Eppendorf microfuge and the protein concentrations of supernatants (cytosolic protein fraction) were determined by the Bradford assay.

Immunoblotting was performed by 10% SDS-PAGE of samples in buffer containing 62.5 mM Tris pH 6.8, 2% SDS, 10% glycerol and 0.7 M β-mercaptoethanol (1 x SDS sample buffer) using a Biorad Mini Protean apparatus. Running buffer was 1 x Laemmli buffer (25 mM Tris, 192 mM glycine, 1% SDS). To verify that samples contained equal amounts of protein, gels were stained in a solution of Coomassie Blue (0.25% Coomassie Brilliant Blue G-250 in 25% isopropanol and 10% acetic acid) and destained in 10% isopropanol/10% acetic acid. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) with a Biorad Mini Transblot apparatus in a buffer of 25 mM Tris, 192 mM glycine and 20% methanol for 1.5 hours at 100V (constant voltage) at 4°C. Nonspecific binding sites on membranes were blocked overnight at 4°C in a solution of 7% dry milk and PBST (150 mM NaCl, 16 mM Na₂HPO₄·7H₂O, 4 mM NaH₂PO₄, pH 7.5 and .05% Tween-20). Membranes were incubated with diluted primary antibodies in 7% milk/PBST for 2 hours at room temperature. After several washes with PBST blots were incubated for 1 hour with either horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson Immunoresearch) or horseradish peroxidase-conjugated protein A (Sigma) diluted 1000-fold in 7% milk/PBST. Membranes were washed several times in PBST and developed using an

enhanced chemiluminescence reaction (Amersham).

e. Indirect immunofluorescence

Adult C3H mice were anesthetized and perfused through the left ventricle with 4% phosphate-buffered paraformaldehyde. Tissues were dissected, post-fixed in 4% phosphate-buffered paraformaldehyde for 1 hour, placed in 15% sucrose overnight at 4°C and then frozen in OTC embedding medium with -80°C isopentane. Sections (8 μ m) were cut on a cryostat and thaw mounted on gelatin coated slides. Sections were permeabilized with 1% Tween 80 (v/v) in PBS (pH 7.5) for 15 min. at room temperature and non-specific sites were blocked with 3% BSA, fraction V (Sigma Chemicals) in PBS for approximately 2 hours at room temperature. Sections were incubated in a humid chamber with primary antibodies diluted in 3% BSA/PBS for 2 hours at room temperature. Slides were washed six times for 5 min. each in PBS. Secondary antibodies (fluorescein-conjugated donkey anti-rabbit IgG or Texas Red-conjugated goat anti-mouse IgG (Jackson Immunoresearch)), were diluted 1:100 in 3% BSA/PBS and applied to sections for 30 min. at 37°C in a humid chamber. Slides were washed in PBS six times for 5 min. each. Sections were covered with Slow Fade (Molecular Probes) in 50% glycerol prior to mounting and sealing coverslips. Sections were photographed using a Polyvar fluorescence microscope.

f. Kinase assays for endogenous PFTAIR activity

Tissues from adult mice were dissected and homogenized immediately with either a mechanical Teflon tissue grinder or a Polytron tissue grinder in an ice cold tissue homogenization buffer of 50 mM Tris pH 7.4, 200 mM NaCl, 5 mM EDTA, 1 mM Na₃VO₄, 10 mM NaF, 25 mM β -glycerophosphate, 1 mM PMSF and 1 μ g/ml leupeptin, aprotinin and pepstatin A. The homogenates were centrifuged at 100,000 x g at 4°C for 20 min. and the supernatants saved. The pellets were re-homogenized and centrifuged as for the first homogenization. Supernatants were pooled and centrifuged at 100,000 x g at 4°C for 30 min.. The protein concentrations of the cleared cytosolic fractions were determined by the Bradford assay. For immunoprecipitations, cytosolic protein fractions (5-8 mg of protein) were combined with antibodies pre-adsorbed to protein A-Sepharose and mixed at 4°C to form immune complexes. Following several washes at 4°C in 50 mM Tris pH 7.4, 200 mM NaCl, 5 mM EDTA, 1 mM Na₃VO₄, 10 mM NaF, 25 mM β -glycerophosphate, 1 mM PMSF and .01% Triton X-100 and one wash in kinase buffer, the kinase activity of proteins bound to the protein A-Sepharose was assayed by adding 20 mM Tris, pH 7.4,

1 mM DTT, 10 mM MgCl₂, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA and 5 μ Ci [γ -³³P]-ATP (1000 Ci/mmol, NEN Dupont) to each sample and incubating 1 hour at 30°C. Reactions were stopped by the addition of 4 x SDS sample buffer and samples were electrophoresed on 10% SDS-polyacrylamide gels (1.0 mm x 120 mm). Gels were stained with Coomassie Blue, destained and dried before exposing to film (Kodak, Biomax MR) at -80°C with intensifying screens.

g. Kinase assays with *in vitro* -translated proteins

The plasmids pc3PF and pc3D228N were used to synthesize PFTAIRE and the D228N mutant *in vitro* to demonstrate that kinase activity in PFTAIRE immunoprecipitations was due to PFTAIRE and not to the activity of another kinase that may have associated with the PFTAIRE immune complex. The plasmids were linearized by restriction digest with *NotI* and used as templates for transcription reactions with T7 RNA polymerase as described in Section 2.7c. The transcribed mRNAs were quantitated and analyzed on agarose gels to confirm transcript size and concentrations. The mRNAs were translated *in vitro* for 1 hour at 30°C in a 100 μ l reaction containing 2 μ g mRNA (heated 10 min. at 67°C), 2 units RNAGuard, 20 μ M amino acid mix minus methionine (Promega), 70 μ l nuclease-free rabbit reticulocyte lysate (Promega) and 80 μ M L-methionine (Sigma Chemicals). Each *in vitro* translation reaction was diluted to 600 μ l with tissue homogenization buffer (50 mM Tris pH 7.4, 200 mM NaCl, 5 mM EDTA, 1 mM Na₃VO₄, 10 mM NaF, 25 mM β -glycerophosphate, 1 mM PMSF and 1 μ g/ml leupeptin, aprotinin and pepstatin A) and divided into three samples (200 μ l each) for immunoprecipitation with antibodies pre-adsorbed to protein A-Sepharose (Pharmacia) at 4°C. Following several washes *in vitro*-translated protein immune complexes were mixed at 4°C with the cytosolic protein fraction (5-8 mg of protein) from a mouse brain homogenate (prepared as described in Section 2.7f) to allow binding of PFTAIRE-interacting proteins. After 2 hours of mixing at 4°C, the cytosolic protein fraction was removed and samples were washed and processed as described in Section 2.7f, to assay the kinase activity of proteins bound to the protein A-Sepharose beads. Samples were analyzed by electrophoresis on 10% SDS-polyacrylamide gels (1.0 mm x 120 mm). The gels were processed as described in Section 2.7f. After autoradiograms of dried gels were obtained, segments of the dried gels corresponding to proteins phosphorylated by PFTAIRE were excised and phosphate incorporation was quantitated by liquid scintillation counting (Harlow and Lane, 1988). A schematic diagram of this experimental procedure is presented in Figure 2.11.

h. Kinase assays with PFTAIRE and D228N transfected cells

Confluent monolayers of cells transfected with the pc3PForf or pc3D228Norf plasmid were briefly rinsed several times with PBS. The cells were removed from tissue culture plates following treatment with trypsin and collected by centrifugation for 5 min. at 500 x g. Cell pellets from four confluent 100 mm tissue culture plates were resuspended in 1.2 ml of lysis buffer containing 50 mM Tris pH 7.4, 200 mM NaCl, 5 mM EDTA, 1.5 mM MgCl₂, 0.5% Nonidet P-40, 0.5 mM DTT, 1 mM Na₃VO₄, 10 mM NaF, 25 mM β-glycerophosphate, 1 mM PMSF and 1 μg/ml leupeptin, aprotinin and pepstatin A. Lysates were kept on ice for 10 min. then centrifuged for 15 min. at 4°C at 12,000 x g in an Eppendorf microfuge to pellet nuclei. Cytosolic protein fractions were used for immunoprecipitations with antibodies pre-adsorbed to protein A-Sepharose and immunoprecipitated proteins were assayed for kinase activity as described in Section 2.7f.

i. Kinase assays with bacterial fusion proteins

The plasmids pGEX1λT, PGPF19 (Figure 2.6) and PGD228N were used to produce GST, GST-PFTAIRE and GST-D228N proteins in bacteria to study PFTAIRE kinase activity. Bacterial pellets from one liter of IPTG-induced cultures were processed, as described in Section 2.7a, in ice cold PBS containing 1 mM Na₃VO₄, 10 mM NaF, 25 mM β-glycerophosphate, 1 mM PMSF and 1 μg/ml leupeptin, aprotinin and pepstatin A. One-tenth of each lysate preparation was coupled to glutathione -Sepharose and eluted proteins were quantitated by Bradford protein assays and by comparison with a known quantity of BSA on Coomassie Blue stained protein gels to estimate the amount of soluble GST or fusion protein. The remainder of each lysate was flash-frozen in an ethanol-dry ice bath and stored at -80°C until use in kinase assays.

A volume of lysate estimated to contain 10-15 μg of GST or soluble fusion protein was mixed with one-tenth the volume of a 50% glutathione agarose bead slurry (ie. 2.7 ml of lysate+270 μl of 50% glutathione agarose bead slurry) for 2 hours at 4°C. To determine if the GST-fusion proteins could bind to and phosphorylate proteins in a cytosolic protein preparation from mouse brain, after GST or the fusion proteins were coupled to glutathione-Sepharose beads, the beads were washed several times with tissue homogenization buffer (50 mM Tris pH 7.4, 200 mM NaCl, 5 mM EDTA, 1 mM Na₃VO₄, 10 mM NaF, 25 mM β-glycerophosphate, 1 mM PMSF and 1 μg/ml leupeptin, aprotinin and pepstatin A) and a mouse brain cytosolic protein preparation was applied to the prepared beads. GST- or fusion protein-coupled beads were

mixed for 2 hours at 4°C with the mouse brain cytosolic protein preparation. To assay the autophosphorylation kinase activities of GST and the GST fusion proteins coupled to the beads, control samples were incubated with homogenization buffer only for 2 hours at 4°C. At the end of 2 hours all samples were washed several times at 4°C with 50 mM Tris pH 7.4, 200 mM NaCl, 5 mM EDTA, 1 mM Na₃VO₄, 10 mM NaF, 25 mM β-glycerophosphate, 1 mM PMSF and .01% Triton X-100 and once in kinase buffer. The kinase activity of proteins bound to the glutathione-Sepharose beads was assayed by the addition of 20 mM Tris, pH 7.4, 1 mM DTT, 10 mM MgCl₂, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA and 5 μCi [³²P]-ATP (1000 Ci/mmol, NEN Dupont) to each sample and incubating 1 hour at 30°C. Kinase assay samples were analyzed by SDS-PAGE as described in Section 2.7f. This experiment is presented schematically in Figure 2.12.

j. Two dimensional gel electrophoresis

Proteins in immunoprecipitation-kinase assays samples were separated by two dimensional gel electrophoresis to improve separation of the components of immune complexes. For separation of proteins by charge, isoelectric focussing was performed according to the method of O'Farrell (Ausabel et al., 1992, O'Farrell 1975). After kinase assays were performed, proteins in the immune complexes were eluted from the protein A-Sepharose in a solubilization buffer of 9.5 M urea, 2% Nonidet P-40, 2% broad-range ampholytes (Biorad, pH 3-10) and 5% β-mercaptoethanol. For samples that would be used to prepare western blots, 15 μg of a brain cytosolic protein preparation was added at this step so that spots representing phosphorylated proteins could be aligned with proteins detected by antibodies. The samples were loaded on 1.5 mm x 125 mm tube gels of 3.8% acrylamide (30% acrylamide: 1.8% bis-acrylamide stock), 9.25 M urea, 0.19 % Nonidet P-40 and 1.9 % ampholytes. Proteins were separated electrophoretically by charge in a Hoeffer cylindrical isoelectric focussing apparatus, containing an anode buffer of 10 mM phosphoric acid and a cathode buffer of 20 mM NaOH. Samples were pre-focussed for 1 hour at 150V (constant voltage) and 1 hour at 300V (constant voltage) before beginning overnight (~16 hour) electrophoresis at 500V (constant voltage).

Samples were extruded from gel tubes and stored at -20°C in a sample buffer of 62.5 mM Tris pH 6.8, 3% SDS, 5% β-mercaptoethanol and 10% glycerol. For separation of proteins by molecular mass a single tube gel was thawed in sample buffer, placed on top of a 10% SDS-polyacrylamide gel (1.5 mm x 120 mm) and sealed into place with a solution of 1% agarose, 62.5

mM Tris pH 6.8, 3% SDS, 5% β -mercaptoethanol and 10% glycerol. Following migration gels were transferred to nitrocellulose membranes as described in Section 2.7d in a Biorad Transblot apparatus overnight (16 hours) at 30 milliamps (constant current). Nitrocellulose membranes were exposed to Biomax film at -80°C with intensifying screens to obtain an autoradiogram of proteins phosphorylated in the kinase reactions. The membranes were then immunoblotted with the PFTAIRE antibody PFC5 (1:2500) to determine the position of PFTAIRE.

2.8 Cell culture and transfections

a. Maintenance of cell lines

Several different cell lines were analyzed for expression of PFTAIRE mRNA or protein prior to beginning transfection experiments. All cell lines were grown in monolayer cultures at 37°C in a humidified atmosphere containing 5% CO₂. Stocks of NIH-3T3, Ltk⁻, COS7, Vero, 293 and P19 embryonic carcinoma cells were maintained on 100 mm Falcon tissue culture plates in Dulbecco's Modified Eagle Medium with high glucose, supplemented with 5-10 % heat inactivated fetal bovine serum (Gibco, Life Technologies). Cells were harvested by rinsing with Dulbecco's PBS and a brief incubation at 37°C with .05 % trypsin-.02 % EDTA (Gibco, Life Technologies). Stocks of PC12 cells were maintained on 100 mm Falcon tissue culture plates coated with 2 μ g/ml rat tail collagen (Boehringer) in RPMI 1640 supplemented with 10 % heat inactivated horse serum and 5 % heat inactivated fetal bovine serum. To differentiate PC12 cells to a neuronal cell phenotype, medium was supplemented with 50 ng/ml 2.5S nerve growth factor (NGF) for a period of 7-8 days (Green and Tischler, 1976).

b. Transfections

Various cell lines were transfected transiently or for selection of clones that stably expressed plasmids containing the neomycin-resistance gene. Plasmid DNA for transfection of pcPF, pc3PF, pc3D228N, pc3PF_{orf}, pc3D228N_{orf}, pHermes-L and pHermes-L/PF plasmids was purified from overnight bacterial cultures following alkaline lysis of bacteria, precipitation and CsCl density gradients (Sambrook et al., 1989). Either the cationic liposome reagent Lipofectamine (Gibco, Life Technologies) or calcium phosphate precipitation was used to transfect plasmid DNA. For either transfection method, the plating density of cells varied with the cell type and the experiment for which the cells would be used.

Lipofectamine-mediated transfection

For transfections using the Lipofectamine reagent, DNA molecules are incorporated into liposomes that are endocytosed, allowing entry of the plasmid DNA into the cytoplasm. The protocol provided with the reagent by the supplier (Gibco, Life Technologies) and a report by (Hawley-Nelson et al., 1993) served as guidelines for these transfection experiments. All transfections with Lipofectamine were in the serum-free medium Optimem (Gibco, Life Technologies). For most cell lines the ratio of DNA to Lipofectamine reagent resulting in the highest transfection efficiency was approximately 10 µg DNA to 45 µl Lipofectamine for a 100 mm tissue culture plate of cells at 50-70 % confluence (transient transfection). For deriving permanent cell lines the transfection solution was added to cells that were approximately 20-30% confluent. Cells were incubated for 6 hours at 37°C with the transfection solution. The transfection solution was replaced with serum-containing growth medium, without rinsing cells. For transient transfection experiments cells were maintained for 12-24 hours after transfecting and then fixed for X-gal staining (β-galactosidase expression) or for indirect immunofluorescence labelling with antibodies to detect expression of transfected genes.

For deriving stably transfected clones, transfected cells were maintained in growth medium for 16-24 hours before the selection of transfectants was started. Transfected cells were selected by the addition of the antibiotic geneticin (G418) (Gibco, Life Technologies) to growth medium. The concentration of G418 required to kill the mock-transfected population of cells within a period of 5-7 days varied with each cell line used. Until the cell death of mock-transfected cells was complete, all cells were maintained in growth medium containing concentrations of G418 ranging from 500 µg/ml to 2 mg/ml. When cell death was complete and transfectants began forming individual clones, the concentrations of G418 in the growth medium were reduced. After approximately 2-3 weeks individual clones containing more than 100 cells were transferred to a well in a multiwell tissue culture plate and maintained in G418-supplemented growth medium. Individual clones were expanded in order to freeze a sample of each and to analyze each for expression of the transfected mRNA by Northern blot.

Calcium phosphate-mediated transfections

Transfections using the calcium phosphate precipitation method rely on the formation of a precipitate of plasmid DNA with calcium phosphate (Gorman et al., 1983). The plasmid DNA enters cells when the DNA-calcium phosphate precipitate is endocytosed by cells. All calcium

phosphate-mediated transfections were performed following the method of Chen and Okayama (1987) with modifications made to the buffer used. A Hepes buffer (2 x HBS) of 50 mM Hepes pH 7.0, 280 mM NaCl, 1.5 mM NaPO₄ pH 7.0 was substituted for the 2 x BBS buffer used by Chen and Okayama.

To derive permanent cell lines that expressed PFTAIR and the neomycin-resistance gene, 5 µg of pc3PF, pc3D228N (Figure 2.8), pc3PF_{orf} and pc3D228N_{orf} plasmids were used to transfect 100 mm tissue culture plates of cells grown to approximately 20-30 % confluence. DNA-calcium phosphate precipitates were allowed to form for approximately 10-15 min. at room temperature and then gently pipetted on to cells. Cells were incubated with the DNA-calcium phosphate solutions for 6 hours at 37°C. The precipitates were removed from cells by aspiration followed by brief rinses with HBBS (20 mM Hepes pH 7.2, 10 mM D-glucose, 120 mM NaCl, 4.6 mM KCl) containing 5 mM EDTA. Clones were selected in growth medium containing G418 as described above for cells transfected using Lipofectamine.

For transient transfections using the calcium phosphate method cells were plated at high density (60-80% of confluence). Twenty-four hours after transfection cells were either fixed for indirect immunofluorescence labelling with antibodies, or harvested to prepare cell lysates for SDS-PAGE, immunoblotting or kinase assays.

c. Detection of transiently transfected cells by X-gal staining and indirect immunofluorescence

The transfection efficiency of cells transfected transiently with the pHermes-L and pHermes-L/PF plasmids was monitored by staining fixed cells with a solution of the β-galactosidase substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). Cells were fixed with a solution of 2% formaldehyde and 0.2% glutaraldehyde in PBS for 10 min. at room temperature. Fixed cells were rinsed four times with PBS and incubated at 37°C in a humid chamber with a solution of 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl₂, 1 mg/ml X-gal in PBS for 4-16 hours. Cells expressing β-galactosidase were stained blue in the presence of X-gal solution and were visualized and photographed using a Zeiss Axiovert inverted microscope.

Cells transfected transiently with pHermes-L, pHermes-L/PF, pcPF and pc3PF plasmids were also fixed for indirect immunofluorescence labelling with antibodies. Cells were fixed on tissue culture plates with a mixture of 50% methanol/50% acetone for 1 x 10 min. and 1 x 5 min. at -20°C. Fixed cells were permeabilized with 1% Tween 80 in PBS for 15 min. at room temperature.

Blocking of non-specific sites and antibody incubations were performed as described for tissue sections in Section 2.7e. Cells were visualized and photographed using a Zeiss Axiovert inverted fluorescence microscope.

Table 2.1. Oligonucleotides used for cloning and subcloning

Oligonucleotide	Sequence	Derived from	Position in sequence	Use
cdc809	5' CCGTGCTAACCTGTTGAGTAAC 3'	mouse cdc2 kinase 5'	2-23	RT-PCR, generate cdc2 kinase cDNA
cdc808	5' GCAGACAGGGACATCCATCCAG 3'	mouse cdc2 kinase 3'	944-923	RT-PCR, generate cdc2 kinase cDNA
PF925	5' CCAAGTTTTTCGTATGAGTC 3'	PFTAIRE	395-376	PCR, generate cDNA 1-397bp for subcloning <i>EcoRI</i> - <i>DraI</i> 290bp insert
PF2603A	5' CGTCG <u>GGAATTC</u> ATGTCTACCCGGAAGTCCAG 3'	PFTAIRE initiation for translation	119-138	subcloning PFTAIRE orf in pGex1 λ T at <i>EcoRI</i> site
PF2603B	5' GGACGGAATTCCTGTGCTCTCCTGAGAGTGCC 3'	PFTAIRE 3'UTR	1398-1418	subcloning PFTAIRE orf in pGex1 λ T at <i>EcoRI</i> site
PF0809A	5' GGACGGAATTCCTTTAGGTGACTCTTTGCCAGC 3'	PFTAIRE amino-terminal	322-303	used with oligo PF2603A to subclone region encoding aa 1-68 in pGex1 λ T at <i>EcoRI</i> site
PF2701A	5' AGCTGGCA <u>A</u> ATTCGGTCTG 3'	PFTAIRE kinase domain VII, (Mg ²⁺ ATP binding domain)	792-811	mutagenesis, to change G800 to A, aspartate 228 to asparagine (D228N)

Oligonucleotides are written in the 5' to 3' direction. The numbers indicating the position in sequence define the first and last nucleotide of each, and indicate whether the oligonucleotide sequence is derived from the coding or non-coding strand of the cDNA. Also refer to Figures 2.1 and 2.4 for positions of the oligonucleotides in the cdc2 kinase and PFTAIRE cDNAs.

The oligonucleotides PF2603A, PF2603B and PF0809A were synthesized with *EcoRI* restriction sites (underlined) for subcloning PCR generated cDNA fragments.

The oligonucleotide PF2701A contains a point mutation changing guanosine 800 of PFTAIRE to adenosine (bold type, underlined).

Figure 2.1 Positions of oligonucleotides derived from the mouse *cdc2* kinase cDNA sequence. The cDNA sequence of mouse *cdc2* kinase reported by Spurr et al. (1989) was used to derive oligonucleotides *cdc609* (5') and *cdc608* (3') for reverse transcription and PCR. The 3' oligonucleotide, *cdc608*, was used for the reverse transcription reaction with mouse spleen total RNA to generate the full length non-coding strand of the cDNA. A PCR reaction containing the cDNA non-coding strand and the 5' and 3' oligonucleotides resulted in synthesis of the second (coding) strand of the cDNA and amplification of the product for purification and subcloning into a plasmid vector.

cdc609 →
 tccgtcgttaacctgttgagtaactatggaagactatatcaaaatagagaaaattggagaa 60
 M E D Y I K I E K I G E
 ggtacttacgggtgtggtgtataagggtagacacagagtcactggccagatagtggcaatg 120
 G T Y G V V Y K G R H R V T G Q I V A M
 aagaagatcagacttgaaagcgaggaagaaggagtgccagtgcaattcgggaaatc 180
 K K I R L E S E E E G V P S T A I R E I
 tctctattaaaagaacttcgacatccaaatatagtcagcctgcaggatgtgctcatgcag 240
 S L L K E L R H P N I V S L Q D V L M Q
 gactccaggctgtatctcatctttgagttcctgtccatggacctcaagaagtacctggac 300
 D S R L Y L I F E F L S M D L K K Y L D
 tccatccctcctgggcagttcatggattcttcactcggttaagagttacttacaccaaatac 360
 S I P P G Q F M D S S L V K S Y L H Q I
 ctccaggaattgtgttttgcactcccggcgagttcttcacagagacttgaaacctcaa 420
 L Q G I V F C H S R R V L H R D L K P Q
 aatctattgattgatgacaaaggaacaatcaaactggctgatttcggccttgccagagcg 480
 N L L I D D K G T I K L A D F G L A R A
 tttggaataccgatacgagtgtagacacacgaggtactgacgctgtggtaccgatctcca 540
 F G I P I R V Y T H E V L T L W Y R S P
 gaagtgttgctgggctcggtctactccactccggttgacatctggagtatagggacc 600
 E V L L G S A R Y S T P V D I W S I G T
 atatttgcagaactggccaccaagaagccgcttttccacggcgactcagagattgaccag 660
 I F A E L A T K K P L F H G D S E I D Q
 ctcttcaggatcttcagagctctgggcactccctaacaacgaagtgtggccagaagtcgag 720
 L F R I F R A L G T P N N E V W P E V E
 tccctgcaggactacaagaacacctttcccaagtgaagccggggagcctcgcatccac 780
 S L Q D Y K N T F P K W K P G S L A S H
 gtcaagaacctggacgagaacggcttgatttgcctctcaaaaatgctagtctatgatcct 840
 V K N L D E N G L D L L S K M L V Y D P
 accaaacgaatctctggcaaaatggccctgaagcaccctgactttgatgacttggacaat 900
 T K R I S G K M A L K H P Y F D D L D N
 cagattaagaagatgtagccctctggatggatgacccctgtctgctggtcgtaggggaaga 960
 Q I K K M *
 ← cdc608
 tcgtgttgtttaccgttggtctctctcctgtcttgtatagttttctttgtttgtaaattgt 1020
 cattggactttttcttaatttctacgtataacttaattacatgtaaatattattccggaat 1080
 tc 1082

Figure 2.2 PFTAIRE-pBluescript SK+, the 2.5 kb cDNA in pBluescript SK+.

Lambda DNA from the λ gt11 clone obtained from low stringency screening of the mouse brain cDNA library with cdc2 kinase was digested with *Eco*RI to release a 2.5 kb insert that was ligated to *Eco*RI digested pBluescript SK+. The T3 and T7 oligonucleotide primers were used to sequence the 5' and 3' ends of the 2.5 kb insert in pBluescript SK+. The plasmid was also used to make unidirectional deletions into the cDNA by exonuclease III/S1 nuclease digestion, in order to obtain small fragments of the cDNA for sequencing. The restriction sites shown in the PFTAIRE-pBluescript SK+ plasmid map have been confirmed by restriction digests and sequence analysis.

T3 .Sac1 .Sac2 .Not1 .Xba1 .BamH1 .Sma1 .Pst1 .EcoR1

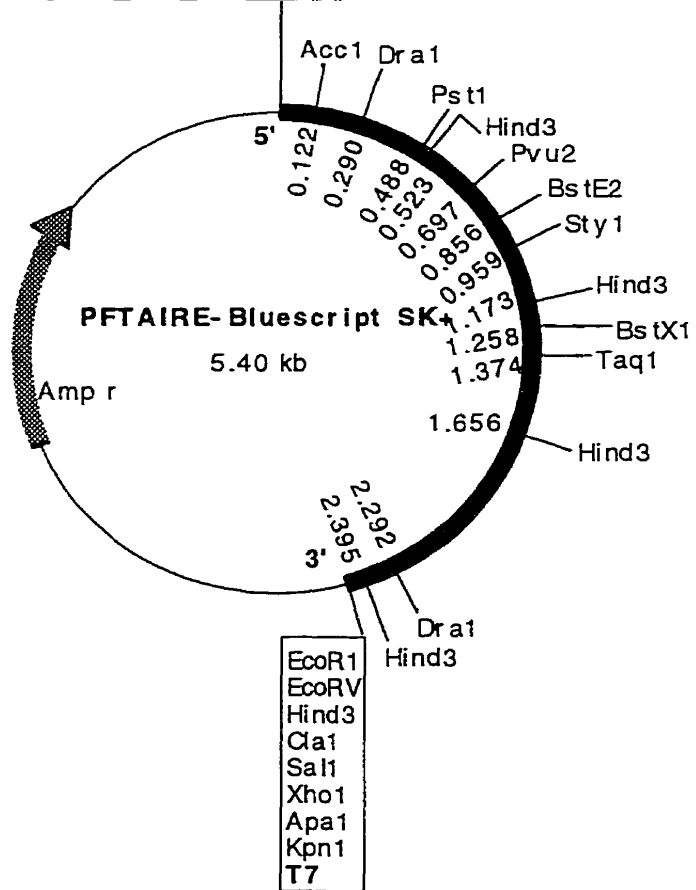


Figure 2.3 H312, H334, H323 and H345, *Hind*III fragments derived from the 2.5 kb cDNA and subcloned in pBluescript SK+.

Restriction digest analysis of the 2.5 kb cDNA in pBluescript SK+ identified four *Hind*III restriction sites, producing fragments of approximately 550 bp, 650 bp, 450 bp and 750 bp. Each fragment was subcloned in pBluescript SK+ and sequenced using the T3 and T7 oligonucleotide primers. Results of the sequencing revealed that H312 (**A**) was a 523 bp fragment that spanned nucleotides 1-523 and contained 118 bp of 5' untranslated sequence; H334 (**B**) was a 650 bp fragment spanning nucleotides 523-1173; H323 (**C**) was a 482 bp fragment spanning nucleotides 1173-1655; and H345 (**D**) was a 740 bp fragment spanning nucleotides 1655-2395 and contains only 3' untranslated sequence. Positions of restriction sites in the *Hind*III subclones refer to their positions in the full length PFTAIRE cDNA.

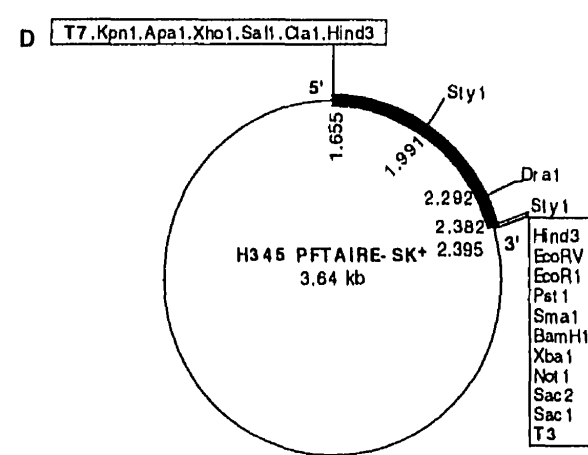
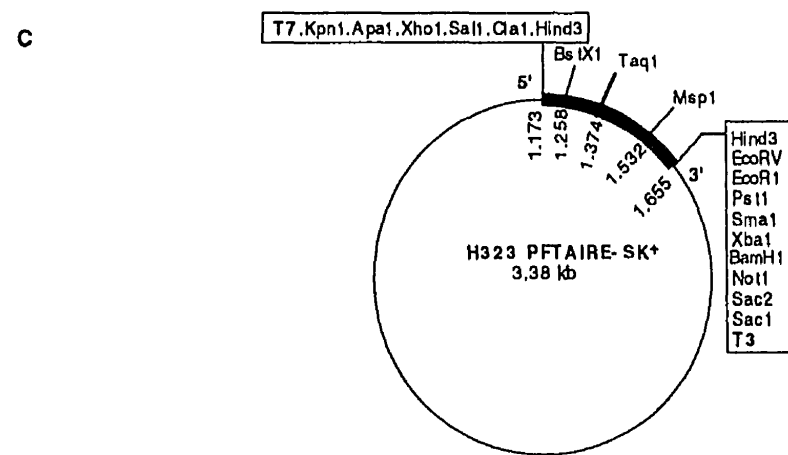
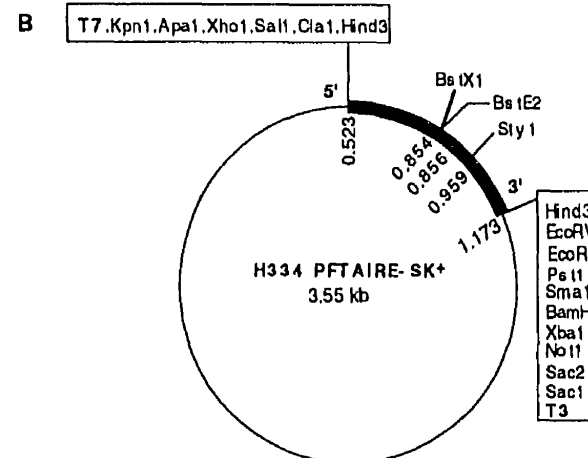
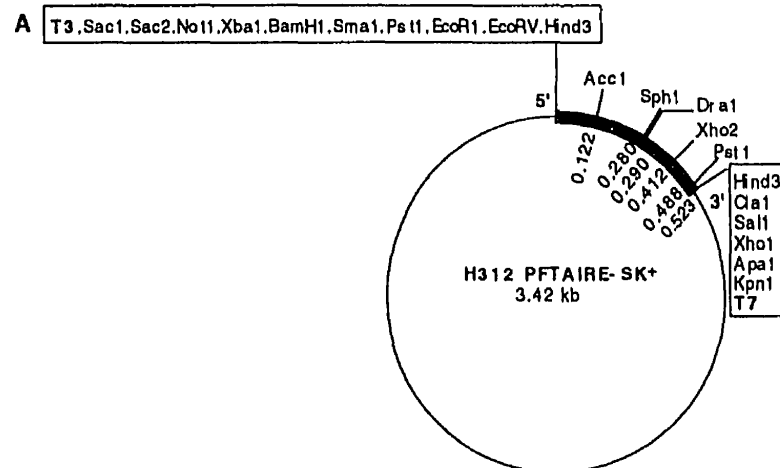


Figure 2.4 Positions of oligonucleotides derived from the PFTAIRE cDNA sequence.

The oligonucleotides PF2603A, PF2603B, PF0809A, PF925 and PF2701A are underlined in the PFTAIRE cDNA sequence. Arrows indicate whether oligonucleotides were derived from the coding strand (pointing right) or non-coding strand (pointing left). Sequences of the oligonucleotides and descriptions of their uses are presented in Table 2.1.

GAATTCAGTTGCTGCTGAGGCTGGCTTGGTGCAAAACACATTGCTAGAGATATGTTAAGGAGTTCCAGGTGAATCCGGCTCAAGAGAGACAACAGCATAAACGATATGTCACAAAG 118
PF2603A →
 ATGTTTACCCGGAACCTCCAGGGGACAGATTCAAGTATCAAGCACCTGGACACAATTCCTGAAGACAAGAAAGTCAGGGTTCAGAGGACGCAGAGCACTTTGGACCCATTTGAGAAACCA 238
 M S T R N C Q G T D S V I K H L D T I P E D K K V R V Q R T Q S T F D P F E K P 40
 ← **PF0809A**
 GCCAACCAAGTCAAAGGGTCCATTCGAGAACAATGCATGCATTAACTTTAAATCCTCCCTCTGCTGGCAAGAGTCACTTAAAGTTCCGGCGGCACCTCCAGCCCCAGCTCGCCAACGAGT 358
 A N Q V K R V H S E N N A C I N F K S S S A G K E S P K V R R H S S P S S P T S 80
 ← **PF925**
 CCCAAATTTGGAAAAGCTGACTCATACGAAAACCTGGAAAAACCTGGGGAAGGATCTTATGCAACAGTGTACAAAGGAAAAAGCAAAGTGAATGGGAAGCTGGTGGCTCTAAAGGTSATC 478
 P K F G K A D S Y E K L E K L G E G S Y A T V Y K G K S K V N G K L V A L K V I 120
 CGGCTGCAGGAAGAAGAGGGCACACCTTTACAGCCATCAGGGAAGCTTCCCTGTTGAAAGGACTAAAGCACGCCAACATCGTGTGCTTCACGACATCATCCACACTAAGGAAACCCCTG 598
 R L Q E E E G T P F T A I R E A S L L K G L K H A N I V L L H D I I H T K E T L 160
 ACCCTTGTCTTTGAATACGTGCACACTGATTTATGTCTAGTACATGGACAAGCACCCCTGGAGGACTCCATCCAGATAATGTGAAGTTGTTTATTTTACGCTGCTGCGAGGACTGCTTTAC 718
 T L V F E Y V H T D L C Q Y M D K H P G G L H P D N V K L F L F Q L L R G L S Y 200
 → **PF2701A**
 ATCCACCAGCGTTATATTTTGCACAGAGACCTGAAACCCGAGAACCCTTCTCATCAGCGATACGGGGGAGTTGAAGCTGGCAGATTTCGGTCTGGCAAGAGCAAAATCCGTCCTTAGCCAC 838
 I H Q R Y I L H R D L K P Q N L L I S D T G E L K L A D F G L A R A K S V P S H 240
 ACATACCTCAATGAAGTGGTTACCTTTGTTGTACAGACCTCCAGATGTTCTTCTGGGCTCTACAGAAATATTCACCTGCCTTGACATGTGGGGAGTTGGCTGTATCTTCTGTTGAGATGATC 958
 T Y S N E V V T L W Y R P P D V L L G S T E Y S T C L D M W G V G C I F V E M I 280
 CAAGGAGTTGCTGCGTTTCCAGGAATGAAAGACATTTCAGGATCAACTTGAACGGATATTTCTGGTCTTGGAAACACCGAATGAGGACACGTGGCTTGGAGTTTCAATTTTACCACATTTT 1078
 Q G V A A P P G M K D I Q D Q L E R I F L V L G T P N E D T W P G V H S L P H F 320
 AAGCCAGAACGCTTTACCGTGTACAACCTTAAAGCCCTTAGACAAGCATGGAATAAGCTCAGCTATGTAAATCATGCTGAAGACTTGGCTTCCAAGCTTCTCCAGTGTTCCTCCAAAGAAC 1198
 K P E R F T V Y N S K S L R Q A W N K L S Y V N H A E D L A S K L L Q C S P K N 360
 AGGCTATCAGCACAGGCCGCTTGAGCCATGAGTATTTTACGCGATCTGCCCTCCACGGCTATGGGAGCTGACTGATATGCTTCTATTTTACCCTGCCAAATGTGAGATTGCAACCAGAA 1318
 R L S A Q A A L S H E Y F S D L P P R L W E L T D M S S I F T V P N V R L Q P E 400
 ← **PF2603B**
 GCTGGAGAGAGCATGAGGGCCTTTGGAAAAACAATAGTTATGGGAAAAGCCTATCGAACAGCAAACACTGACAAGCCCGGCACCTCAGGAGAGCACAGGATTAAGTTGTCAATCATCT 1438
 A G E S M R A F G K N N S Y G K S L S N S K H *
 GGGAAGAAGAAGAAGAGATAAAAAATAATCAATCAATAAAAAACCACATAATGAAGTGGCTCAGTGCAGGGGCCCTGAATCGGTTCTTCTGCCCCGGTGCAGTGGATTTCAGTGCATGAA 1558
 AATGGAAGCTGGCAAGAACCCTGTTCTCTGCAATTTATTTAAACCTTGACGCAATTTGGATACCTTGCTGACTTCCAAGAAGTACGTGACGATTAAGCTTGGCATGTATCTTTTCCGT 1678
 CTTTGTGCTGCTGGTTCGTTTGTCTTCCCCTGCCGCACAGTGTCCATCGCACGGTGTCTCTGTGAAGGGTTTGTCTGCTTTCAGCCAGCTCCCTCTTAAGTACATTAAGACAACACATTTG 1798
 GTGTTACACATTTCTCAGCCATGCTGCACTTGAAGCCACACAGCGGCAGGAGACAAATGTGTGATTTCCCTGAGAGCAGAGTGCATTTGCAACCTCCAGGAAGAACATCTTTTGTCT 1918
 AAAACAACTCCATGCTTTCTGACAGACTAGACAGCGTGGTCCAGACAGTGGGGCCCTACCTGTTGCCCTGCCCTGGAGGACATTTAGAAATGTAAGGGTTTTTTTGTTTGTTTTGGTT 2038
 TTCGTTTATTTATGTGAAGTAGCACACATTTACACAGGAGACCAAAATCCACTTGCATTTTCAATAGCCTGACTGTTTCTGAGCAGGTAGAGAAATAAACATGGAACAAAGACAGGGT 2158
 GACTGAAGGTTAATAAGCATTACTATCATTTTCTTTAGCCGTGTAGAAAGCAAGGCAACTTTTTCATGTGCTCTTGTCCACATCTTACCTTTTCCCATCAGCTCTGTATTATTAACATGTT 2278
 ACACCTTATTTTAACTGGAGAAAAATAGTAGCAAAATATCATTTCTTAAACCTGGGTACATAGAAAAAAAATCTGCTGCTCAGGGTAGGTCTTGAATGCAGCCTTGGCTGATTAAGCT 2398
 TAGAAATCACATTTTAAATTTATCCAGAGTTAAAAATGAGCTTTATTTACGACAAAGGACCTTTTGAATTC

T7 .Kpn1 .Apa1 .Xho1 .Sal1 .Cla1 .Hind3 .EcoRV .EcoR1

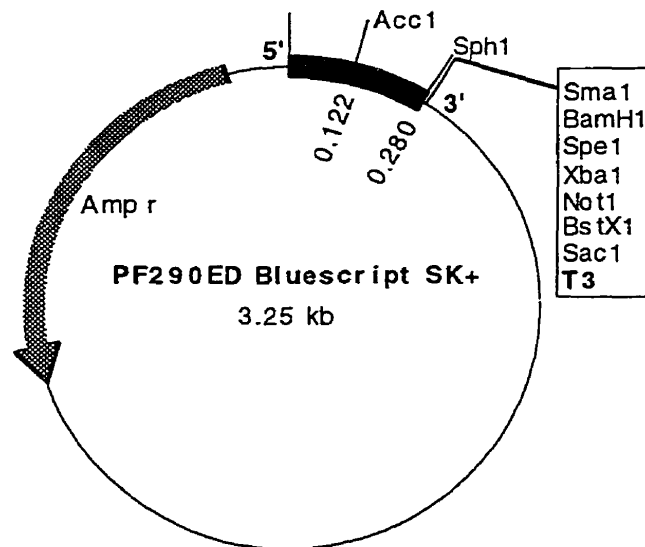


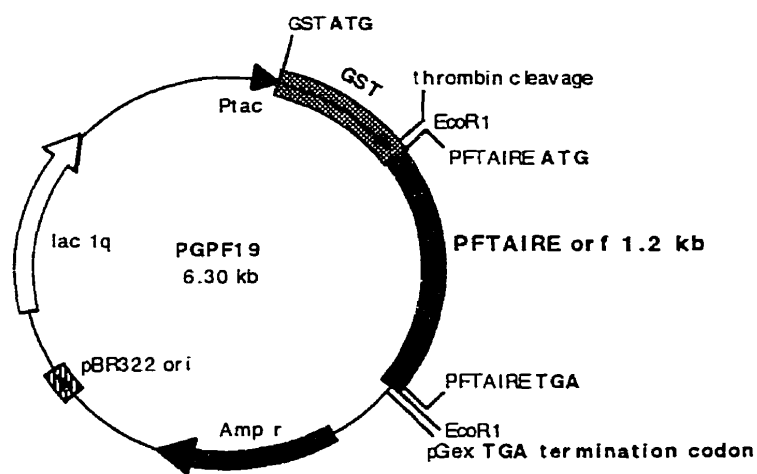
Figure 2.5 PF290ED-pBluescript SK+, a 5' 290 bp *EcoRI*-*DraI* fragment from the 2.5 kb cDNA subcloned in pBluescript SK+.

DNA sequence analysis of the 2.5 kb cDNA revealed a *DraI* restriction site at 290 bp. This region of the cDNA contained 118 bp of 5' untranslated sequence and 172 bp of amino-terminal sequence. The plasmid was constructed by using the PFTAIRE-pBluescript SK+ plasmid as a template for a PCR reaction with the PF925 and T3 oligonucleotide primers. The PCR product, a 397 bp fragment containing only one *DraI* site (at 290 bp), was digested with *EcoRI* and *DraI* to obtain the 290 bp 5' fragment. The 290 bp fragment was ligated to pBluescript SK+ digested with *EcoRI* and *SmaI*.

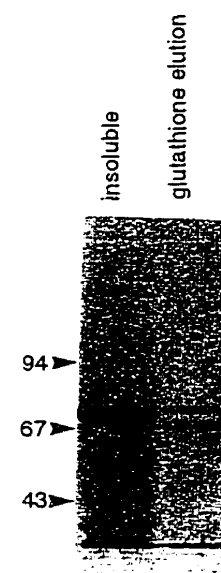
Figure 2.6. GST-PFTAIRE bacterial fusion protein expression plasmids.

A) PGPF19, the entire coding region of the PFTAIRE cDNA was inserted at the *EcoRI* cloning site of the pGex1 λ T plasmid vector. The subcloned insert was generated by PCR using the PFTAIRE-pBluescript SK+ plasmid and oligonucleotides PF2603A (5') and PF2603B (3'), which contained *EcoRI* restriction sites. Sequence analysis confirmed that PFTAIRE would be translated in the same open reading frame as the glutathione S-transferase cDNA and the pGex1 λ T termination codon. **B)** The fusion protein GST-PFTAIRE produced upon expression of the PGPF19 plasmid in *E.coli*. Lane 1, the insoluble bacterial pellet contained most of the GST-PFTAIRE. Lane 2, soluble GST-PFTAIRE eluted from glutathione-Sepharose beads with 10 mM reduced glutathione. Both of the major protein bands reacted with the PFC5 antibody on immunoblots, suggesting that some of the soluble fusion protein had been degraded. The size of the GST-PFTAIRE fusion protein encoded by the PGPF19 plasmid is 74.5 kDa (27 kDa GST + 47.5 kDa PFTAIRE). **C)** PGN5, a 204 bp fragment of the PFTAIRE cDNA encoding amino acids 1-68 was inserted at the *EcoRI* cloning site of the pGex1 λ T plasmid vector. The subcloned insert was generated by PCR using the PFTAIRE-pBluescript SK+ plasmid and oligonucleotides PF2603A (5') and PF0809A (3'). Sequence analysis confirmed that amino-terminal PFTAIRE fragment, the glutathione S-transferase cDNA and the pGex1 λ T termination codon were all in the same open reading frame. **D)** The fusion protein GST-N-PFTAIRE produced upon expression of the PGN5 plasmid in *E.coli*, eluted from glutathione-Sepharose beads with 10 mM reduced glutathione. The size of the GST-N-PFTAIRE fusion protein encoded by the PGN5 plasmid is 34 kDa (27 kDa GST + 7 kDa PFTAIRE). The eluted fusion protein was used to raise PFTAIRE antibodies.

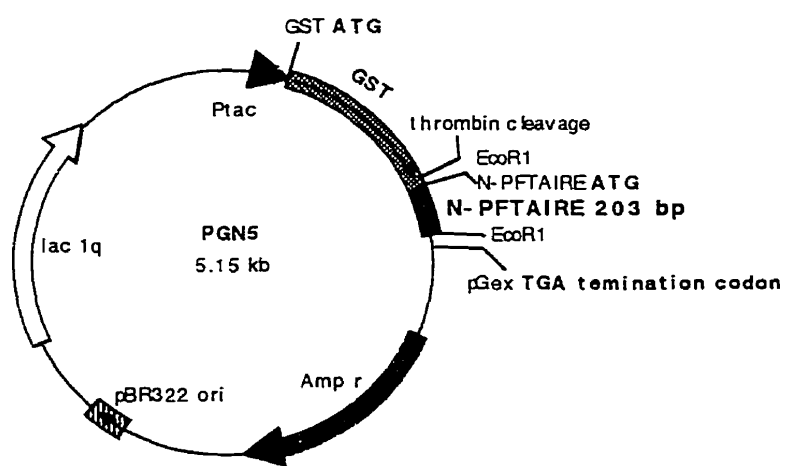
A



B



C



D

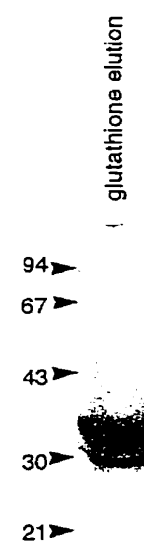
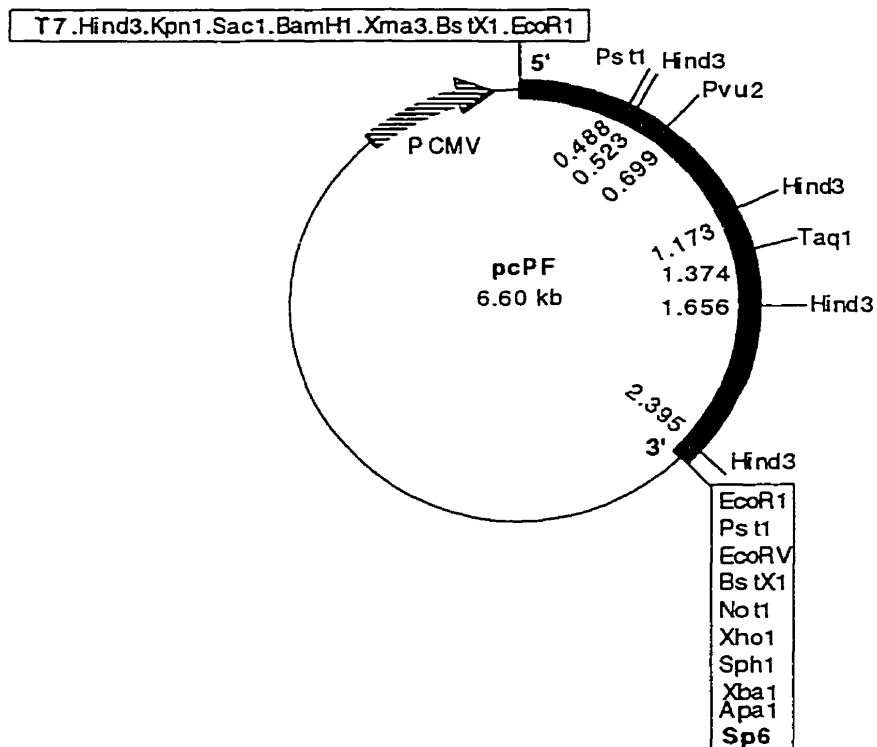


Figure 2.7 pcDNA1-PFTAIRE eukaryotic expression plasmids.

The entire PFTAIRE cDNA (2.5 kb) was subcloned at the *EcoRI* site of the pcDNA1 plasmid for PFTAIRE expression in eukaryotic cells controlled by the human cytomegalovirus (CMV) promoter. **A)** pcPF, the CMV promoter controls expression of the sense strand of the PFTAIRE cDNA. **B)** pcPF-AS, the CMV promoter controls expression of the antisense strand of PFTAIRE.

A



B

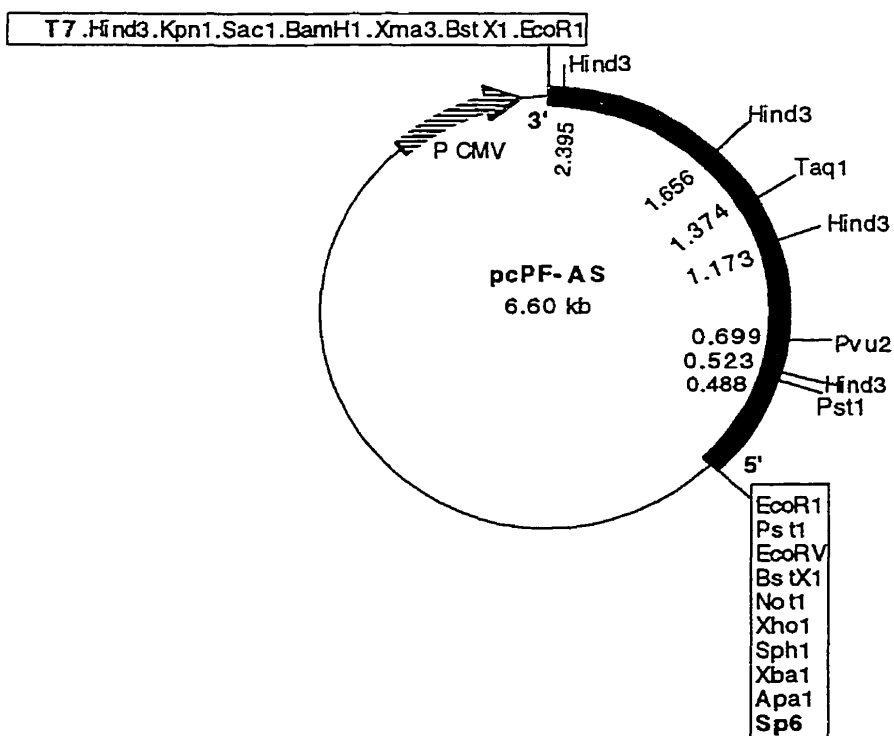


Figure 2.8 pcDNA3-PFTAIRE eukaryotic expression plasmids.

The entire PFTAIRE cDNA (2.5 kb) was subcloned at the *EcoRI* site of the pcDNA3 plasmid for PFTAIRE expression in eukaryotic cells controlled by the CMV promoter. The pcDNA3 plasmid vector contains a neomycin-resistance gene, enabling selection of transfected clones with the antibiotic G418 (geneticin). **A)** pc3PF, the CMV promoter controls expression of the wild-type PFTAIRE (sense strand). **B)** pc3D228N, the CMV promoter controls expression of the PFTAIRE "kinase inactive" mutant, D228N. **C)** pc3PF-AS, the CMV promoter controls expression of antisense PFTAIRE.

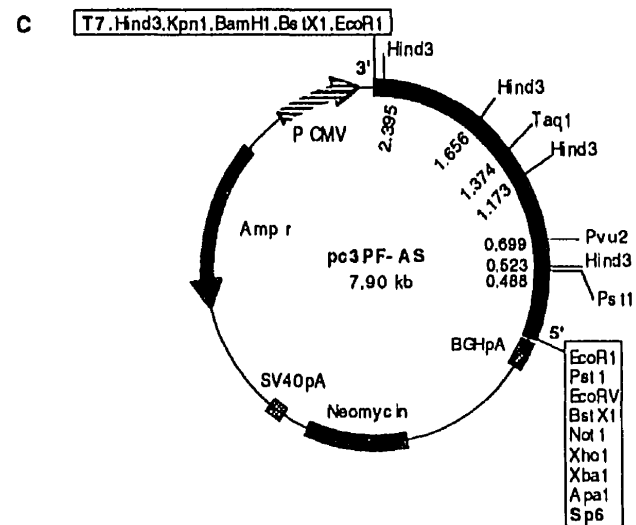
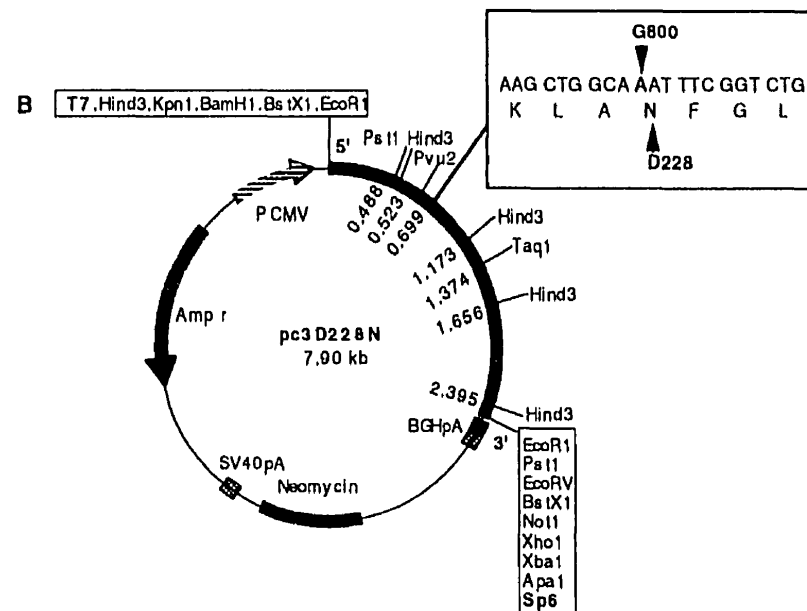
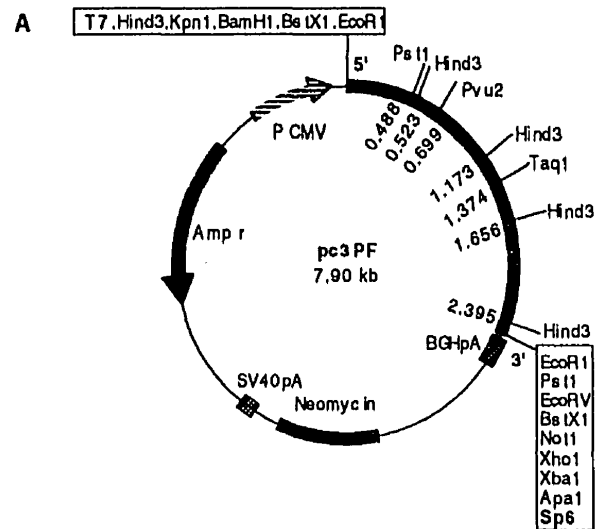
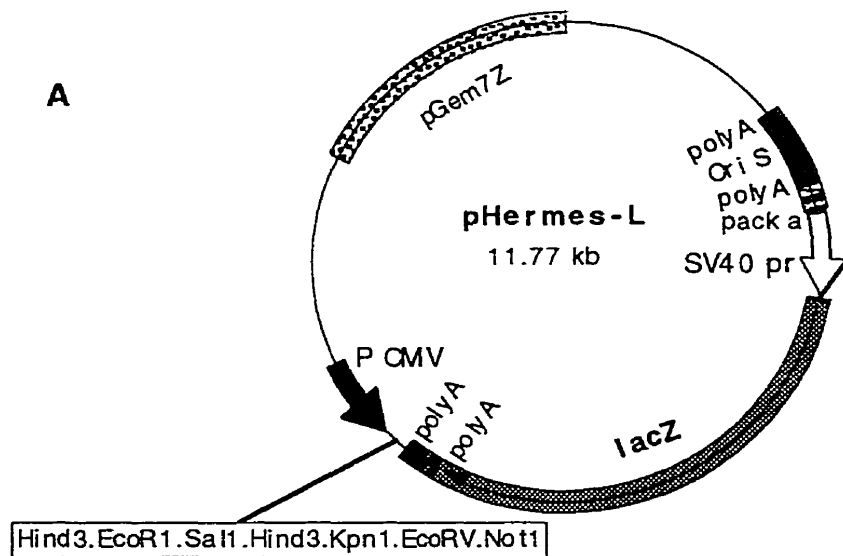


Figure 2.9 pHermes-L and pHermes-L/PFTAIRE expression vectors.

The pHermes-L vector, designed for infecting mammalian cells with HSV-1 virus stocks carrying a cDNA of interest was used as an expression plasmid for transient transfection of mammalian cells.

A) pHermes-L, the cloning site of pHermes-L was modified by inserting an oligonucleotide containing *EcoRI*, *SalI* and *HindIII* restriction sites. Expression of the *lacZ* gene is controlled by the SV40 promoter. **B)** pHermes-L/PF, the entire 2.5 kb PFTAIRE cDNA was inserted at a unique *SalI* restriction site in the pHermes-L vector, for expression controlled by the CMV promoter. The SV40 promoter controls expression of the *lacZ* gene and transfection efficiency could be monitored by examining β -galactosidase expression.

A



B

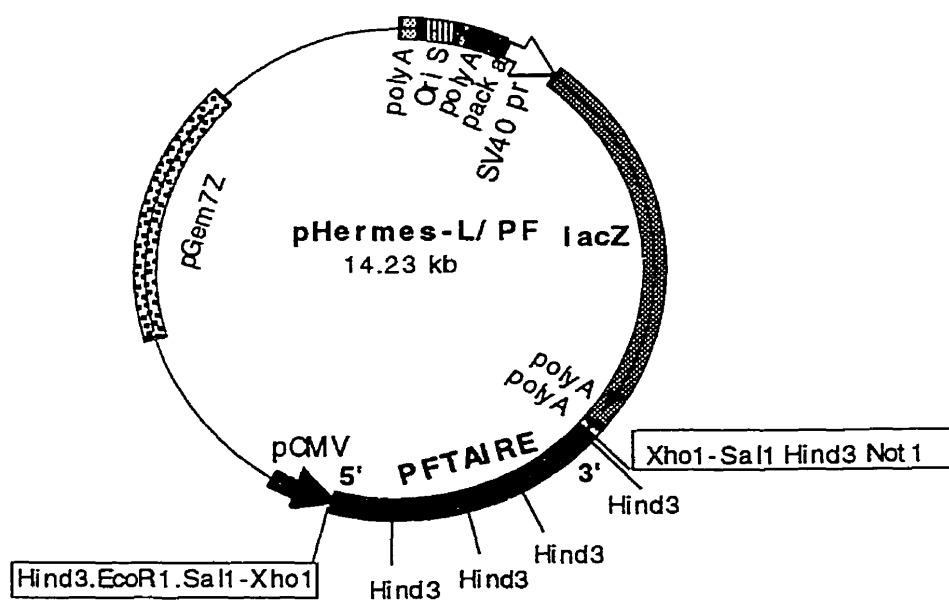


Figure 2.10 Location of PFTAIRE peptides used to raise antibodies.

Alignment of the PFTAIRE amino acid sequence with other CDKs and cdc2-related kinases demonstrates the low amino acid homology between PFTAIRE and other kinases in the amino- and carboxy-terminal regions of PFTAIRE, which were used to produce PFTAIRE antibodies. Amino acids 1-68 of PFTAIRE, fused in frame to glutathione S-transferase, to generate the PGN5 plasmid (Figure 2.6) produced a 34 kDa fusion protein used to raise antibodies (PGN5) against the amino-terminal region of PFTAIRE. A synthetic peptide spanning amino acids 409-422 was used to raise antibodies (PFC5) against the carboxy-terminal region of PFTAIRE. Amino acid sequences of PFTAIRE peptides are shown in underlined bold text.

PCTAIRE-1	MDRMKKIKRQLSMTLRGGRGIDKT.NGAPEQIGLDES	36
PCTAIRE-2	<MKKFKRRLSLTLRGSQTIDESLSELAEQMTIEEN	34

PFTAIRE	MSTRNC	6
PCTAIRE-1	GGGGGSDPGEAPTRAAPGELRS.....ARGPLSSAPEIVHEDLKMGSDESDQAS	86
PCTAIRE-2	SSKDNEP IVKNGRPPTSHSMHSLHQYTGSFKKPLRRPHSVIGGSLGSFMAMPNGSRDLIVHENLKMGSDESDQAS	114

PFTAIRE	OGTDSVLKGLDTPEDKKVRVORTOSTFDPFEKPANOVKRVHSENNACINFKSSSAGKESPKVRRHSSPSSPTSPKFGK	85
PCTAIRE-1	ATSSDEVQSPVRVVRMRNHPRKISTEDINKRLSLPADIRLPEGYLEKLTLSNPIFDKPLSRRLRRVSL....SEIGFGK	161
PCTAIRE-2	GTSSDEVQSPGTGVLNRRIHRRISMEDLNKRLSLPADIRIPDGYLEKLTLSNPIFDKPLSRRLRRVSL....SEIGFGK	189
PCTAIRE-3	<KRLSLPMDIRLPQEFQLQKQMESPDLPKPLSRMSRRASL....SDIGFGK	46

	**	
CDC2	MEDYIKIEKIGEGTYGVVYKGRHRVTGQIVAMKKIRLESEEEGVFSTAIREISLLKELRHPNIVSLQDVLQMDSRLYL	79
CDK2	MENFQKVEKIGEGTYGVVYKARNKLTGQVVALKKIRLDTETEGVPSTAIREISLLKELNHPNIVKLLDVHTENKLYLV	79
PFTAIRE	ADSYEKLEKLGEGSYATVYKGSKVNGKLVALKVIRLQ.EEETPFTAIREASLLKGLKHANIVLLHDIHTKETLT	163
PCTAIRE-1	LETYIKLDKLGEGTYATVYKGSKLTDNLVALKEIRLEHE.EGAPCTAIREVSSLKDLKHANIVTLHDIHTEKSLTLV	239
PCTAIRE-2	METYIKLEKLGEGTYATVYKGRSKLTENLVALKEIRLEHE.EGAPCTAIREVSSLKDLKHANIVTLHDIHTEKSLTLV	267
PCTAIRE-3	LETYVKLDKLGEGTYATVFHGRSKLTENLVALKEIRLEHE.EGAPCTAIREVSSLKDLKHANIVTLHDIHTEKSLTLV	124
CDK5	MQKYEKLEKIGEGTYGVVYKARNKLTGQVVALKKIRLDTETEGVPSTAIREISLLKELRHPNIVSLQDVLQMDSRLYL	79

CDC2	FEFLSMDLKKYLDSPPGQFMDSSLVKSYLHQILQGVFCHSRRLVHRDLKPQNLLIDDKGTIKLADFGLARAFGIP	158
CDK2	FEFLHQDLKKFMDASALTG.IPLPLIKSYLFQLLQGLAFCHSRRLVHRDLKPQNLLINTEGAIKLADFGLARAFGVP	157
PFTAIRE	FEYVHTDLCQYMDKH..PGGLHPDNVKLFLFQLLRGLSYIHQRYILHRDLKPQNLLISDTGELKLADFGLARAKSV	240
PCTAIRE-1	FEYLDKDLKQYLDHC..GNIINMHNVKLFLFQLLRGLAYCHRRKVLHRDLKPQNLLINERGERKLADFGLARAKS	316
PCTAIRE-2	FEYLDKDLKQYMDHC..GNIMSMHNVKLFLYQILRGLAYCHRRKVLHRDLKPQNLLINERGERKLADFGLARAKS	344
PCTAIRE-3	FEYLDKDLKQYLDHC..GNLMSMHNVKLFLFQLLRGLAYCHRRKVLHRDLKPQNLLINERGERKLADFGLARAKS	201
CDK5	FEPCDQDLKNFDC..NGDLDEIVKSLFQLLKGLGFCHSRNLVHRDLKPQNLLINERGERKLADFGLARAFGIP	155

	*	
CDC2	VYTHEVTLWYRSPPEVLLGSARYSTPVDIWSIGTIFAELA.TKKPLFHGDSEI.DQLFRIFRALGTPNNEVWPEV	235
CDK2	TYTHEVVTWYRAPEILLGCKYYSTAVDIWSLGCIFAEMV.TRRALFPGDSEI.DQLFRIFRTLGTPDEVVWPEV	234
PFTAIRE	TYSNEVVTWYRPPDVLLGSTYESTCLDMWGVGCIYFEMA.QGVAAFPGMKDIQDQLERIFLVLGTPNEDTWPGV	318
PCTAIRE-1	TYSNEVVTWYRPPDILLGSTDYSTQIDMWGVGCIYFEMA.TGRPLFPGSTVE.EQLHFIFRILGTPTEETWPG	393
PCTAIRE-2	TYSNEVVTWYRPPDVLLGSSEYSTQIDMWGVGCIYFEMA.SGRPLFPGSTVED.ELHLIFRLLGTPSQTETWPG	421
PCTAIRE-3	TYSNEVVTWYRPPDVLLGSTYESTPIAMWGVGCIYFEMA.TGRPLFPGSTVKE.ELHLIFRLLGTPTEETWPGV	278
CDK5	CYBAEVVTWYRPPDVLFAGAKLYSTSIDMWSAGCIFAELANAGRPLFPGNUVDD.QLKRIFRLLGTPTEEQWP	233

CDC2	DYKN.TFPKW.KPGSLASHVKNLDENGLD..LLSKMLVYDPTKRISGKMLKHPYFDDLDNQIKKM	297
CDK2	DYKP.SFPKW.ARQDFSKVVPPLDEDGRS..LLSQMLHYDPNKRISAKAALAHPPFQDVTKPVPHRL	298
PFTAIRE	HPKPERFTVYNSKSLRQAWNK.LSYVNHAEDLASKLLQCSPKNRLSAQAALSHYFSDLPPRLWELTDMSSIFTVP	400
PCTAIRE-1	EFKTYNYPKY.RAEALLSHAPRLDSDGADL..LTKLQFEGRNRIASADAMKHPFSLGERIHKLPDTSIFALKEI	473
PCTAIRE-2	EFKTYNYPKY.KPQPLINHAPRLDSEGIEL..IRKFLQYESKNRVSAEEAMKHVYFRSLGPRIHAPESVSIFSLKEI	500
PCTAIRE-3	EFRTYNYPKY.LPQPLINHAPRLDTGIDL..LSSLLVYESKSRMSAEALSHSYFRSLGERVHQLEDTASIFSLKEI	358
CDK5	DYKP..YPMYPATTSLVNVPKLNATGRDL..LQNLKCNVPQIRISAEALQHPYFSDFCPP	291

PFTAIRE	LQPEAGESMRAFGKNSYKSLNSKH	423
PCTAIRE-1	LQKEASLRSSMPDSGRPAFRVVDTEF	496
PCTAIRE-2	LQKDPGFRNSSYPETGH.GKNRRQSMFL	523
PCTAIRE-3	LQKDPGYRGKAFQPGR.GKNRRQSIF	380

Figure 2.11 Scheme for *in vitro* binding-kinase assay.

In vitro-translated PFTAIRE and D228N were immunoprecipitated with protein A-Sepharose pre-adsorbed with the PFC5 antibody. Immune complexes formed with *in vitro*-translated proteins were mixed with a brain cytosolic protein preparation to allow binding of PFTAIRE-interacting proteins. After 2 hours beads were washed several times in buffer containing .01% Triton X-100 and kinase buffer. The kinase activities of proteins bound to the protein A-Sepharose beads were assayed by the addition of 50 μ l kinase buffer containing [γ 33P]ATP.

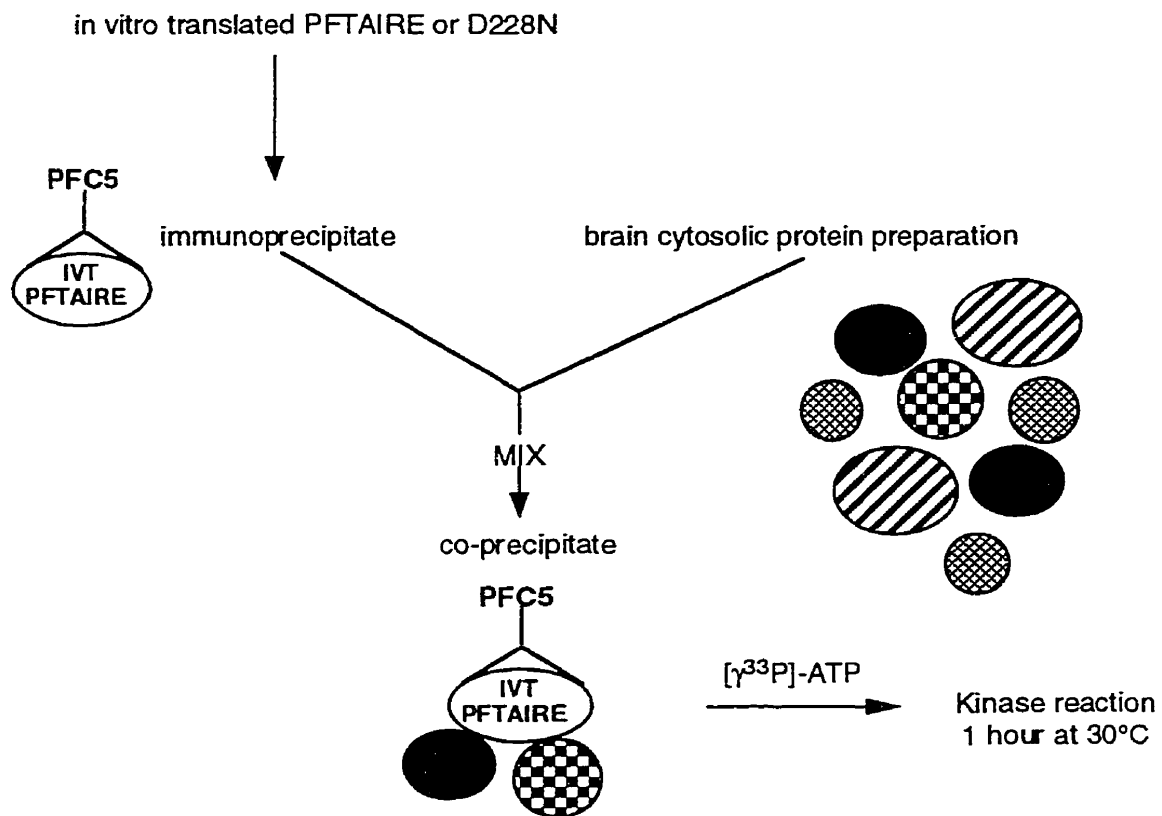
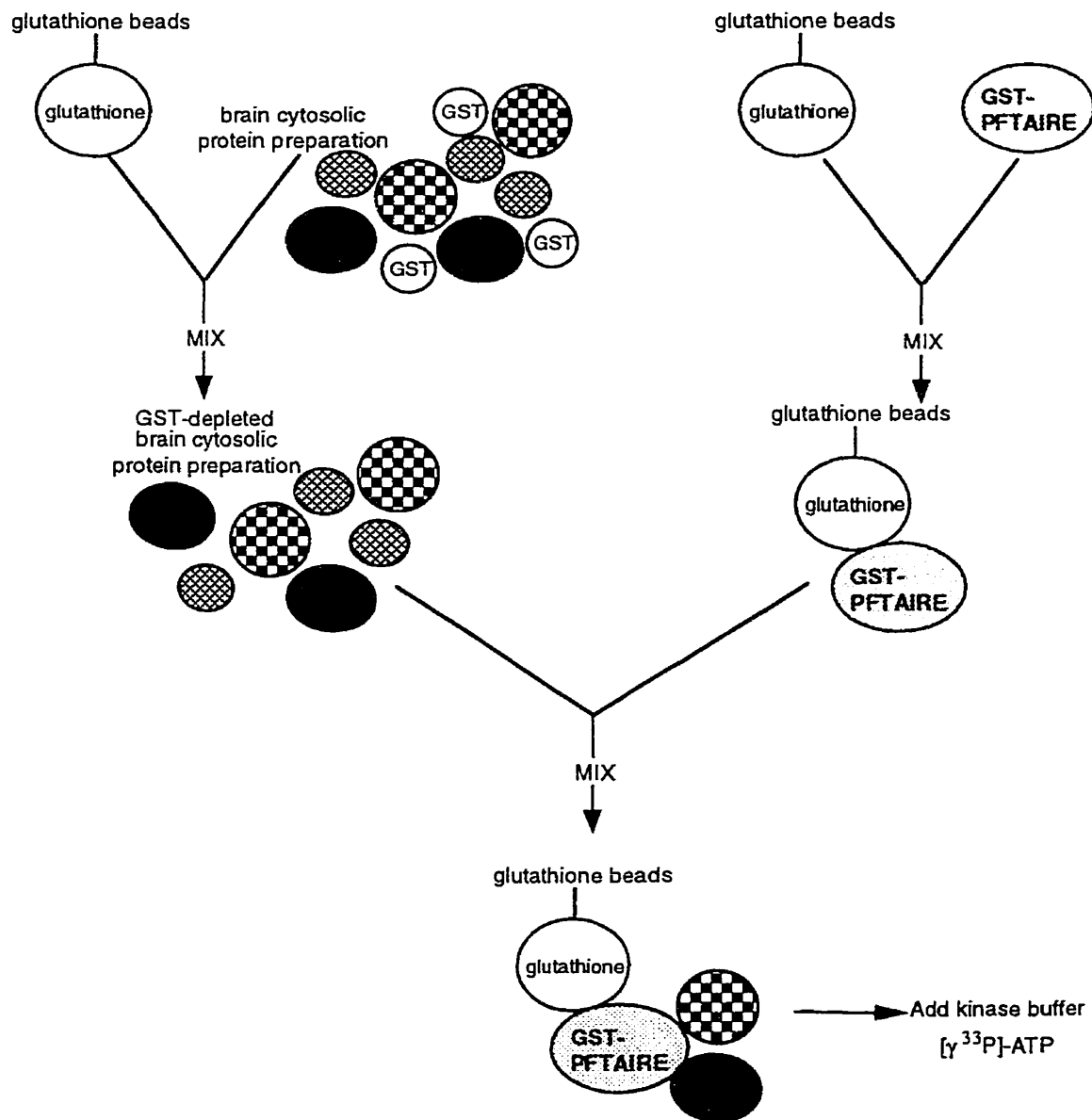


Figure 2.12 Scheme for kinase assay with GST-PFTAIRE and GST-D228N.

The soluble protein fractions from lysates of bacteria expressing GST, GST-PFTAIRE and GST-D228N were mixed with glutathione-Sepharose beads to bind each of the GST proteins. A brain cytosolic protein preparation was mixed with glutathione-Sepharose beads to deplete endogenous glutathione S-transferase from this preparation. The GST-depleted brain preparation was mixed with GST or GST fusion proteins bound to glutathione beads to allow binding of proteins from brain. After 2 hours, the brain protein preparation was removed, glutathione-Sepharose beads were washed and kinase buffer containing [$\gamma^{33}\text{P}$]-ATP was added to assay the kinase activities of proteins bound to the beads.



CHAPTER 3

A cDNA ENCODING A NOVEL cdc2-RELATED PROTEIN KINASE and ITS mRNA EXPRESSION IN THE MOUSE NERVOUS SYSTEM

3.1 Introduction

The ability of p34^{cdc2} kinase purified from starfish oocytes to phosphorylate *in vitro* purified, dephosphorylated neurofilament subunit proteins, NF-M and NF-H, at a level similar to the *in vivo* phosphorylation state of these proteins (Hisanaga et al., 1991) was a provoking observation for many investigators of neurofilament protein phosphorylation. As a serine/threonine protein kinase required for regulation of the eukaryotic cell cycle, p34^{cdc2} kinase is present at very low levels in the tissues of the adult nervous system, where the majority of cells are postmitotic. This suggested that p34^{cdc2} kinase was not likely to be the kinase responsible for phosphorylation of neurofilament proteins *in vivo*, but that other kinases related to p34^{cdc2} kinase may exist in the nervous system to mediate this function. Based on this hypothesis I have used the cdc2 kinase cDNA as a probe for low stringency hybridization screening, to isolate putative cdc2-related kinases in the postnatal/adult nervous system. The results presented in Chapter 3 describe the novel cdc2-related kinase that was cloned by this approach. Experiments performed to determine that the cDNA represents a single gene and the chromosomal location of the mouse gene are also described here. Results of mRNA analysis demonstrate that this kinase is expressed predominantly in the nervous system of postnatal and adult mice, in several populations of neurons and in some glia.

3.2 Cloning of a novel cdc2-related kinase from mouse brain

For low stringency hybridization screening a mouse cdc2 kinase cDNA was generated in a reverse transcription reaction and amplified by PCR, as described in Chapter 2 (Section 2.2). Screening of approximately 5 X 10⁵ clones from an 18 day postnatal mouse brain cDNA library with the cdc2 probe using low stringency conditions yielded a positive clone that was plaque purified. Restriction digest of DNA purified from the λ gt11 clone with *EcoRI* yielded an insert of approximately 2.5 kb.

The entire cDNA was sequenced and found to contain a full length open reading frame of 1269 nucleotides encoding a novel cdc2-related gene. The nucleotide and deduced amino acid sequences for the gene are shown in Figure 3.1. The invariant purine in the -3 position (relative to A of the AUG codon) of the consensus sequence ACCAAUGG, required for initiation of

translation (Kozak, 1986), was present in this new cDNA. Three stop codons located 21 bp, 63 bp and 102 bp upstream of this methionine and a putative polyadenylation signal beginning 82 bp downstream of the first 3' stop codon were identified in this reading frame, suggesting that this was the full length open reading frame.

3.3 Physical features of the protein kinase encoded by a novel cDNA

The predicted amino acid sequence encoded a protein of 423 amino acids (47.5 kDa) and the central 300 amino acids represent a conserved catalytic domain common to all protein kinases. Within this region there were many of the conserved subdomains and residues characteristic of serine/threonine protein kinases, including the consensus motif G-X-G-XX-G-X-A (residues 96-101) which is predicted to form part of the ATP binding region (Hanks et al., 1988; Hanks and Hunter, 1995). A PSTAIRE sequence, the highly conserved amino acid motif characteristic of the cdc2 kinase family, was identified at residues 129-135 and found to contain a single residue substitution of phenylalanine for serine (PFTAIRE). Cdc2-related kinases that have been shown to associate with regulatory cyclin proteins are referred to as cyclin-dependent kinases (ie. CDK1, CDK2, CDK3 etc.). Other cdc2-related kinases that have not been shown to associate with cyclins have been named according to the amino acid sequences found at the PSTAIRE regions (1991 Cold Spring Harbor Symposium on the Cell Cycle). In keeping with this nomenclature this new cdc2-related kinase cDNA has been tentatively named PFTAIRE.

Alignment of the amino acid sequence of PFTAIRE with murine p34^{cdc2}, other CDKs and cdc2-related kinases showed the many highly conserved domains and residues among these proteins (Figure 3.2), suggesting that PFTAIRE was a putative cdc2-related protein kinase. PFTAIRE had 48% amino acid identity with p34^{cdc2}, 51% with CDK2, 55% with PCTAIRE-1 and PCTAIRE-2, 54% with PCTAIRE-3 and 54% with CDK5. Sequence similarity searches of the EMBL and GenBank databases indicated that the complete PFTAIRE nucleotide sequence had highest homology to PCTAIRE-1 and PCTAIRE-2. Like the PCTAIRE kinases, PFTAIRE has extended amino- and carboxy-termini relative to p34^{cdc2}. The PFTAIRE amino-terminal extension consisted of 85 amino acids and shared few residues in common with PCTAIRE-1 and PCTAIRE-2 amino-terminal extensions. Shifting the PFTAIRE reading frame during sequence

alignments, to use the ATG codons upstream of what I have identified as the initiation codon, did not increase the homology level of the amino-terminal regions of PFTAIRE and the PCTAIRE kinases. The carboxy-terminal extensions of PFTAIRE, PCTAIRE-1 and PCTAIRE-2 were of similar length, approximately 40 amino acids, but also showed little homology to each other (20% identity for PFTAIRE vs. PCTAIRE-1 and PCTAIRE-2). The functional significance of the extended amino- and carboxy-termini in these *cdc2*-related protein kinases is presently unknown.

Three major phosphorylation sites corresponding to Thr14, Tyr15 and Thr161 (Thr167 in yeast) in p34^{cdc2} homologues from higher eukaryotes, regulate the activities of p34^{cdc2} and CDKs during the cell cycle (Gould and Nurse, 1989; Gould et al., 1991; Norbury et al., 1991; Krek and Nigg, 1991; Solomon, 1993 et al.; Fesquet et al., 1993; Poon et al., 1993; Kato et al., 1994). The activities of p34^{cdc2}, CDK2 and CDK4 are positively regulated by a CDK-activating kinase (CAK) that phosphorylates threonine 161 of p34^{cdc2} (T160 of CDK2, T172 of CDK4) (Solomon, 1993 et al.; Fesquet et al., 1993; Poon et al., 1993; Kato et al., 1994). The CDK-activating kinase is a CDK7-cyclin H complex (Fisher and Morgan, 1994; Mäkelä et al., 1994) that is constitutively expressed and active throughout the cell cycle of proliferating cells (Tassan et al., 1994), possibly reflecting its function as a regulator of multiple CDKs that are active at different times in the cell cycle. In G2 phase, phosphorylation of p34^{cdc2} on tyrosine 15 by wee1 kinase (Parker et al., 1992; Parker and Piwnicka-Worms, 1992; McGowan and Russell, 1993) and threonine 14 by Myt 1 kinase (Mueller et al., 1995) results in a delay in mitosis, by inhibiting p34^{cdc2}-cyclin B kinase activity, until cells have grown to the appropriate size. At the G2-M phase transition dephosphorylation of these two residues by the dual-specificity phosphatase, *cdc25*, results in the activation of the p34^{cdc2}-cyclin B complex and progression through mitosis (Kumagai and Dunphy, 1991; Strausfeld et al., 1991). These three phosphorylation sites corresponded to serine 99, tyrosine 100 and serine 243 in the sequence predicted from the PFTAIRE cDNA. The conservative substitution of serine for threonine at two of these sites in the PFTAIRE sequence was similar to the substitutions found in other *cdc2*-related protein kinases shown in the sequence alignments in Figure 3.2.

In addition to identifying features suggesting that the PFTAIRE cDNA encoded a *cdc2*-related kinase, sequence analysis of the predicted protein also demonstrated the presence of a

putative nuclear localization signal in the amino-terminal region and consensus motifs for phosphorylation by other kinases. The putative nuclear localization signal PEDKKVR, located at amino acids 20-26, contained the minimal nuclear localization signal (NLS) consensus sequence K/R-K/R-X-R/K. In general, the most conserved feature of the NLS consensus sequence, generated from analysis of homologues of the SV40 T antigen nuclear localization signal (PKKKRKV), is the presence of several positively charged amino acids associated with a proline residue (Garcia-Bustos et al., 1991). Although the sequence found in the amino-terminal region of PFTAIRE had these features it is not known whether this sequence actually acts as a nuclear localization signal. Consensus motifs for phosphorylation by cAMP-or cGMP-dependent protein kinase, protein kinase C and tyrosine kinases were also identified in the PFTAIRE sequence at positions 70-73 (PKA), 64-68 and 331-334 (PKC), and 94-100 (tyrosine kinase). Table 3.1 summarizes the locations of the putative nuclear localization signal and consensus motifs for phosphorylation by other kinases found in the PFTAIRE sequence. The presence of consensus motifs for phosphorylation by other kinases and conservation of specific regulatory sites required for activation of CDKs suggest that PFTAIRE activity may be regulated similarly, by other protein kinases.

3.4 Genomic PFTAIRE

a. The PFTAIRE cDNA represents a single gene

To determine whether the cloned PFTAIRE cDNA represented a single gene, different regions of the cDNA were used to probe Southern blots of mouse genomic DNA digested with various restriction endonucleases. The same genomic Southern blot was hybridized with three different PFTAIRE cDNA probes: 1) the 290 bp 5' region of PFTAIRE (*EcoRI-DraI* fragment); 2) a 740 bp *HindIII* fragment of 3' untranslated sequence (1655-2395 bp) or 3) the entire 2.5 kb cDNA. The results of these hybridizations are shown in Figure 3.3. High molecular weight *XbaI* (~9-10 kb) and *EcoRI* (~7.2 kb) genomic fragments (indicated by vertical arrowheads) were detected by all three probes, suggesting that these cDNA fragments belonged to a single gene. Genomic fragments common to the 5' 290 bp probe and the entire cDNA are indicated by small arrowheads pointing left while fragments that were detected by the 3' 740 bp probe and the 2.5 kb cDNA probe are indicated by larger arrowheads pointing right. The sizes of genomic fragments

detected on the Southern blots suggested the presence of large introns in the PFTAIRE gene. However, the genomic structure of PFTAIRE has not been determined by extensive restriction digest analysis of cloned fragments of genomic DNA. Restriction digest analysis of DNA purified from a clone isolated from a mouse genomic DNA library (strain 129Sv), screened with the 5' 290 bp *EcoRI-DraI* PFTAIRE cDNA probe, indicated the presence of a large intron in the 5' region of the gene close to the translation initiation codon. The Southern blot analysis presented in Figure 3.3 and the restriction digests of the 5' genomic PFTAIRE clone suggest that the cloned cDNA represented a region of the PFTAIRE gene corresponding to approximately 10 kb and that introns were located within this region.

b. PFTAIRE restriction fragment length polymorphisms and chromosomal localization of the mouse PFTAIRE gene, *Pftk1*

The identification of restriction fragment length polymorphisms in genomic DNA from different inbred strains of mice provides a way of determining the chromosomal location of a gene when the inheritance of the polymorphic alleles is examined in an interspecific backcross DNA panel. Information about the chromosomal location of mouse genes can ultimately be used to determine the chromosomal location of human homologues and provide genetic markers that may be used to map genes involved in human diseases.

To determine the chromosomal location of the PFTAIRE gene and its proximity to other genetically mapped loci, the 5' 290 bp PFTAIRE cDNA fragment was used for Southern blot analysis to identify restriction fragment length polymorphisms in genomic DNA from the mouse strains C57BL/6J*Ei* and SPRET/*Ei*. The PFTAIRE cDNA probe contained 5' untranslated sequence and amino-terminal coding sequence, which showed little homology to other known CDKs and *cdc2*-related kinases. When hybridized to genomic DNA digested with several restriction endonucleases the 290 bp cDNA did not detect more than three genomic fragments (Figures 3.3 and 3.4), suggesting that it recognized a single gene and did not cross-react with related genes. Polymorphic fragments detected by the PFTAIRE probe were identified in *MspI*, *EcoRI* and *TaqI* digested genomic DNA from the two parental mouse strains, C57BL/6J*Ei* and SPRET/*Ei* (Figure 3.4). The 94 progeny of the BSS backcross panel ((C57BL/6J*Ei* x SPRET/*Ei*)F₁ x SPRET/*Ei*, The Jackson Laboratory, Bar Harbor, ME) were examined for inheritance of the PFTAIRE *TaqI* polymorphic fragments (approximately 2.1 kb and 7.2 kb for

C57BL/6J*Ei*; 1.6 kb and 10 kb for SPRET/*Ei*) by hybridizing the 290 bp PFTAIRE cDNA probe to Southern blots prepared from *TaqI* digested genomic DNA obtained from the animals. The inheritance of the PFTAIRE *TaqI* polymorphic fragments was compared with the inheritance of approximately 2300 markers that have been typed with the BSS backcross panel (Rowe et al., 1994; The Jackson Laboratory World Wide Web site address). Based on this analysis the PFTAIRE gene (*Pftk1*) was positioned close to the centromere of chromosome 5 (Figure 3.5B). The haplotype distribution of the 94 progeny in the BSS backcross shown in Figure 3.5A demonstrates how the C57BL/6J*Ei* and SPRET/*Ei* alleles for *Pftk1* and four other markers in the centromeric region of chromosome 5 have been inherited. Each column in Figure 3.5A represents a chromosomal haplotype, and the number of animals observed with each haplotype is given below. Forty-five of 94 animals were heterozygous for all five markers included in the analysis, while 40 animals inherited only the SPRET/*Ei* alleles for these markers. The numbers of recombinant progeny are shown in the remaining five columns and provide information about the distances between markers. A higher frequency of recombination (R) between two markers indicates a greater distance in centimorgans (cM) between them. *Pftk1* showed closest linkage with the markers *D5Bir4*, and *D5Mit48* (also known as *Pgy1*, a multidrug resistance gene homologue) *Pftk1* had 0/94 and 2/94 recombinants with the *D5Bir4* and *D5Mit48* markers, respectively (Figure 3.5A). The position of *Pftk1* with respect to other chromosome 5 loci is shown in a map of the proximal region proximal region of chromosome 5 (Figure 3.5B).

Recently the chromosomal location of another cdc2-related kinase gene, the mouse *Cdk5* gene, was reported (Oshima et al., 1995). Based on analysis of mapping panels from (NFS/N x *M. m. musculus*) F₁ x *M. m. musculus*, (C58/J x *M. m. musculus*) F₁ x *M. m. musculus* and (NFS/N x *M. spretus*) F₁ x *M. spretus*, the mouse *Cdk5* gene was also shown to map to the centromeric region of chromosome 5. The *Cdk5* gene was reported to have 0/184 recombinants with the *Dpp6* marker (dipeptidyl aminopeptidase-like protein 6) (Oshima et al., 1995). The close linkage of *Cdk5* with *Dpp6* suggests that *Cdk5* and *Pftk1* are not located in the same position on chromosome 5, because *Pftk1* is approximately 9 cM proximal to *Dpp6*. However, both *Pftk1* and *Cdk5* are located in the proximal region of mouse chromosome 5.

3.5 PFTAIRE mRNA expression

a. PFTAIRE mRNA tissue distribution and developmental expression

The mRNA transcripts of most CDKs and *cdc2*-related kinases identified in higher eukaryotic species are not expressed in a tissue-specific manner (Meyerson et al., 1992; Hellmich et al. 1992; Lew et al., 1992; Okuda et al, 1992; Xiang et al., 1994), possibly reflecting functions as regulators of the cell cycle in cells capable of undergoing mitosis. Because I sought to identify a *cdc2*-related kinase that functioned primarily in the postmitotic cells of the nervous system, specifically as a NF-H kinase, it was important to determine whether the PFTAIRE mRNA transcript showed tissue-specific expression in the nervous system and whether its expression was developmentally regulated.

A Northern blot of total RNA prepared from various adult mouse tissues and hybridized with the full length PFTAIRE cDNA probe (2.5 kb) showed that a PFTAIRE transcript of approximately 5.0 kb was expressed predominantly in tissues from the central nervous system (Figure 3.6). The PFTAIRE transcript was strongly expressed in the brain, cerebellum and spinal cord (lanes 1, 2 and 3) and in lung (lane 9). Longer exposure of the same Northern blot revealed weak expression of PFTAIRE mRNA in the heart. The same RNA blot was hybridized with a mouse β -actin cDNA probe to determine that approximately equal amounts of total RNA were present in each lane (lower panel). In a separate experiment the same transcript size and pattern of expression were obtained when a single blot was hybridized with either the 5' 290 bp cDNA fragment, the 2.5 kb cDNA or with the 740 bp *HindIII* cDNA fragment from the 3' untranslated region of the cDNA (Figure 3.7). These results indicate that the cloned cDNA represented a single PFTAIRE mRNA transcript. The size of the mRNA transcript expressed in mouse tissues was approximately twice the size of the PFTAIRE cDNA clone that was isolated from a mouse brain library, indicating that although the cDNA clone contained a complete open reading frame, it was lacking a significant amount of untranslated sequence. Although most CDKs and *cdc2*-related kinases show widespread expression in mitotic tissues some of these kinases, such as PCTAIRE-1, PCTAIRE-2, PITALRE, PISSLRE and CDK5 are present or enriched in tissues considered to be largely postmitotic, such as the brain (Meyerson et al., 1992; Hellmich et al. 1992; Lew et al., 1992; Okuda et al, 1992; Ino et al., 1993; Graña et al, 1994a; Graña et al., 1994b; Brambilla and Draetta, 1994). However, the size of the PFTAIRE mRNA transcript and its tissue distribution

differed from other CDK and *cdc2*-related kinase mRNA transcripts that have been identified in nervous tissue. The PFTAIRE mRNA transcript was restricted primarily to postmitotic tissues of the nervous system. Although PFTAIRE mRNA appeared similarly abundant in lung and nervous tissue it is unclear why lung, which is not considered terminally differentiated, would be the only tissue outside the nervous system to express a similar amount of the PFTAIRE mRNA. Subsequent immunoblot analysis using PFTAIRE antibodies did not detect the PFTAIRE protein in lung (Chapter 4, Section 4.3a), suggesting that PFTAIRE may not function outside the nervous system.

In order to determine the developmental pattern of PFTAIRE mRNA expression in the nervous system, RNA was prepared from mouse embryos on days 12.5 and 15.5 of gestation, from neonatal pups and at various times during the first four weeks of postnatal development. Figure 3.8 shows that prior to birth on days E12.5 and E15.5 (lanes 1 and 2) expression of PFTAIRE mRNA occurred at very low levels. Expression of PFTAIRE mRNA remained equally low on E18.5 (not shown). However, a marked increase in expression was seen at birth, postnatal day 0.5 (lane 3). The maximum level of PFTAIRE expression, comparable to what was seen in adult animals, was observed by approximately postnatal day 10.5 (lane 7). Hybridization of the same RNA blot with a mouse β -actin cDNA probe suggested that similar amounts of total RNA were present in each lane (lower panel). The marked increase in PFTAIRE mRNA expression at birth, followed by its progressive upregulation during the first two weeks of postnatal development demonstrate that the putative kinase is developmentally regulated. It has been demonstrated previously by others that the expression of *cdc2* kinase mRNA is downregulated in rat cerebral cortex and cerebellum during neurogenesis. Between 16-20 days gestation (E16-E20), as neuronal precursors in the rat cortex undergo their last mitoses to become terminally differentiated, *cdc2* mRNA expression declines to a very low level that is maintained in the postnatal and adult cortex (Hayes et al, 1991). The upregulation of PFTAIRE mRNA expression at birth occurs well after most cells in the mouse nervous system have become postmitotic (Miale and Sidman, 1960; Angevine and Sidman, 1961; Normes and Carry, 1978), making it unlikely that PFTAIRE would be involved in neuronal or glial cell cycle control or exit from the cell cycle to a terminally differentiated state. The temporal and spatial expression patterns of PFTAIRE mRNA expression suggest that, unlike most other CDKs, PFTAIRE may not function in the cell cycle but may be involved in processes related to the formation or functioning of the mature nervous

system.

b. In situ hybridization studies

To determine which populations of cells in the central nervous system expressed PFTAIRE mRNA, 290 bp and 523 bp PFTAIRE cDNA fragments were hybridized *in situ* to 10 μ m sections from adult mouse brain, cerebellum and spinal cord. The 290 bp PFTAIRE cDNA probe included the 5' untranslated region and the unique amino-terminal region of the gene product. The 523 bp probe included the 5' untranslated region and the first three kinase domains of the PFTAIRE gene product. Although hybridizations with both probes produced similar results, the 523 bp probe generated more background. The results presented in Figures 3.9 and 3.10 represent the results of hybridizations with the 290 bp PFTAIRE probe. For comparison of mRNA localization and abundance, cDNA fragments of mouse cdc2 kinase (550 bp, beginning at the ATG codon) and mouse neurofilament light (NF-L) protein (300 bp corresponding to the α -helical region of NF-L) were used as probes. Each probe was hybridized to corresponding control sections that had been pretreated with RNAase A (Figure 3.9B, F and J; Figure 3.10B, F and J) to ensure the specificity of hybridization. Additional controls to verify the specificity of *in situ* hybridizations with PFTAIRE and NF-L cDNA probes included 1) using each probe for hybridizations to sections of liver and skeletal muscle, tissues that do not express either mRNA and 2) using a rat prolactin cDNA probe for hybridizations to sections of brain cerebellum and spinal cord, tissues that do not express prolactin mRNA. Neither the NF-L probe nor the PFTAIRE probe hybridized specifically to mRNA in sections of skeletal muscle or liver. The prolactin probe hybridized strongly to mRNA in sections of pituitary gland, but did not hybridize specifically with mRNA in the brain, cerebellum or spinal cord. The control hybridizations suggested that conditions for *in situ* hybridizations with cDNA probes yielded results that could be considered representative of the actual localization of each mRNA transcript that was analyzed.

The results of the *in situ* hybridization experiments demonstrate that PFTAIRE mRNA expression was widespread in the central nervous system, though not uniform. A comparison of hybridizations of PFTAIRE, cdc2 and NF-L probes to brain sections is presented in Figure 3.9. PFTAIRE mRNA was widely distributed in the brain although certain regions displayed higher levels of expression (Figure 3.9A). In the cerebral cortex PFTAIRE expression was strongest in the outer cellular layers and no labelling was detected in layer 1 which contains primarily axons and

dendrites. PFTAIRE mRNA was expressed in the hippocampus, in pyramidal neurons but not in the molecular layer. PFTAIRE mRNA was most abundant in the pyramidal neurons in area CA3 (Figure 3.9C and D). Granule cells of the dentate gyrus also expressed PFTAIRE mRNA. In the thalamus strongly labelled cells were located in the medial habenula (seen in Figure 3.9A). PFTAIRE mRNA was also highly expressed in the primary olfactory cortex (not visible in the section shown in Figure 3.9A). No PFTAIRE mRNA was detected in the corpus callosum. The expression of *cdc2* mRNA in the adult brain was low in comparison to PFTAIRE (Figure 3.9E). Pyramidal neurons of the hippocampus (Figure 3.9G and H) and granule cells of the dentate gyrus demonstrated weak expression of *cdc2* mRNA. A low level of *cdc2* mRNA was also detected in the medial habenula. It is possible that the *cdc2* probe used may have cross-hybridized to PFTAIRE mRNA (or another *cdc2*-related kinase mRNA) in these neurons. However, the same *cdc2* probe did not hybridize in the cerebellar cortex or deep cerebellar nuclei, where PFTAIRE mRNA was strongly expressed, as described below. In addition, hybridization of the *cdc2* and PFTAIRE probes to sections of olfactory bulb demonstrated that *cdc2* mRNA was moderately expressed in the mitral cell layer, where others have detected *cdc2*-immunoreactivity (Okano et al., 1993), and PFTAIRE mRNA was strongly expressed in the mitral cell and granule cell layers. The NF-L mRNA was highly expressed throughout the brain as shown in Figure 3.9 I. The NF-L mRNA was more uniformly distributed throughout the cellular layers of the cortex compared to PFTAIRE, (compare 3.9A and I) and was usually the more abundant mRNA species in neurons. Comparison of PFTAIRE and NF-L mRNA expression also shows that both were most strongly expressed in hippocampus and in the medial habenula while neither was expressed in the corpus callosum.

In the spinal cord (Figure 3.10A and C) the 290 bp PFTAIRE probe hybridized with mRNA in several different populations of neurons throughout the gray matter, in the posterior and anterior horns. Higher magnification of motor neuron cell bodies in the anterior horn shows that PFTAIRE mRNA was well expressed in these cells (Figure 3.10D). The PFTAIRE probes also hybridized specifically to mRNA in a small population of cells in the white matter, perhaps oligodendrocytes, but the remainder of the labelling in the white matter appeared to be nonspecific and was not localized over cell bodies or axons. Hybridization of spinal cord sections with the NF-L probe shows that NF-L expression in the spinal cord was more restricted than that of PFTAIRE. Neurons located in the posterior horn that expressed PFTAIRE mRNA did not express

NF-L mRNA (compare 3.10C and G). However, in anterior horn NF-L mRNA was more abundant than PFTAIRE in motor neuron cell bodies (compare 3.10D and H).

In the cerebellum, PFTAIRE mRNA expression was restricted to cells in the layers of the cerebellar cortex and cerebellar deep nuclei (Figure 3.10 I). A higher magnification of the region marked by arrowheads in Figure 3.10 I shows the hybridization of the PFTAIRE probe to mRNA in Purkinje cells and in some cells in the molecular and granule layers (Figure 3.10K). In contrast, no cells were labelled when the *cdc2* probe was hybridized to cerebellum sections.

3.6 Discussion

a. PFTAIRE is a new member of the *cdc2* kinase family

A cDNA encoding a putative kinase was isolated from 18 day postnatal mouse brain by low stringency hybridization with a cDNA probe encoding the cell cycle regulatory kinase p34^{cdc2}. The predicted amino acid sequence of the 2.5 kb cDNA clone encoded a novel protein of 423 amino acids with homology to serine/threonine protein kinase catalytic domains. Most of the conserved residues and subdomains characteristic of all protein kinases, including a G-X-G-XX-G sequence motif (residues 96-101) which is predicted to form the ATP binding domain of protein kinases (Hanks et al., 1988; Hanks and Hunter, 1995), were present in this new cDNA clone. The putative protein kinase shared extensive homology with *cdc2* homologues, cyclin-dependent kinases (CDKs) and *cdc2*-related kinases, having approximately 48-54% amino acid identity with *cdc2* and other related kinases (Figure 3.2), in the 300 amino acid region corresponding to the *cdc2* kinase catalytic domain.

In addition to the high homology shared with members of the *cdc2* kinase family, the predicted amino acid sequence of the cDNA contained two features indicating that this cDNA represented a new member of the *cdc2* kinase family. Like the other *cdc2* kinase homologues, CDKs and *cdc2*-related kinases identified to date, the new cDNA contained a sequence motif analogous in position and context to the highly conserved PSTAIRE motif (amino acids 44-50 in mouse *cdc2*, Figure 3.2) which is unique to this family of kinases. Mutagenesis of the Arg and Glu residues in the PSTAIRE region of p34^{cdc2} has demonstrated that this region is involved in the binding of regulatory cyclin proteins (Ducommun et al., 1991) that positively regulate p34^{cdc2}

kinase activity (Solomon et al., 1990; Lees and Harlow, 1993). The substitution of phenylalanine for serine in this region of the new cDNA forms a PFTAIRE sequence (residues 129-135, Figure 3.1 and 3.2), which has been used to tentatively name this putative kinase. The PFTAIRE cDNA also contains three sites for potential regulation by phosphorylation that are highly conserved in the *cdc2* kinase family. The phosphorylation of threonine 14 and tyrosine 15 of p34^{cdc2} inhibits kinase activity until the onset of mitosis when dephosphorylation of these residues by *cdc25* causes the abrupt activation of the kinase (Kumagai and Dunphy, 1991; Strausfeld et al., 1991). The homologous residues in PFTAIRE were serine 99 and tyrosine 100. In addition to positive regulation by cyclin binding, activation of p34^{cdc2} is also dependent on phosphorylation of threonine 160 by another member of the *cdc2* kinase family, the CDK-activating kinase (CAK), CDK7. CDK2 and CDK4 are also positively regulated by phosphorylation of threonine residues in the corresponding position (threonine 160 of CDK2, threonine 172 of CDK4) by the CDK-activating kinase (Fesquet et al., 1993; Poon et al., 1993; Kato et al., 1994). In the PFTAIRE sequence a conservative substitution of serine for threonine occurred at this site, serine 243, which is similar to the substitution that occurs in other CDKs and *cdc2*-related kinases (Figure 3.2). Kato et al. (1994) demonstrated that mutating threonine 172 of CDK4 to a serine residue did not alter the ability of the CDK-activating kinase to regulate CDK4 activity. This observation suggests that there is not a strict requirement for a threonine residue at this position, and that the serine 243 residue of PFTAIRE may function as a phosphoacceptor for activation of the kinase.

The cDNA sequences of p34^{cdc2} and closely related CDKs, such as CDK2 and CDK3 encode proteins of approximately 300 amino acids (32-34 kDa), containing little more than the highly conserved kinase subdomains. While several CDKs and *cdc2*-related kinases are similarly small proteins, a subset of *cdc2* kinase family, that includes PCTAIRE -1, -2 and -3, KKLIARE, PISSLRE, PITSLRE and CDK8, contains proteins with amino- and carboxy-terminal regions that extend beyond the p34^{cdc2} catalytic domain (Meyerson et al., 1992; Okuda et al., 1992; Graña et al., 1994; Xiang et al., 1994; Tassan et al., 1995). PFTAIRE, with an amino-terminal region of 85 amino acids and a carboxy-terminal extension of approximately 40 residues, is also included in this group. Nucleotide and amino acid sequence comparisons demonstrate that PFTAIRE is most similar to the PCTAIRE *cdc2*-related kinases. Although PFTAIRE, PCTAIRE-1, -2 and -3 have amino- and carboxy-terminal extensions of similar length, the amount of homology or amino acid

identity among the kinases in these regions is low (Figure 3.2). The function of the amino- and carboxy-terminal extensions of these kinases has not been determined. It was proposed that these regions may form the regulatory components of these putative kinases, providing a function similar to cyclin proteins (Meyerson et al., 1991; Pines, 1993). However, the recent demonstration that human CDK8, which also has amino- and carboxy-terminal extensions, associates with cyclin C in immunoprecipitates from HeLa cells (Tassan et al., 1995) suggests that *cdc2*-related kinases like PFTAIRE may associate with regulatory proteins similar to other CDKs.

b. The PFTAIRE gene and the chromosomal locations of PFTAIRE, CDKs and other *cdc2*-related kinases

Southern blot analysis has shown that large fragments of mouse genomic DNA generated by digestion with *Xba*I and *Eco*RI hybridized similarly with three different PFTAIRE cDNA probes, suggesting that the PFTAIRE cDNA corresponded to a single gene (Figure 3.3). Although the exon-intron structure of the PFTAIRE gene was not determined by extensive restriction digest analysis of cloned genomic DNA fragments, restriction digests and partial sequence analysis of a single 5' genomic clone and the Southern blots presented in Figure 3.3. indicate that the PFTAIRE gene is large and contains introns.

The chromosomal locations of a number of human CDK and *cdc2*-related kinase genes have been determined by others in an effort to examine the possible involvement of rearrangement of cell cycle-related genes in human malignancies (Demetrick et al., 1994; Bullrich et al., 1995). The *CDK2*, *CDK4*, *CDK3*, *CDK6*, *PITALRE*, *PISSLRE*, *PCTK1* and *PCTK3* (*PCTAIRE-1* and *PCTAIRE-3*) genes have been mapped to 12q13, 17q22, 7p13, 16q24, 9q34.1, Xp11.3-p11.23 and 1q31-q32, respectively (Demetrick et al., 1994; Bullrich et al., 1995; Knight et al., 1995; Okuda et al., 1994). Both *CDK2* and *CDK4* localized to 12q13, a region associated with amplifications and translocations in solid tumors, such as the MDM2/GLI amplicon in human sarcomas (Demetrick et al., 1994; Khatib et al., 1993). A polymorphism or rearrangement of *CDK3* was identified in the breast cancer cell line MDA-MB-543, suggesting a possible involvement of *CDK3* in breast cancer malignancies (Bullrich et al., 1995). *CDK6* mapped to a region of chromosome 9 (q34) that showed abnormalities in T-cell non-Hodgkin's lymphoma while *PITALRE* was mapped to a region on chromosome 7 (p13) where deletions have been reported in association with non-Hodgkin's lymphoma (Bullrich et al., 1995). Presently there

is no information to suggest the involvement of *PISSLRE*, *PCTK1* or *PCTK3* in human diseases.

The identification and inheritance of PFTAIRE polymorphic fragments generated by digestion of mouse genomic DNA with *TaqI* was used to determine the chromosomal position of the PFTAIRE gene in mouse. The results of this analysis indicated that the PFTAIRE gene, *Pftk1*, was located close to the centromere of mouse chromosome 5 (Figure 3.5B). The human PFTAIRE gene has not yet been identified, so its chromosomal location is not known. However, the *Pgy1* gene (D5Mit48), which is homologous to the multidrug resistance gene (*mdr*) and positioned 2 cM distal to *Pftk1* on mouse chromosome 5, maps to 7q21 (Jackson Laboratory World Wide Web site address). The proximity of *Pftk1* and *Pgy1* on mouse chromosome 5 suggests that the human PFTAIRE gene may also be located in the 7q21 region. The 7q21 region has been reported as a site of gene amplification in several glioblastoma cell lines and in primary and recurrent glioblastoma tumors (Fischer et al., 1994; Kim et al., 1995). In addition, an autosomal dominant split hand/split foot locus designated SHFD maps to the 7q21 region, and chromosomal alterations in this region are associated with the limb development malformation split hand/split foot, or ectrodactyly, which is characterized by the absence of middle rays of the hand or foot (Palmer et al., 1994).

Cdk5 was also recently mapped to mouse chromosome 5, where it showed close linkage with the *Dpp6* gene (Oshima et al., 1995). The close linkage of *Cdk5* with *Dpp6* indicates that *Cdk5* is positioned approximately 9cM distal to *Pftk1* on mouse chromosome 5. The region of mouse chromosome 5 that *Cdk5* mapped to is homologous to human chromosome 7q36, where the human CDK5 gene (*CDK5*) has recently been localized (Demetrick et al., 1994). This region (7q36) does not appear to be a site of chromosomal alterations in solid tumors (Demetrick et al., 1994), and it has not been established whether this gene may be altered in some other disease process. The *engrailed* gene, *en2*, is located close to *Cdk5* on mouse chromosome 5 and the human homologue *EN2* has also been mapped to the 7q36 region. Recently the gene responsible for a congenital limb deformity, polysyndactyly, was mapped to 7q36, suggesting that genes involved in limb pattern formation are located in this region (Tsukurov et al., 1994). Although CDK5 is expressed in several tissues and cell types (Meyerson et al, 1992; Hellmich et al., 1992; Ino et al., 1994) no cell cycle-related function has been determined for this kinase (van den Heuvel and Harlow, 1993). CDK5 is enriched in nervous tissue and it appears to be active

only in postmitotic cells of the nervous system (Tsai et al., 1993). PFTAIRE expression is restricted predominantly to postnatal and adult nervous tissue (Section 3.5), where it also demonstrates kinase activity (Chapter 4, Section 4.4a). Based on these findings polymorphisms or chromosomal rearrangements of either gene would not be expected to contribute to limb defects even though both genes may be located in regions where genes involved in limb formation have been mapped (7q21 and 7q36). It remains to be determined whether chromosomal alterations in either gene are linked to defects in the nervous system.

c. Expression of PFTAIRE mRNA and cell cycle proteins in the nervous system

PFTAIRE mRNA expression was both spatially and temporally restricted, predominantly to tissues of the postnatal and adult nervous system. By Northern blot analysis I have demonstrated that an abundant PFTAIRE mRNA transcript of 5.0 kb was present in total mRNA preparations of adult mouse brain, cerebellum and spinal cord. Among the tissues outside the nervous system examined for PFTAIRE mRNA expression, including thymus, kidney, liver, spleen, skeletal muscle, heart and lung, only lung was found to express a similar level of PFTAIRE mRNA (Figure 3.6). The tissue distribution and size of the PFTAIRE mRNA transcript was examined using three different PFTAIRE probes to determine that the cloned cDNA represented a single mRNA. The same pattern of expression and transcript size was observed with each probe indicating that the cDNA did correspond to a single mRNA transcript (Figure 3.7). Although the PFTAIRE cDNA clone that was isolated was 2.5 kb, the presence of a 5.0 kb mRNA transcript indicates that a large amount of untranslated sequence is lacking in this cDNA clone. The discrepancy in size between the PFTAIRE cDNA clone and mRNA transcript may be due to the use of a polyadenylation signal located further downstream from the polyadenylation signal identified in this clone. During development, expression of PFTAIRE mRNA was extremely low in embryos at 12.5, 15.5 and 18.5 day gestation. However, on the day of birth PFTAIRE mRNA expression was upregulated significantly and continued to increase steadily during the first two weeks of postnatal development (Figure 3.8). This finding indicates that PFTAIRE expression in the nervous system is developmentally-regulated. By in situ hybridization I have demonstrated that PFTAIRE mRNA was expressed in several neuronal populations in the brain, cerebellum and spinal cord. Relative to the expression of *cdc2* kinase, PFTAIRE mRNA is more abundant and more widely distributed in adult nervous tissue (Figure 3.9).

In general, the mRNA transcripts for cdc2 kinase and many CDKs and cdc2-related kinases do not appear to localize in specific tissue systems in adult animals (Meyerson et al., 1992; Hellmich et al., 1992; Okuda et al., 1992; Graña et al., 1994a; Graña et al., 1994b; Brambilla and Draetta, 1994; Xiang et al. 1994). This lack of tissue specific expression by most CDKs perhaps reflects the involvement of many of these kinases in regulation of the mitotic cell cycle. PFTAIRE appears to be the first member of the cdc2 kinase family identified to date to demonstrate such a restricted pattern of mRNA expression in the central nervous system. CDK5, which is expressed in several tissues but enriched in the nervous system (Meyerson et al., 1992; Hellmich et al., 1992; Ino et al., 1994), is the first example of a cdc2-related kinase to demonstrate histone H1 kinase activity only when immunoprecipitated from brain (Tsai et al., 1993). Although CDK5 was initially shown to associate with cyclin D1 in immunoprecipitates from the normal human fibroblast cell line WI38, no kinase activity was detected in this complex (Xiong et al., 1992). The specificity of CDK5 activity in the nervous system is the result of its association with non-cyclin regulatory proteins, p35 and p67, that are expressed specifically in nervous tissue (Tsai et al., 1994; Lew et al., 1994; Shetty et al., 1995). These findings indicate that there is a precedent for cdc2-related kinases that may function only in the nervous system in postmitotic neurons.

Several laboratories have examined the expression of cell cycle related proteins, including CDKs, cyclins and CDK inhibitors, in the nervous system or in neuronal cultures to determine if the presence or absence of these proteins can be correlated with exit from cell cycle, the differentiated state or programmed cell death. During development the expression of cdc2 mRNA in the rat brain and cerebellum is downregulated as neuronal precursor cells undergo neurogenesis to become differentiated neurons. This change in cdc2 mRNA expression occurs in the embryonic cortex between E16 and E20 and postnatally in the cerebellum between 5 and 22 day after birth (Hayes et al., 1991). At the completion of neurogenesis cdc2 mRNA is barely detectable and remains expressed at a very low level throughout adult life (Hayes et al., 1991). In developing mouse spinal cord cdc2 mRNA expression has been demonstrated exclusively in the proliferating cells of the ventricular zone during the period of neurogenesis, between E10.5 and E13.5. By E15, when spinal cord neurogenesis is complete, cdc2 mRNA was not detected (Zhao et al., 1995). ³H-thymidine labelling has been used to demonstrate that as neuronal precursor cells in the adult rat brain, originating in the subependymal layer of the lateral ventricle or the

subgranular region of the dentate gyrus, become differentiated the cdc2 kinase immunoreactivity in these cells declines (Okano et al., 1993). In the adult rat brain p34^{cdc2} kinase immunoreactivity has also been detected in discrete populations of postmitotic cells such as the pyramidal cells of the hippocampus, hilar cells of the dentate gyrus, mitral cells of the olfactory bulb, and in the cerebellar cortex and deep cerebellar nuclei (Okano et al., 1993; Wood and Zinsmeister, 1993). This is in partial agreement with the localization of cdc2 mRNA that I detected in the adult mouse brain in the hippocampus, dentate gyrus (Figure 3.9) and olfactory bulb. Wood and Zinsmeister reported robust anti-PSTAIRE immunoreactivity in nuclei and lower levels of cytoplasmic immunoreactivity in adult rat hippocampal pyramidal neurons, cortical neurons, neurons in all three layers of the cerebellar cortex and in the deep cerebellar nuclei. The results of in situ hybridization analysis presented in Figure 3.9 demonstrated that cdc2 mRNA was present at a low level in the hippocampal pyramidal neurons and the dentate gyrus. The same cdc2 probe did not detect cdc2 mRNA in the cerebellum. It is possible that the intense immunoreactivity detected in the hippocampal and cerebellar neurons by Wood and Zinsmeister did not represent p34^{cdc2} entirely and that the anti-PSTAIRE antibody also cross-reacted with another cdc2-related kinase expressed in these neurons, such as PFTAIRE. The absence of cdc2 mRNA expression in the cerebellum that I observed was in agreement with results obtained by Hayes et al. (1991) demonstrating that p34^{cdc2} immunoreactivity was barely detectable with a C-terminal p34^{cdc2} antibody in postnatal day 16 rat cerebellum or in cultures of immortalized cerebellar neurons that had undergone differentiation. Together all of these data indicate that in most populations of neurons the downregulation of p34^{cdc2} kinase is correlated with terminal differentiation and the postmitotic state.

Although cdc2 kinase mRNA and protein expression in the nervous system is downregulated during neurogenesis, terminal differentiation does not necessarily reflect only the loss of key cell cycle regulators like cdc2 or CDK2. A multitude of mRNAs and proteins are upregulated and downregulated during the differentiation process, but the upregulation of other cell cycle-related proteins may be a key component of the mechanism controlling cell cycle arrest during differentiation. Recently it has been demonstrated that induction of G₁ CDK inhibitors, such as p21^{CIP1} and p16^{INK4a}, occurs during terminal differentiation of skeletal muscle and neurons (Halevy et al., 1995; Parker et al., 1995; Lois et al., 1995). This suggests that in addition

to the decreased expression and loss of *cdc2* and CDK2 activity during differentiation, cell cycle inhibitor proteins may be involved in regulating terminal differentiation by inhibiting the activities of CDK4 and CDK6, which do not show a decrease in expression during the differentiation process (Yan and Ziff, 1995). Constitutive expression of p16^{INK4a} in differentiated cells may lead to inhibition of CDK4 and CDK6 activities, which would prevent phosphorylation of retinoblastoma protein and progression through the G₁/S transition. In addition, p21^{CIP1} may regulate terminal differentiation by binding to and inactivating the proliferating cell nuclear antigen (PCNA), the processivity subunit of DNA polymerase δ , which is required for DNA replication.

Expression of CDK5 mRNA and protein during development begins at approximately embryonic day 12. The mRNA and protein levels of CDK5 progressively increase throughout development and are maintained at a high level in the adult (Ino et al., 1994; Tsai et al., 1993). By *in situ* hybridization Ino et al. (1994) demonstrated that at early embryonic stages CDK5 mRNA is expressed at a low level in almost all cells of the neural tube. During late embryonic and early postnatal development the expression of CDK5 becomes less uniform and specific populations of neurons begin to accumulate CDK5 mRNA (Ino et al., 1994). CDK5 histone H1 kinase activity is also detected at E12, and increases in CDK5 activity coincide with the increases in CDK5 expression and the decrease in *cdc2* expression and kinase activity (Tsai et al., 1993). The mRNA of p35, a CDK5 regulatory protein, shows a similar increase in expression in the developing nervous system (Tsai et al., 1994). These findings suggest the possibility that CDK5 may have an early role in the establishment of the neuronal phenotype.

In contrast, the low level of PFTAIRE mRNA expression prior to birth and its marked upregulation beginning at birth suggests that PFTAIRE is probably expressed too late in the developmental program to have a role in the establishment of the postmitotic state of neurons in the central nervous system, where the majority of neurogenesis is completed during embryonic development (Angevine and Sidman, 1961; Miale and Sidman, 1961; Nornes and Carry, 1978). The temporal expression pattern of PFTAIRE mRNA and its widespread expression in many different types of neurons suggests that its function may be less related to neuronal differentiation but possibly involved in processes related to the maturation of the nervous system.

At birth the majority of neurons in the mouse nervous system have differentiated, except for granule cell populations in the olfactory bulb, dentate gyrus and cerebellar cortex that continue

to undergo neurogenesis for the first 2-3 weeks of postnatal life (Jacobson, 1991), cells in the cerebellar molecular layer (Miale and Sidman, 1961) and the neuronal precursor cells that continue to exist throughout the adult life of the animal. However, during early postnatal development the nervous system undergoes major structural reorganization. Widespread programmed cell death of neurons plays a major role in establishing the form of the adult nervous system, but the vast majority occurs prior to birth. Much of the reorganization of the nervous system that occurs shortly after birth is due to the formation and elimination of synapses that will form the mature neuronal circuitry. Studies of the postnatal development of the rat cerebellum have demonstrated that during the first 2-3 weeks after birth while the granule cell and molecular layers are still undergoing differentiation, immature Purkinje cells, formed prior to birth, undergo a period of cell growth and elaboration of dendritic processes. During this time the Purkinje cells establish synaptic contacts in the molecular layer to become functionally mature neurons. (Altman, 1972) Dramatic increases in the number of synapses in the rat parietal cortex and visual cortex during early postnatal development have also been reported. By the third week of postnatal development, the number and distribution of synapses in the rat cortex are similar what is found in adult animals (Aghajanian and Bloom, 1967; Blue and Parnevelas, 1983).

In addition to being a period of synapse formation, the early postnatal period is also a time when a many synapses are eliminated. Although neuronal cell death results in elimination of synapses, synapse elimination also occurs in the absence of cell death in many regions of the nervous system during early postnatal development. The elimination or withdrawal of axon terminals occurs much later than the period of cell death for a many neuronal populations (Cowan et al., 1984). The elimination of synapses during postnatal development was first demonstrated in studies of the developing neuromuscular junction. Shortly after birth, each muscle cell is innervated by several separate axons, but during the first 2-3 weeks of postnatal development there is a progressive elimination of supernumerary axons, so that ultimately each cell is innervated by a single axon (Bennett and Pettigrew, 1974; Korneliussen and Jansen, 1976). Another example of postnatal synapse elimination is the elimination of all but one of the many synapses formed by climbing fibers from the inferior olivary nucleus on Purkinje cells in the cerebellum by approximately postnatal day 15 in the rat (Crepel et al., 1976).

Although synapse formation and elimination help form the mature neuronal circuitry

during the early development of the nervous system, these processes also occur in the mature adult nervous system, though mostly in response to injury or degenerative diseases. Synapse elimination also occurs during normal aging. Following injury, the activation of synapses which are normally functionally ineffective or the stimulation of dendritic or axonal sprouting to form new synapses are properties that are maintained in certain regions of the adult CNS (reviewed in Jacobson, 1991). It is likely that the molecular mechanisms underlying these processes, that occur during postnatal development or in adult organisms in response to injury, require changes in the expression and activity of protein kinases such as PFTAIRE, in postmitotic neurons.

d. Conclusions

A cDNA encoding a novel putative protein kinase named PFTAIRE has been identified by screening a mouse brain cDNA library at reduced stringency with a *cdc2* kinase cDNA probe. The primary sequence predicted from the PFTAIRE cDNA encodes a protein kinase of 423 amino acids which shares all of the features characteristic of serine/threonine protein kinase catalytic domains and contains a PSTAIRE motif that is a unique feature of the *cdc2* kinase family.

PFTAIRE shows on average 50% amino acid identity with p34^{*cdc2*} (CDK1) and other related CDKs or *cdc2*-related kinases. Although the entire PFTAIRE gene was not isolated, Southern blot analysis of mouse genomic DNA indicated that the isolated cDNA represents a single gene that contain introns. The PFTAIRE gene has been mapped to the centromeric region of mouse chromosome 5. One other member of the *cdc2* kinase family, CDK5, has also been mapped to chromosome 5 at approximately 9 cM distal to the location of PFTAIRE. Other genes in the region that PFTAIRE was mapped to are located on human chromosome 7q21, suggesting that the human PFTAIRE gene may also map to 7q21.

The PFTAIRE mRNA transcript was approximately 5.0 kb and was detected by three different PFTAIRE cDNA probes in adult mouse nervous tissue, such as brain, cerebellum and spinal cord. Among the non-neuronal tissues examined only lung expressed PFTAIRE mRNA. Although other CDKs and *cdc2*-related kinases are present or enriched in nervous tissue, all of these kinases are also expressed in many other tissues. This result suggested that PFTAIRE may be the most nervous system-specific member of the *cdc2* kinase family identified to date. During development PFTAIRE mRNA is expressed at very low levels in embryos but shows a dramatic upregulation of expression at birth. During the first two weeks of postnatal development,

expression of PFTAIRE mRNA progressively increases to a level that is maintained in adult animals. The developmentally regulated expression of PFTAIRE mRNA in nervous tissue differs from that of *cdc2* mRNA, which is reduced to very low levels during neurogenesis in embryos and maintained at low levels in adult animals (Hayes et al., 1991). Expression of PFTAIRE mRNA during development also differs from that of CDK5, which begins at approximately embryonic day 12 (Ino et al., 1994). Analysis of PFTAIRE mRNA expression by in situ hybridization demonstrated that PFTAIRE is expressed in many populations of neurons throughout the CNS and in a population of glia in the spinal cord white matter. Some of the same populations of postmitotic neurons also express *cdc2* kinase mRNA, but at a much lower level than PFTAIRE mRNA. In addition, many of these neurons also express CDK5 mRNA (Ino et al., 1994).

In summary the results presented in this chapter indicate that the anatomical, cellular and ontogenic patterns of PFTAIRE expression in the nervous system differ from those of *cdc2* and CDK5, suggesting that the function of this novel protein kinase may differ from the functions of other related proteins. The onset of PFTAIRE mRNA expression at birth occurs later than the period of neurogenesis for most populations of neurons, which suggests that the function of PFTAIRE in postmitotic neurons may not be related to exit from cell cycle or terminal differentiation. In contrast, the earlier onset of CDK5 expression and the recent demonstration that mice lacking the CDK5 gene showed abnormal brain development (Oshima et al., 1996) suggest that CDK5 could be involved in neurogenesis and terminal differentiation. PFTAIRE mRNA expression is upregulated in the nervous system at a time when the nervous system undergoes extensive reorganization to form the mature neuronal circuitry. Thus PFTAIRE may be required to establish or maintain properties that are acquired by neurons as they mature.

Table 3.1 Consensus motifs found in the PFTAIRE sequence

Consensus sequence type	Consensus sequence	Position in PFTAIRE sequence	PFTAIRE sequence
Nuclear localization signal	K/R-K/R-X-R/K ^a	20-26	PEDKKVR
cAMP- dependent protein kinase	R-(R/K)-X-(S/T) ^b	70-73	RRHS
protein kinase C (PKC)	(R/K) ₁₋₃ (X) ₀₋₂ (S/T)(X) ₀₋₂ (R/K) ₁₋₃ ^b	64-68, 331-334	KESPK, KSLR
tyrosine kinase	K-X ₃ -E-X ₃ -Y	94-100	KLGEGSY

^aGarcias-Bustos et al., 1991

^bKennelly and Krebs, 1991

Figure 3.1 Nucleotide and deduced amino acid sequence for murine PFTAIRE.

The 2467 bp PFTAIRE clone isolated by low stringency screening with a *cdc2* kinase cDNA probe from a cDNA library prepared from 18 day postnatal mouse brain mRNA. Three in frame stop codons located 21 bp, 63 bp and 102 bp upstream from the AUG initiation codon are indicated by arrowheads and a putative polyadenylation signal located 82 bp downstream from the termination codon (*) is underlined. The eleven kinase subdomains (37) are underlined and numbered. The sequence corresponding to the consensus motif for ATP binding (G-X-G-XX-G-X-A), located at amino acids 96-100 is underlined and identified as kinase subdomain 1. The PSTAIRE motif (kinase subdomain 3), characteristic of the *cdc2* kinase family, is located at amino acids 129-135 and contains a substitution of phenylalanine for serine at residue 130. A putative nuclear localization signal at amino acids 20-26 is double underlined.

GAATTCAGTTGCTGCTCTGAGGCTGGCTTGGTGCAAAACACATTGCTAGAGATATGTTAAGGAGTTCAGGTGAATCCGGCTCAAGAGAGACAACACGATATGTCACAAAG 115
 ATGCTTACCCGAACTGCCAGGGACAGATTCACTGATCAAGCACCTGACACAAATTCCTGAAGACAAGAAAGTCAGGGTTCAGAGGACGCAGAGCACTTTTGACCCATTTGAGAAACCA 238
 M S T R N C Q G T D S V I K H L D T I P E D K K V R V Q R T Q S T F D P F E K P 40
 GCCAACCAAGTCAAAAGGGTCCATTCTGAGAACAATGCATGCACTTAATTTAAATCCTCTCTGCTGCAAAAGAGTCACTAAAGTTCGGCGGCACTCCAGCCCCAGCTCGCCAAACGAGT 358
 A N Q V K R V H S E N N A C I N F K S S S A G K E S P K V R R H S S S P S S P T S 80
 CCACAAATTTGGAAAAGCTGACTCATACGAAAACTGGAAAACTGGGGGAAGGATCTTATGCAACAGTGTACAAAGGAAAAGCAAAGTGAATGGGAAGCTGGTGGCTCTAAAGGTGATC 478
 P K F G K A D S Y E K L E K L G E G S Y A T V Y K G K S K V N G K L V A L K V I 120
 CGGCTGCAGGAAGAAGAGGOCACACCTTTTACAGCCATCAGGGAAGCTTCCCTGTTGAAAGGACTAAAGCACGCCAACATCGTGTGCTTCACGACATCATCCACACTAAGGAAACCTCG 598
 R L Q E E E G T E F T A I P E A S L L K G L K H A N I V L L H D I I H T K E T L 160
 ACCCTTGTCTTTGAATACGTGCACACTGATTTATGTCAGTACATGCAAGCAACCTCGGAGGACTCCATCCAGATAATGTGAAGTGTGTTTTATTTTACAGTGTGCGAGGACTGTCTTAC 718
 T L V P E Y V H T D L C Q Y M D K H P G G L H P D N V K L F L F Q L L R G L S Y 200
 ATCCACCAGCGTTATATTTTGCACAGAGACTGAAACCCGAGAACCTTCTCATCAGCGATACGGGGAGTTGAAGCTGGCAGATTTCGGTCTGGCAAGCAAAAATCCGTCCCTAGCCAC 838
 I H Q R Y I L H R D L K P O N L L I S D T G E L K L A D E G L A B A K S V P S H 240
 ACATACTCCAATGAAGTGGTACCTTGTGCTACAGACCTCCAGATGTTCTTCTGGGCTCTACAGAATATCCACCTGCGCTTACATGTGCGGAGTGGCTGTATCTTCTGTGAGATGATC 958
 T Y S N E V V T L W Y R P P D V L L G S T E Y S T C L D M W G V G C I F V E M I 280
 CAAGGAGTTGCTGCGTTTCCAGGAATGAAAGACATTCAGGATCAACTTGAACGGATATTTCTGTTCTTGGAACCCGAATGAGGACACGTGGCTCGAGTTCATCTTTACCAATTTT 1078
 Q G V A A F P G M K D I Q D Q L E R I F L V L G T P N E D T W P G V H S L P H F 320
 AAGCCAGAAGCTTTACCGTGTACAACCTTAAAGCCCTTAGACAAGCATGGAATAAGCTCAGCTATGTAATCATGCTGAAGACTTGGCTCCCAAGCTTCTCCAGTGTTCCTCCAAAGAAC 1198
 K P E R F T V Y N S K S L R Q A W N K L S Y V N H A E D L A S K L L Q C S P K N 360
 AGGCTATCAGCACAGCGCGCTTGAAGCATGAGTATTTTCAAGCATCTGCTCCACGGCTATGGGAGCTGACTGATATGCTTCTATTTTACCCTCCCAATGTGAGATTGCAACCAGAA 1318
 R L S A Q A A L S H E Y F S D L P P R L W E L T D M S S I F T V P N V R L Q P E 400
 GCTGAGAGAGCATGAGCGGCTTTGGAAAAACAATAGTTATGGAAAAAGCCTATCGAACAGCAAAACACTGACAAGCCCGGCACTCTCAGGAGAGCACAGATTAAAGTTGTCACTATTCT 1438
 A G E S M R A F G K N N S Y G K S L S N S K H *
 GGGAGAAGAAGAAGATAAAAATAAATCAATCAATATAAAACCACTAATGAAGTGGCTCAGTCCGAGGGCCCTGAATCGGTCTCTTCTGCCCCGGTGCAGTGGATTTCACTGACATGAA 1558
 AATGGAAGCTGGCAAGAACCTGTTTCTCTGCAATTTATTTAAACCTTGACAGCATTTGGATACCTTGTGACTTCCAAGAAGTACGTGACGATTAAAGCTTGGCATGTATTCTTTTCCGT 1678
 CTTTGTGTCTGTTCTGTTTGTCTTCCCTGCCGACAGTGTCCATGCCAGGCTGTCTCTGTGAAGGGTTTTGCTGCTTTTCAAGCACTCTCTTAAGTACATTAAAGACAACACATTG 1798
 GTGTTCACTTCTCAGCCATGTCTGCACTTGAAGGCCACACAGCGGAGGAGACAATGTGTGATTTCCTTGAGAGCAGAGTGCATCTGCAACCTCCAGGAAGAATCTTTTGTGCT 1918
 AAAACAACTCCATGCTTTCTGACAGACTAGACAGCGTGGTCCAGACAGTGGGGCCCTACCTGTTGCTTGCCTTGGAGGACATTTAGAAATGTAAGGGTTTTTTGTTTTGTTTTGGTT 2038
 TTCGTTTTTATGTGAAAAGTAGCACACATTTACACAGGAGACCAAAATCCACTTGTCTATTTCAATAGCCTGACTGTTTCTGAGCAGGTAGAGAAATAAACATGGAAAAACAAGACAGGGT 2158
 GACTGAAGTTAATAAGCAATTACTATCATTTTATTTAGCTGTAGAAAGCAAGGCAACTTTTTCATGTGCTCTGTCCACATCTTACCTTTTCCCATCAGCTCTGTATTATTAAGTGT 2278
 ACACCTCTATTTTAACTGGAGAAAAATAGTAACAAATATCATTTTAAACCTGGGTACATAGAAAAAAAATCTGTCTGTCAAGGTAGGTCCTGAATGCAGCCTTGCTGATTAAAGCT 2398
 TAGAAATCACATTTTAAATTTATCCAGAGTTAAAAATGAGCTTATTTACGACAAAGACCTTTGAATTC

Figure 3.2 Alignment of deduced amino acid sequences of PFTAIRE with p34^{cdc2} and cdc2-related kinases.

Boxed segments indicate regions where amino acid residues are identical or highly conserved in all sequences. Asterisks are placed above conserved phosphorylation sites corresponding to Thr14, tyrosine 15 and threonine 161 of p34^{cdc2}. Dots indicate where no residue is present and < represents the predicted continuation PCTAIRE-2 and PCTAIRE-3 open reading frames. p34^{cdc2} is the murine sequence reported by Spurr et al. (1990) and CDK2, PCTAIRE-1, PCTAIRE-2, PCTAIRE-3 and CDK5 are human sequences reported by Meyerson et al. (1992).

PCTAIRE-1	MDRMKKIKRQLSMTLRGGRGIDKT.NGAPEQIGLDES	36
PCTAIRE-2	<MKKFKRRLSLTLRGSQTIDESLSELAEQMTTEEN	34

PFTAIRE	MSTRNC	6
PCTAIRE-1	GGGGGSDPGEAPTRAAPGELRS.....ARGPLSSAPEIVHEDLKMGSDGESDQAS	86
PCTAIRE-2	SSKDNPIVKNGRPPTSHSMHSFLHQYTGSFKKPLRRPHSVIGGSLGSFMAMPNGSRDLIVHENLKMGSDGESDQAS	114

PFTAIRE	QGTDSVIKHLDTIPEDKKVRVQRTQSTFDPFEKPANQVKRVHSENNACINFKSSSAGKESKVRHSSPSSPTSPKFGK	85
PCTAIRE-1	ATSSDEVQSPVRVRMRNHPKRKISTEDINKRLSLPADIRLPEGYLEKLTNSPIFDKPLSRRLRRVSL....SEIGFGK	161
PCTAIRE-2	GTSSDEVQSPGTGVLNRRIHRPISMEDLNKRLSLPADIRIPDGYLEKLIQNSPPFDQPMSPRRRASL....SEIGFGK	189
PCTAIRE-3	<KRLSLPMDIRLPQEFQLQOMESPDLPKPLSRMSRRASL....SDIGFGK	46

CDC2	MEDYIKIEKIGEGTYGVVYKGRHRVTGQVAMKKIRLESEEGVPSTAIREISLLKELRHENIVSLQDVLMODSRFVLI	79
CDK2	MENFCKMEKIGEGTYGVVYKGRHRVTGQVAMKKIRLDTETEGVPSTAIREISLLKELRHENIVSLQDVLMODSRFVLI	79
PFTAIRE	ADSYEKLEKLGECSYATVYKGSKVNKFLVALKVIRIQ.EEEGTPTFAIREASLLKGLKHANIVLHLDIHTKEITLTLV	163
PCTAIRE-1	LETYIKLDKLGECSYATVYKGSKLTNMLVALKEIRLEHE.EGAPCTAIREVSLLKGLKHANIVLHLDIHTKEITLTLV	239
PCTAIRE-2	METVYIKLEKLGECSYATVYKGRSKLTENLVALKEIRLEHE.EGAPCTAIREVSLLKGLKHANIVLHLDIHTKEITLTLV	267
PCTAIRE-3	LETVYIKLDKLGECSYATVYKGRSKLTENLVALKEIRLEHE.EGAPCTAIREVSLLKGLKHANIVLHLDIHTKEITLTLV	124
CDK5	MQYMEKLEKIGEGTYGVVYKGRHRVTGQVAMKKIRLESEEGVPSTAIREISLLKELRHENIVSLQDVLMODSRFVLI	79

CDC2	FEFLSMDIKKYLDOSIPPGQFMDSSIVKSYLHDILOGIVFCHSRVVLHRDLKPQNLLIDKGTIKLADFGLARAFGIEIR	158
CDK2	FEFLSMDIKKYLDOSIPPGQFMDSSIVKSYLHDILOGIVFCHSRVVLHRDLKPQNLLIDKGTIKLADFGLARAFGIEIR	157
PFTAIRE	FEYVHTDLCQYMDKH..FGGLHPDINVKLFLFQLLRGLSYIHOYIILHRDLKPQNLLISDTGELKLADFGLARAKSVPSH	240
PCTAIRE-1	FEYLDKDLKQYMDCC..GNIINMHNVKLFLFQLLRGLSYIHOYIILHRDLKPQNLLINERGELKLADFGLARAKSVPTK	316
PCTAIRE-2	FEYLDKDLKQYMDCC..GNIMSMHNVKLFLYQILRGLSYIHOYIILHRDLKPQNLLINERGELKLADFGLARAKSVPTK	344
PCTAIRE-3	FEYLDSDLKQYMDHC..GNLMSMHNVKLFLFQLLRGLSYIHOYIILHRDLKPQNLLINERGELKLADFGLARAKSVPTK	201
CDK5	FECDQDLKNEFDC..NGDLDPEIVKSLFQLLKGLGECHSRVVLHRDLKPQNLLINERGELKLADFGLARAFGIEIR	155

CDC2	TYTHEVTLWYRPPDVLLGSEYSTQIDMWGVCIFVEMT.QGVAAFPGMKDIDQLERIFLVLGTFNEDTWPGVHSL	235
CDK2	TYTHEVTLWYRPPDVLLGSEYSTQIDMWGVCIFVEMT.TREAFPGDSEI.DQLFRIFRILGTFNEDTWPGVHSL	234
PFTAIRE	TYSNEVTLWYRPPDVLLGSEYSTQIDMWGVCIFVEMA.TGRPLFPGSTVE.EQLHIFRILGTFNEDTWPGVHSL	318
PCTAIRE-1	TYSNEVTLWYRPPDVLLGSEYSTQIDMWGVCIFVEMA.TGRPLFPGSTVE.EQLHIFRILGTFNEDTWPGVHSL	393
PCTAIRE-2	TYSNEVTLWYRPPDVLLGSEYSTQIDMWGVCIFVEMA.SGRPLFPGSTVED.EQLHIFRILGTFNEDTWPGVHSL	421
PCTAIRE-3	TYSNEVTLWYRPPDVLLGSEYSTQIDMWGVCIFVEMA.TGRPLFPGSTVKE.EQLHIFRILGTFNEDTWPGVHSL	278
CDK5	CYSAEVTLWYRPPDVLLGSEYSTQIDMWGVCIFVEMANAGRPLFPGNDVDD.QLKRIFRILGTFNEDTWPGVHSL	233

CDC2	DYKN.TFPPKW.KPGSLASHVKNDLDEGLD..LLSKMLVYDPTKRISGKMAIKHPYFDLQNOIKKM	297
CDK2	DYKP.SFPPKW.ARODFSKVVPFLDEGDS..LLSKMLHYDPNKRISAKAALAHPPFODMTKPVPHRL	298
PFTAIRE	EFKPERFTVYNSKSLRQAWNKL.SYVVAHAEDLASKLLQCSFKNRLSADAALSHEVSDLPRLWELLIDMSSIFTVPNVR	400
PCTAIRE-1	EFKTYNYPKY.RAEALLSHAPRLDSGADL..LTKLLOFEGNRNRSABAMKHPFELSGLERIHKLEDTTSIFALKIEIQ	473
PCTAIRE-2	EFKTYNYPKY.KPQPLINHAPRLDSEGTEL..LTKLLOVESKNRVSABEAMKHPVFRSLGERIHKLEDTTSIFALKIEIQ	500
PCTAIRE-3	EFKTYNYPKY.LPQPLINHAPRLDTGHL..LSSLLVYESKSRMSABAALSHSVFRSLGERVHOLEDTASIFSALKIEIQ	358
CDK5	DYKP..YEMYPATTSLVNVAPKLNATGRDL..LONLLKCNFVCRISABEALCHPVESDFCPP	291

PFTAIRE	LQPEAGESMRAFGKNNSYGKSLNSKH	423
PCTAIRE-1	LQKEASLRSSSMFDSGRPAFRVVDTEF	496
PCTAIRE-2	LQKDPGFRNSSYPETGH.GKNRRQSMFL	523
PCTAIRE-3	LQKDPGYRGKAFQOPGR.GKNRRQSMFL	380

Figure 3.3 The PFTAIRE cDNA represents a single gene.

Southern blot analysis of mouse genomic DNA with three PFTAIRE cDNA probes. Mouse genomic DNA was digested with the restriction endonucleases indicated above each lane and used to prepared a Southern blot. The Southern blot was hybridized with three different PFTAIRE cDNA probes 1) a 290 bp *EcoRI-DraI* fragment containing 5' untranslated sequence and amino-terminal coding sequence, 2) the full length (2.5 kb) PFTAIRE cDNA and 3) a 740 bp *HindIII* fragment from the 3' untranslated region of the cDNA. The locations of the 290 bp and 740 bp fragments are shown in a linear map of the PFTAIRE cDNA. Vertical arrowheads indicate high molecular weight *XbaI* (~9-10 kb) and *EcoRI* (~7.2 kb) genomic fragments that hybridized to all three probes. Small arrowheads pointing left indicate fragments that hybridized with the 2.5 kb and 290 bp PFTAIRE probes. Genomic fragments common to the 2.5 kb cDNA and the 740 bp probes are indicated by arrowheads pointing right. Between hybridizations, the Southern blot was stripped to ensure that each autoradiogram represented the result of a single hybridization.

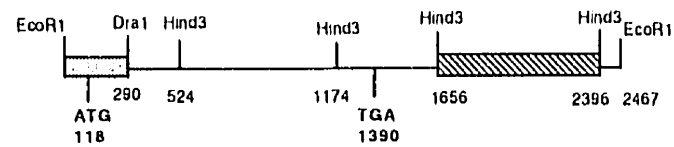
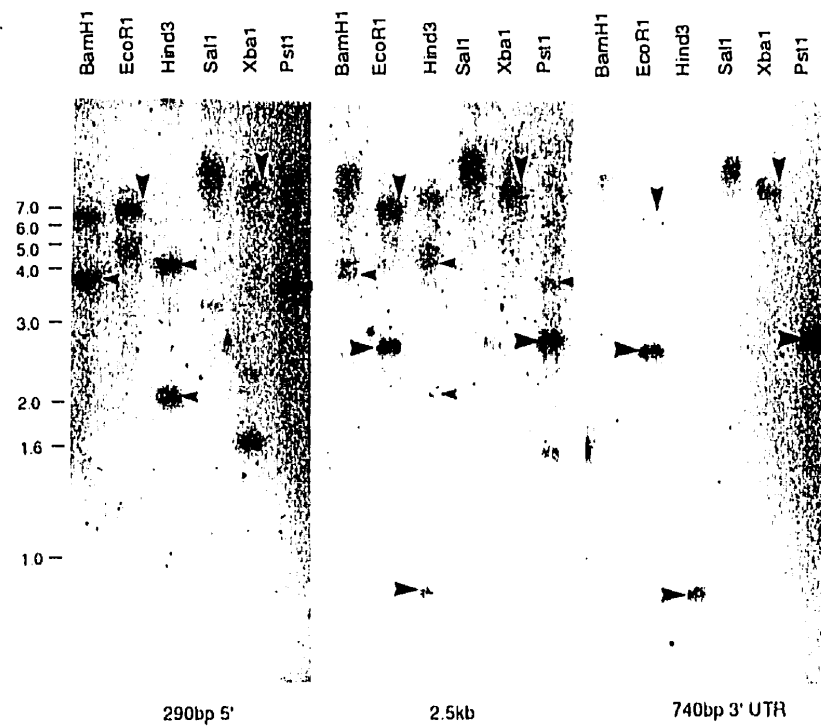


Figure 3.4 PFTAIRE restriction fragment length polymorphisms.

Identification of PFTAIRE restriction fragment length polymorphisms in C57BL/6J*Ei* and SPRET/*Ei* parental strains. The 5' 290 bp *EcoRI-DraI* fragment of the PFTAIRE cDNA was used to detect polymorphic fragments on a Southern blot when the genomic DNA from the two parental strains was digested with *MspI*, *EcoRI*, *PvuII* and *TaqI*. Inheritance of the 2.1 and 7.2 kb *TaqI* fragments from C57BL/6J*Ei* and of 1.6 kb (arrowheads) and approximately 10 kb (not shown) *TaqI* fragments from SPRET/*Ei* was typed in the (C57BL/6J*Ei* x SPRET/*Ei*)F₁ x SPRET/*Ei* backcross to determine the chromosomal location of the PFTAIRE gene.

PFTAIRE RESTRICTION FRAGMENT LENGTH POLYMORPHISMS

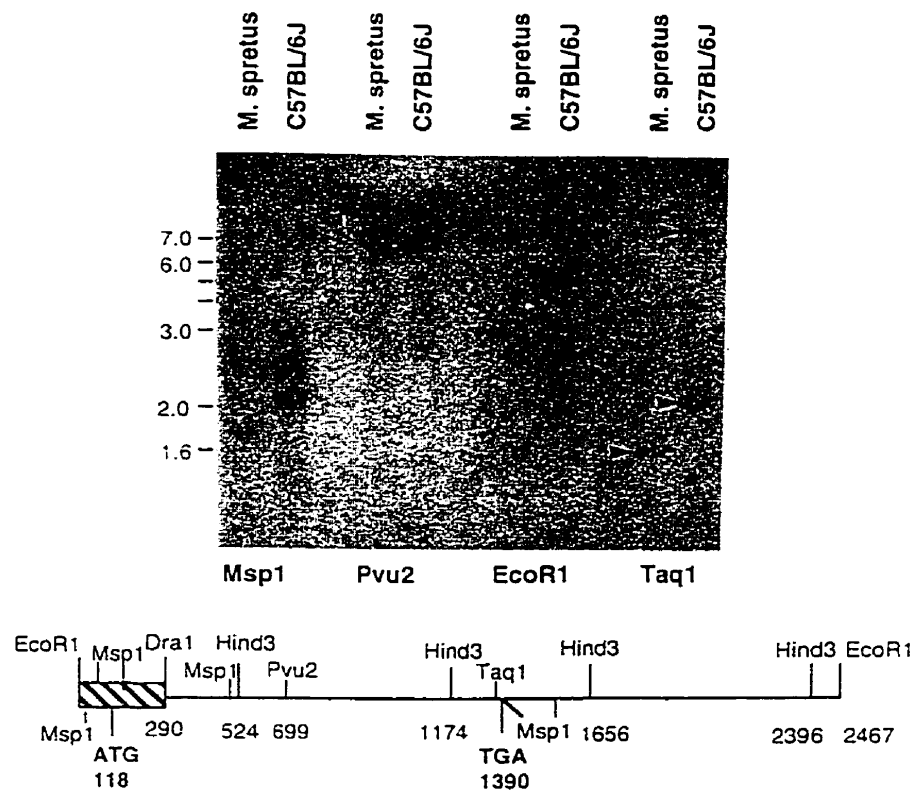


Figure 3.5 Location of the PFTAIRE gene (*Pftk1*) on mouse chromosome 5.

A) Haplotype distribution of 94 progeny in the (C57BL/6J*Ei* x SPRET/*Ei*)F₁ x SPRET/*Ei* backcross. Each column represents a chromosomal haplotype and the number of animals observed with that haplotype is given below. Animals were scored at each locus as heterozygous for the SPRET/*Ei* and C57BL/6J*Ei* alleles (filled boxes) or homozygous for the SPRET/*Ei* alleles (open boxes). R represents the recombination frequency or distance in centimorgans (cM) between adjacent loci. **B)** A genetic map of the proximal region of mouse chromosome 5 constructed from data typing the inheritance of approximately 2300 markers using the BSS backcross panel (Rowe et al., 1994; The Jackson Laboratory World Wide Web Site address) shows the position of *Pftk1* relative to other loci. The map distances in centimorgans (cM) were calculated, as described previously (Rowe et al., 1994), from the recombination frequency observed for each interval. The complete data, including references and notes, for all loci presented in the proximal map are available on the Jackson Laboratory World Wide Web site. The mouse symbol *Pftk1* has been approved by the Mouse Nomenclature Committee. The MGD Accession number for *Pftk1* is MGD-CREX-675.

A

Chromosome 5

							R	SE
<i>D5Bir4, Ptk1</i>	■	□	■	□	□	■	2.13	1.49
<i>D5Mit48</i>	■	□	□	■	□	■	3.19	1.81
<i>D5Mit1</i>	■	□	□	■	■	□	4.25	2.08
<i>Dpp6</i>	■	□	□	■	■	□		
	45	40	1	1	3	3	1	

B

Proximal Chromosome 5

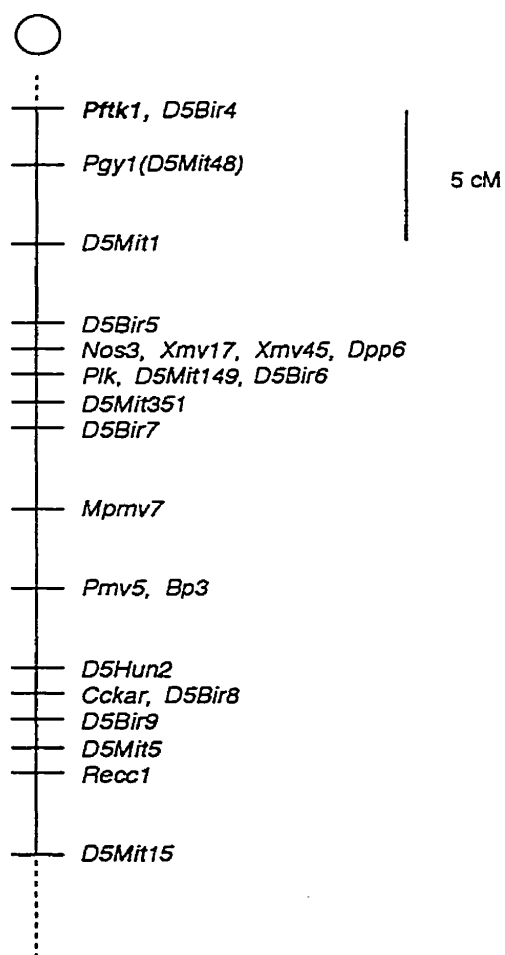
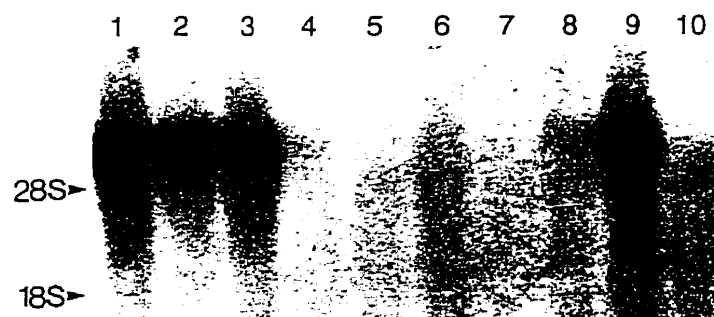


Figure 3.6 Northern blot analysis of PFTAIRE mRNA expression in adult mouse.

Tissue distribution of PFTAIRE mRNA. Total RNA (20 µg per lane) extracted from adult mouse tissues; brain (lane 1), cerebellum (lane 2), spinal cord (lane 3), heart (lane 4), thymus (lane 5), spleen (lane 6), liver (lane 7), kidney (lane 8), lung (lane 9) and skeletal muscle (lane 10). The upper panel shows hybridization with a 2.5 kb PFTAIRE cDNA probe and the lower panel shows hybridization of the same blot with a mouse β -actin cDNA probe.



2.5kb PFTAIRE kinase



actin

Figure 3.7 The PFTAIRE cDNA represents a single mRNA transcript.

Northern blot analysis of the PFTAIRE mRNA with three PFTAIRE cDNA probes. Total RNA (20 µg per lane) extracted from adult mouse tissues was used to prepare a Northern blot. The Northern blot was hybridized with three different PFTAIRE cDNA probes: 1) a 290 bp *EcoRI-DraI* fragment containing 5' untranslated sequence and amino-terminal coding sequence, 2) the full length (2.5 kb) PFTAIRE cDNA and 3) a 740 bp *HindIII* fragment from the 3' untranslated region of the cDNA. Between hybridizations, the blot was stripped and exposed to X-ray film to ensure that each autoradiogram represented the result of a single hybridization.

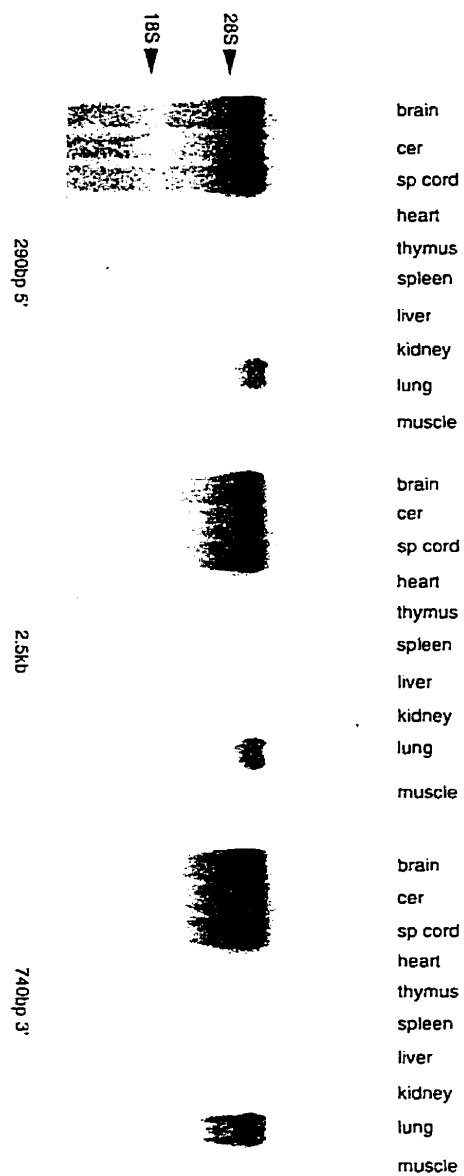


Figure 3.8 Northern blot analysis of PFTAIRE mRNA expression during development.

Developmentally regulated expression of PFTAIRE mRNA. Total RNA (20 µg per lane) extracted from mouse embryos, neonatal and postnatal brains and adult mouse brain; E12.5 whole embryos (lane 1), E15.5 heads (lane 2), P0.5 neonatal (lane 3), P2.5 (lane 4), P3.5 (lane 5), P5.5 (lane 6), P10.5 (lane 7), P15.5 (lane 8), P21.5 (lane 9), P27.5 (lane 10) and adult (lane 11). The upper panel shows hybridization with the 5' 290 bp *EcoRI-DraI* PFTAIRE cDNA fragment and the lower panel shows hybridization of the same blot with a mouse β -actin cDNA probe.

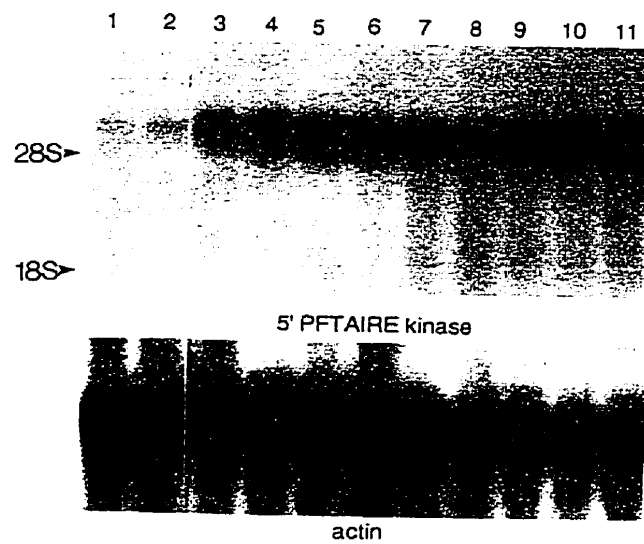


Figure 3.9. In situ localization of PFTAIRE, cdc2 and NF-L mRNA in adult mouse brain.

Shown are autoradiograms of 10 μ m coronal sections from adult mouse brain hybridized with a 5' 290 bp PFTAIRE (A), cdc2 (E) and NF-L (I) cDNA probes. Autoradiograms shown in B, F and J are of sections hybridized with the same probes as A, E and I, following pre-treatment with RNAase A to abolish hybridization with mRNA. Dark field micrographs show hybridization of the PFTAIRE (C), cdc2 (G) and NF-L (K) probes in the hippocampus. Bright field views of pyramidal cells in the CA3 region (marked by white arrowheads in C, G and K) are shown in D, H and L for comparison of mRNA abundance. A, B, E, F, I and J are the same magnification. Scale bar in B, 2 mm. C, G and K are the same magnification. Scale bar in C, 1 mm. D, H and L are the same magnification. Scale bar in D, 10 μ m.

PFTAIRE

cdc2

NFL

A



E



I



B



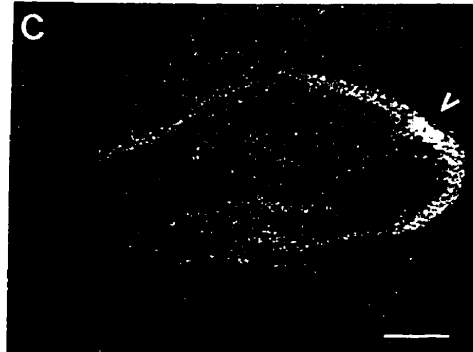
F



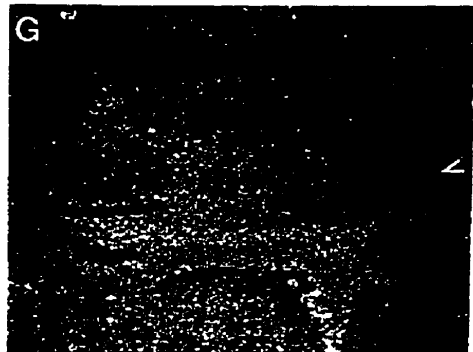
J



C



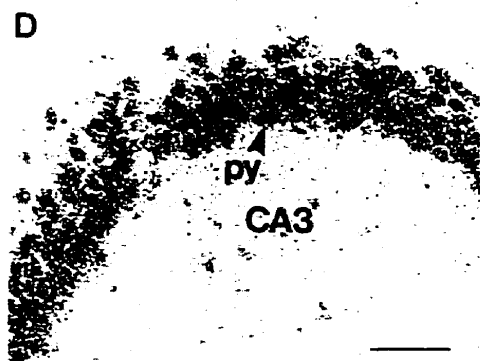
G



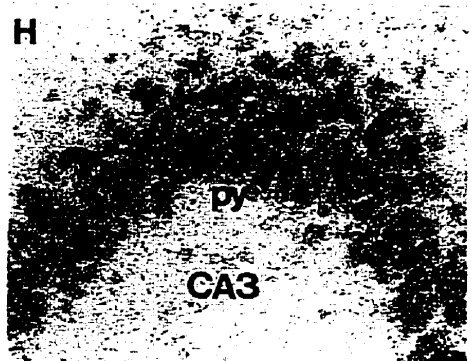
K



D



H



L

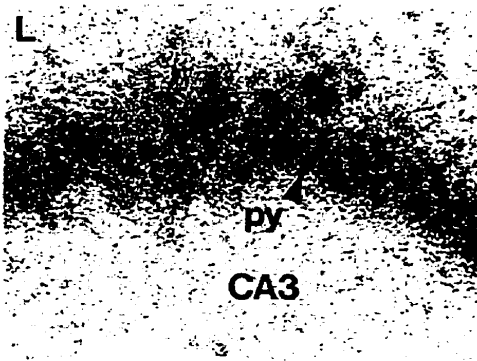
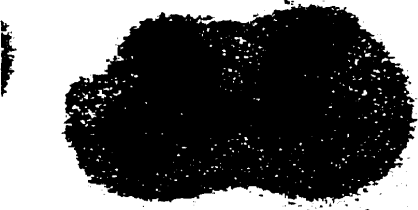


Figure 3.10 Expression of PFTAIRE mRNA in adult mouse spinal cord and cerebellum.

Autoradiograms of 10 μ m sections of spinal cord hybridized with a 5' 290 bp PFTAIRE cDNA probe and a NF-L cDNA probe are shown in A and E. Shown in B and F are autoradiograms of sections hybridized with the same probes as A and E, following RNAase A pre-treatment. Dark field micrographs show spinal cord sections hybridized with PFTAIRE (C) and NF-L (G) probes. Bright field micrographs of the regions marked by white arrowheads in C and G show hybridization of PFTAIRE and NF-L probes to mRNA in the motor neurons. A dark field micrograph of a 290 bp PFTAIRE cDNA probe hybridized to a section of cerebellum is shown in I. A control section of cerebellum pre-treated with RNAase A prior to hybridization with the PFTAIRE probe is shown in J. A bright field micrograph (K) of the region marked by white arrowheads in I shows PFTAIRE mRNA expression in the Purkinje (p) and molecular layers (mol) of the cerebellum. A, B, E and F are the same magnification. Scale bar in F, 2 mm. C and G are the same magnification. Scale bar in G, 1 mm. D and H are the same magnification. Scale bar in H, 10 μ m. I and J are the same magnification. Scale bar, 1 mm. The scale bar in K represents 10 μ m.

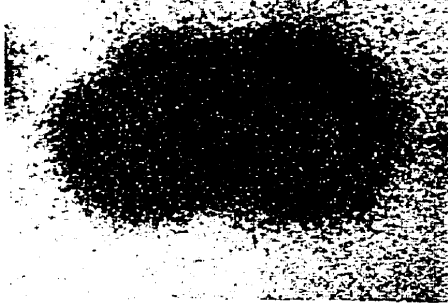
A



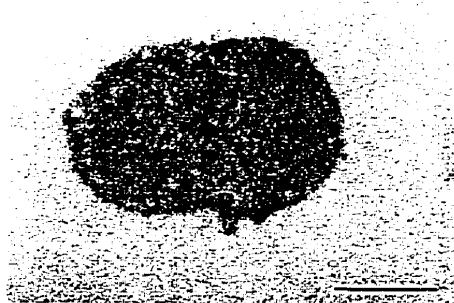
E



B



F



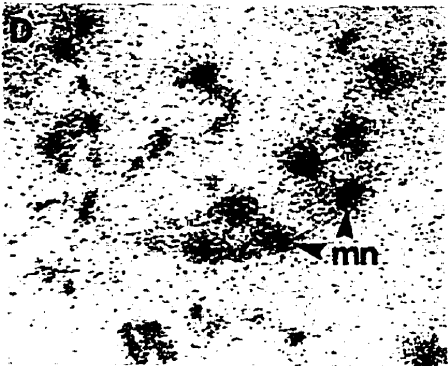
C



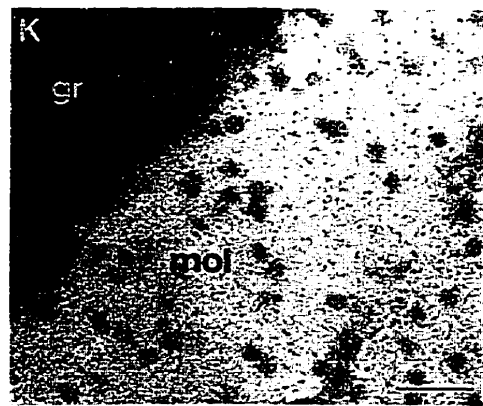
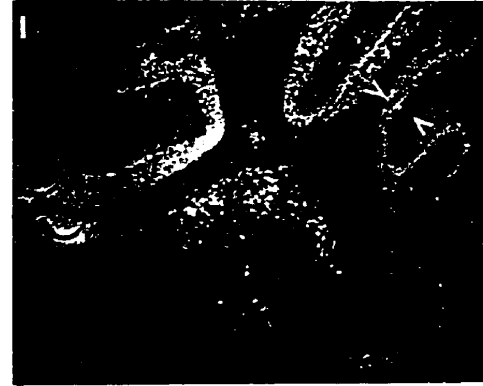
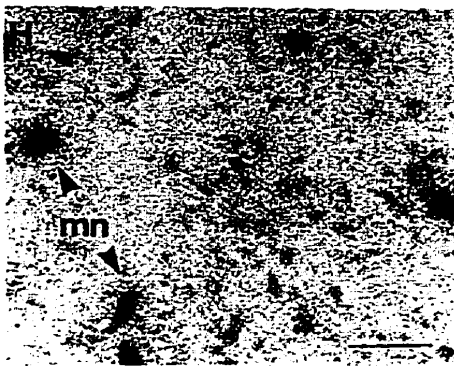
G



D



H



CHAPTER 4
ANALYSIS OF PFTAIRE PROTEIN EXPRESSION
and
KINASE ACTIVITY
IN THE MOUSE NERVOUS SYSTEM

4.1 Introduction

Results presented in Chapter 3 demonstrated that the PFTAIRE cDNA clone encodes a putative protein kinase, related to the *cdc2* family of protein kinases. Analysis of mRNA expression indicated that the PFTAIRE cDNA represents a single mRNA species that is expressed primarily in the postnatal and adult nervous system in many populations of neurons and in some glia. Compared to most of the other CDKs and *cdc2*-related kinases that have been identified, PFTAIRE mRNA demonstrated nervous system specific expression, suggesting that the activity of this putative protein kinase may be involved in processes that are unique to the functions of the maturing nervous system.

To study the PFTAIRE gene product and begin to address PFTAIRE function antibodies were raised against PFTAIRE to specifically detect PFTAIRE in mouse tissues. Polyclonal antibodies to two different domains of PFTAIRE were raised and the results of experiments examining the specificity of the antibodies are presented in this chapter. One of the antibodies was used more extensively to examine the protein distribution, localization and kinase activity of PFTAIRE. Results demonstrating the presence of PFTAIRE in nervous tissue and its subcellular localization are presented in this chapter. In addition, evidence for kinase activity in immunoprecipitates from cytosolic protein preparations of mouse brain or PFTAIRE-transfected cells is presented in this chapter.

4.2 Antibodies specific for PFTAIRE

For the production of PFTAIRE antibodies, peptides derived from the amino- and carboxy-terminal regions of PFTAIRE were selected because neither region showed significant homology to other known *cdc2*-related kinases. The amino-terminal antibody was raised against a bacterial fusion protein of amino acids 1-68 of PFTAIRE fused in frame to GST (refer to Figure 2.6B). The carboxy-terminal antibody was raised against a 15 amino acid synthetic PFTAIRE peptide (amino acids 409-422, refer to Figure 2.10 for sequence) conjugated to keyhole limpet hemacyanin. To determine the ability of each antibody to recognize PFTAIRE, the PFTAIRE mRNA was transcribed and translated *in vitro* using rabbit reticulocyte lysate and the translation product was immunoprecipitated with each antibody. The PFTAIRE translation product was immunoprecipitated by the C-terminal (PFC5) and N-terminal antibodies (PGN5) (Figure 4.1A, lanes 2 and 5), but not by pre-immune serum from either rabbit (lanes 1 and 4). The translation

product migrated close to the molecular mass predicted from the PFTAIRE nucleotide sequence (47.5 kDa). Pre-incubation of each antibody with a molar excess of either the C-terminal peptide or the GST-N-terminal PFTAIRE fusion protein completely inhibited the immunoprecipitation of the PFTAIRE translation product (lanes 3 and 6).

To verify that both antibodies recognized the same protein, COS7 cells were transfected transiently with a CMV-PFTAIRE expression plasmid (pcPF) and cell lysates were prepared for immunoprecipitations with each antibody. The immunoprecipitated samples were used to prepare western blots. When the C-terminal antibody (PFC5) was used for immunoprecipitations, PFTAIRE was detected on the immunoblot by the N-terminal antibody (PGN5) only in the PFTAIRE-transfected sample (Figure 4.1B, lane 4). The N-terminal PFTAIRE antibody did not detect specific proteins in the nontransfected COS7 cell lysate after immunoprecipitation with PFC5 (lane 2). Specific proteins were not detected in nontransfected or PFTAIRE-transfected samples after immunoprecipitation with pre-immune serum (lanes 1 and 3). Figure 4.1C shows that similar results were obtained when the N-terminal antibody was used for immunoprecipitations and the C-terminal antibody was used to detect PFTAIRE on the western blot. These data suggest that both antibodies recognized PFTAIRE.

Preliminary efforts to use each antibody to detect PFTAIRE on western blots of cytosolic protein samples prepared from mouse tissues indicated that the PGN5 antibody recognized more than one protein in these samples. Although the antibody reacted on immunoblots with the PGN5 fusion protein (GST-N-terminal PFTAIRE) and PFTAIRE immunoprecipitated from lysates of transfected cells (Figure 4.1B), the ability to recognize a single protein of the correct size in protein preparations from nervous tissue was inconsistent. In addition, the PGN5 antibody cross-reacted with proteins present in other tissues that do not express PFTAIRE mRNA, such as liver and skeletal muscle. In light of these observations, the PGN5 antibody was not used for studies involving the detection of PFTAIRE in protein samples prepared from mouse tissues. The C-terminal PFTAIRE antibody (PFC5), which reacted with a single protein in nervous tissue on western blots (Section 4.3), was used for all experiments to detect PFTAIRE and for immunoprecipitations to study the kinase activity of the protein.

4.3 Characterization of PFTAIRE protein distribution using the PFC5 antibody

a. Immunoblot analysis of PFTAIRE distribution in adult mouse tissues

Results demonstrating that the expression of the PFTAIRE mRNA transcript in adult mouse was restricted predominantly to tissues of the nervous system and lung were presented in Chapter 3 (Section 3.5a, Figure 3.6). This observation indicated that of the many CDKs and cdc2-related kinases identified to date PFTAIRE displayed the most nervous-system specific mRNA expression. The immunoblot presented in Figure 4.2 demonstrates that the C-terminal PFTAIRE antibody, PFC5, recognized specifically a protein of approximately 52 kDa, which is slightly larger than the size predicted from the PFTAIRE nucleotide sequence. The protein was present in the brain, cerebellum, spinal cord, and sciatic nerve (lanes 1-4) but not in non-neuronal tissues that do not express PFTAIRE mRNA, such as skeletal muscle, kidney and liver (lanes 6-8). In separate experiments, PFTAIRE expression was also detected in dorsal root ganglia dissected from adult mice (Figure 4.3A) and in primary cultures of mouse dorsal root ganglia maintained in NGF-containing growth medium. The presence of PFTAIRE in dorsal root ganglia and sciatic nerve protein preparations indicates that PFTAIRE was expressed in both the central and peripheral nervous systems and suggests that PFTAIRE may be expressed in axons or in Schwann cells. Although PFTAIRE mRNA was as abundant in lung as it was in nervous tissue, the 52 kDa protein was not detected in lung (lane 5). In a separate experiment (Figure 4.3A) the PFC5 antibody detected weakly a protein in lung, but the size of this protein was greater than the 52 kDa protein in nervous tissue. This observation was not reproduced in other experiments, so it is possible that this was not a specific reaction.

To determine whether the PFC5 antibody specifically recognized PFTAIRE in protein samples prepared from mouse tissues, the PFC5 antibody was pre-incubated with C-terminal PFTAIRE peptide prior to using the antibody for immunoblotting. When the peptide and antibody were combined in a ratio of 3 μ g of peptide to 1 μ g of antibody, representing approximately a 35-fold molar excess of peptide, the ability of the antibody to detect the 52 kDa protein in samples prepared from nervous tissue was reduced (compare Figure 4.3A and 4.3B). In addition, pre-immune serum from the same rabbit did not recognize the 52 kDa protein on immunoblots, even when used at concentrations 10-fold higher than the 1:2500 PFC5 dilution used for the immunoblots presented in Figure 4.2 and Figure 4.3. These data suggest that the protein recognized specifically in mouse nervous tissue by the PFC5 antibody, is the endogenous

PFTAIRE protein and that this protein is expressed primarily in the nervous system.

b. The localization of PFTAIRE in adult mouse nervous tissue

To determine whether the PFC5 antibody could recognize PFTAIRE in fixed cells or tissue, the antibody was initially used to detect PFTAIRE in transfected cells by indirect immunofluorescence. Shown in Figure 4.4 are NIH 3T3 and Vero cells transfected transiently with PFTAIRE using the pc3PF and the pHermes-L/PF plasmids. Twenty-four hours after transfection the cells were fixed with 50% methanol/50% acetone and processed for indirect immunofluorescence with the PFC5 antibody (described in Chapter 2, Section 2.8c). Few cells over-expressed PFTAIRE, but the protein was clearly detected in these cells with the PFC5 antibody and fluorescein-conjugated goat anti-rabbit IgG (Figure 4.4B and 4.4D). In transfected cells PFTAIRE expression was both cytoplasmic and nuclear. PFTAIRE expression in Vero cells appeared to be greater in the nucleus than in the cytoplasm (Figure 4.4D), whereas in NIH 3T3 cells PFTAIRE seemed more abundant in the cytoplasm (Figure 4.4B). In both cell lines transfected PFTAIRE was also found in cytoplasmic processes. A low level of endogenous PFTAIRE was detected with the PFC5 antibody in nontransfected cells of both cell lines (compare transfected cells to surrounding cells in the field). Pre-immune serum from the same rabbit that produced the PFC5 antibody did not detect transfected PFTAIRE (Figure 4.4A and 4.4C). These preliminary observations indicated that the PFC5 antibody could be used for indirect immunofluorescence to specifically label PFTAIRE-expressing cells in cultures and that it may be useful for examining expression of endogenous PFTAIRE in sections of mouse nervous tissue.

Indirect immunofluorescence with the PFC5 antibody was used to compare the distribution of PFTAIRE protein with that of PFTAIRE mRNA in mouse nervous tissue. Although indirect immunofluorescence with the PFC5 antibody worked well when transfected cells were fixed with methanol/acetone, post-fixation of frozen tissue sections in methanol/acetone resulted in very high background fluorescence. Considerably less background fluorescence occurred when tissues were fixed in 4% paraformaldehyde and frozen for sectioning. Spinal cord and cerebellum were used to analyze PFTAIRE localization in the central nervous system. Because PFTAIRE was detected by immunoblot in sciatic nerve, the distribution of PFTAIRE in this peripheral nerve was also examined by indirect immunofluorescence. Immunofluorescence labelling of spinal cord sections with PFC5, shown in Figure 4.5B, indicated that PFTAIRE protein was widely distributed in the adult mouse spinal cord, though the protein was present in the same

populations of cells as the mRNA. When the pre-immune serum was used as the primary antibody on another section (Figure 4.5A), the section showed background staining similar to what was obtained when no primary antibody was used. In Figure 4.5C and 4.5D the same section of spinal cord was labelled with PFC5 (4.5C) and the antibody SMI32 (4.5D), which recognizes a hypophosphorylated epitope of the neurofilament heavy protein (NF-H). The high magnification of the anterior gray horn shows that PFTAIRE was present in all populations of neurons that contained NF-H protein (Figure 4.5C and 4.5D). Co-expression of the two proteins was most evident in the motor neurons located in the anterior horn. PFTAIRE was also present in populations of smaller neurons in the anterior gray horn (and posterior horn, not shown) that did not contain neurofilament proteins. These observations were in agreement with the distribution of PFTAIRE and NF-L mRNA in the spinal cord, determined by in situ hybridization (Chapter 3, Section 3.5b, Figure 3.10). Also in agreement with the in situ hybridization studies was the localization of PFTAIRE in the white matter, in glial cell bodies (Figure 4.5B) that were not labelled by SMI32 or by an antibody recognizing a hyperphosphorylated epitope of the NF-H protein present predominantly in axons (not shown). In separate experiments spinal cord sections were labelled with PFC5 and an antibody to detect the intermediate filament protein present in astrocytes, glial fibrillary acid protein (GFAP), to determine if the PFC5 immunoreactive cells in the white matter were astrocytes. The GFAP antibody labelled a much higher proportion of the white matter than the PFTAIRE antibody did and the two proteins did not appear to co-localize. This observation and the morphological appearance of the labelled cells suggests the possibility that PFTAIRE was present in oligodendrocytes in the spinal cord white matter. The identity of these cells was not confirmed with antibodies that specifically recognize oligodendrocytes, such as galactocerebroside or 2', 3'-cyclic nucleotide 3'-phosphodiesterase. The subcellular localization of PFTAIRE within neurons was most evident in the large cell bodies of motor neurons, shown in Figure 4.5C at high magnification. PFTAIRE was detected throughout the cytoplasm and in the nucleus of these neurons. In comparison, labelling of the same section with SMI32 (Figure 4.5D) showed that neurofilaments were present in the cell bodies of the motor neurons, but were excluded from the nucleus. Hypophosphorylated NF-H was also present in axons of the motor neurons (Figure 4.5D, small arrow), while PFTAIRE staining was occasionally detected in axons, close to the neuronal cell body (Figure 4.5C, small arrow). The punctate labelling in the spinal cord that occurred with the SMI32 antibody (Figure 4.5D) represents axons in cross section

labelled with this antibody. Some diffuse labelling occurred with the PFC5 antibody that did not appear to represent neuronal cell bodies in the spinal cord (e.g., the right hand side of Figure 4.5C). This labelling may represent a weak reaction of the PFTAIRE antibody with axons that were also labelled by the SMI32 antibody in Figure 4.5D.

When the PFC5 antibody was used for indirect immunofluorescence with sections of cerebellum PFTAIRE protein was detected in the same neuronal populations as the PFTAIRE mRNA (Figure 4.6). PFTAIRE mRNA and protein were present in all three layers of the cerebellar cortex (compare Figure 4.6A and 4.6C). The distribution of PFTAIRE mRNA and protein in the molecular layer was not uniform, suggesting that a subset of neurons expressed PFTAIRE in this layer. In the granule cell layer the PFC5 antibody staining was diffuse and PFTAIRE appeared to be expressed at a very low level in granule cells. PFTAIRE mRNA and protein were also present in Purkinje cells. The PFC5 labelled section shown in Figure 4.6C was also labelled with the SMI32 antibody (Figure 4.6D). Labelling of the same section with the two antibodies demonstrated which populations of neurons co-expressed PFTAIRE and NF-H and allowed direct comparison of the subcellular localization of each protein. In the Purkinje cells PFTAIRE was localized in the nucleus and cytoplasm while NF-H was present only in the cell body (Figure 4.6C and 4.6D), similar to the distribution of the two proteins in spinal cord motor neurons (Figure 4.5C and 4.5D). The subcellular localization of PFTAIRE in the PFC5-immunoreactive cells located in the molecular layer was not clear. NF-H was not detected in cell bodies in the molecular layer, but the SMI32 antibody did label fibers in this layer that may represent the axons of the PFC5-immunoreactive cells or the parallel fiber axons from granule cells. In addition to the diffuse labelling of PFTAIRE in the granule cell layer there was also punctate labelling in this layer with the PFC5 antibody that closely resembled the distribution of NF-H, suggesting that both PFTAIRE and NF-H may be present in axons in the granule cell layer, possibly in Purkinje cell axons.

Sections of adult mouse sciatic nerve labelled with pre-immune serum, PFC5 and the SMI31 antibody, which recognizes a hyperphosphorylated epitope of NF-H present predominantly in axons, are shown in Figure 4.7. When the same section was labelled with PFC5 (Figure 4.7B) and SMI31 (Figure 4.7C), both proteins were co-localized in the sciatic nerve axons (4.7B and C large arrowheads). NF-H was localized exclusively in axons but PFTAIRE was also detected around the axons, outside the myelin sheath (4.7 B small arrowheads). The localization

of PFTAIRE outside the myelin sheath suggests that it was present in Schwann cells in the sciatic nerve.

The results obtained from indirect immunofluorescence with the PFC5 antibody indicate that in the adult mouse spinal cord and cerebellum PFTAIRE was present in the same populations of cells expressing PFTAIRE mRNA. The subcellular localization of PFTAIRE in CNS neurons was predominantly cytoplasmic and nuclear though some PFTAIRE was also detected in the axon hillock of motor neurons in the spinal cord and in axons located in the granule cell layer of the cerebellum. In the sciatic nerve PFTAIRE was detected in axons and possibly in Schwann cells. It is interesting to note that the two populations of glia that were labelled by the PFC5 antibody in the spinal cord and sciatic nerve may be myelin-producing cells.

4.4 PFTAIRE kinase activity

a. PFTAIRE kinase activity in adult mouse brain

The kinase activities of p34^{cdc2} and several of the CDKs have been demonstrated using in vitro kinase assays. These protein kinases have been purified biochemically or immunoprecipitated from cell lysates and combined with substrates such as histone H1, retinoblastoma protein, microtubule-associated protein tau, or peptides from a wide variety of proteins such as SV40 large T antigen, neurofilament proteins and pp60^{c-src} in the presence of Mg²⁺ and [γ^{32} -P]ATP to demonstrate phosphorylation of these various substrates (Labbé et al., 1989; Kato et al., 1993; Vulliet et al., 1992; Mawal-Dewan et al., 1993; Paudel et al., 1993; Baumann et al., 1993; Shetty et al., 1993; Shenoy et al., 1989). A consensus motif for a p34^{cdc2} kinase phosphorylation site derived from these various studies indicates that p34^{cdc2} kinase phosphorylates serine or threonine residues in the context of (S/T)-P-X-K/R, where proline is the residue immediately carboxy-terminal to the serine or threonine phosphoacceptor site and X is frequently a polar residue (Moreno and Nurse, 1990). Many other CDK/cyclin complexes also phosphorylate serine and threonine residues in this sequence context (Nigg, 1995), suggesting that this may be a conserved feature of this protein kinase family. However, other proline-directed kinases such as mitogen-activated protein kinases (MAP kinases), stress-activated protein kinases (SAP kinases) and glycogen synthase kinase-3 (GSK-3) also act on similar or overlapping consensus sequences which along with structural similarity has led to the classification of these

protein kinases as a superfamily of evolutionarily-related proteins (Hanks and Hunter, 1995).

The PFTAIRE C-terminal antibody, PFC5, was used to immunoprecipitate PFTAIRE from homogenates of adult mouse brain to study the activity of this putative kinase. Initially, I attempted to demonstrate PFTAIRE kinase activity by immunoprecipitating PFTAIRE from brain cytosolic protein preparations and performing *in vitro* kinase assays in the presence of specific exogenous substrates, such as histone H1, neurofilament-heavy protein (NF-H) and bovine tau protein. The histone H1 protein was chosen as potential substrate because p34^{cdc2} kinase and other related kinases strongly phosphorylate this protein *in vitro* (Labbé et al., 1989; Devoto et al., 1992; Lew et al., 1992a; Shetty et al., 1992). PFTAIRE was cloned by low stringency hybridization to *cdc2* in an effort to identify a *cdc2*-related kinase in the nervous system that may function as a neurofilament kinase, that may phosphorylate specifically serine residues located within multiple Lys-Ser-Pro (KSP) repeats of the carboxy-terminal tail of NF-H. To test this hypothesis PFTAIRE was immunoprecipitated from brain cytosolic protein preparations and its ability to phosphorylate NF-H *in vitro* was examined. Others have demonstrated previously that some of the NF-H KSP serine residues are phosphorylated *in vitro* by purified p34^{cdc2} and CDK5 (Hisanaga et al., 1991; Lew et al., 1992a; Shetty et al., 1992). The microtubule-associated protein tau was also considered a candidate substrate for PFTAIRE. Tau occurs in multiple phosphorylation states in the brain and its aberrant hyperphosphorylation results in the conversion of normal tau into the paired helical filament tau found in the neurofibrillary tangles that are associated with Alzheimer's disease pathology (Trojanowski et al., 1993). Like NF-H, tau contains potential sites for phosphorylation by CDKs and other proline-directed kinases such as MAP kinase and glycogen synthase kinase 3 β (Vulliet et al., 1992; Drewes et al., 1992; Baumann et al., 1993; Paudel et al., 1993; Ishiguro et al., 1993). When PFTAIRE was immunoprecipitated from a brain cytosolic protein preparation and used for kinase reactions in the presence of histone H1 (purchased from Boehringer-Mannheim), preparations of mouse neurofilament proteins, the carboxy-terminal tail domain of NF-H (produced in bacteria as a GST fusion protein in a non-phosphorylated form, gift of Dr. W. Mushynski) or bovine tau protein (purchased from Sigma) no specific phosphorylation of these substrates occurred. When CDK5 was immunoprecipitated from the same protein preparation it phosphorylated strongly histone H1, as others have demonstrated (Lew et al., 1992a; Shetty et al., 1992; Tsai et al., 1993), and it phosphorylated weakly the GST-NFH fusion protein. In this immunoprecipitation-kinase assay bovine tau was not phosphorylated by CDK5,

suggesting that this preparation of tau may not have been dephosphorylated or that it contained contaminating phosphatases. The phosphorylation of histone H1 and NF-H by immunoprecipitated CDK5 provided positive controls for the kinase reactions, by confirming the results of others and showing that these exogenously added substrates did not contain contaminants that may have interfered with the PFTAIRE kinase reactions.

Kinase reactions containing immunoprecipitated PFTAIRE consistently demonstrated strong phosphorylation of a few proteins that were present regardless of whether substrate proteins had been included in the kinase reaction. An autoradiogram of kinase reactions performed with immunoprecipitated PFTAIRE is presented in Figure 4.8A. The result shows that in the absence of exogenous substrates PFTAIRE phosphorylated specific proteins that remained associated with it following immunoprecipitation from a brain cytosolic protein preparation. The most highly phosphorylated proteins migrated at an apparent molecular mass of 58-60 kDa (arrowheads) and this broad protein band may represent different phosphorylation states of the same protein. Two other specific, but less phosphorylated proteins migrated at approximately 200-205 kDa (arrowheads). None of the proteins associated with and phosphorylated by the kinase activity in this immune complex were present when immunoprecipitations were performed using pre-immune serum. When the PFC5 antibody was pre-adsorbed with a 100-fold molar excess of C-terminal PFTAIRE peptide prior to the immunoprecipitation reaction, phosphorylation of these proteins was markedly reduced, suggesting that immunoprecipitation of PFTAIRE from the brain cytosolic protein preparation was required for their phosphorylation. In a separate experiment the PFC5 antibody was used for immunoprecipitations with a cytosolic protein preparation from skeletal muscle, a tissue that does not express PFTAIRE. The PFTAIRE-associated phosphoproteins identified in immunoprecipitates from brain preparations were not present in skeletal muscle kinase reaction samples, suggesting that the phosphoproteins in brain samples were associated with immunoprecipitated PFTAIRE and were not contaminants that cross-reacted with the PFC5 antibody (Figure 4.8B). The major phosphoproteins co-immunoprecipitating with CDK5 were different from those associated with PFTAIRE, and migrated at 31-35 kDa and 205 kDa. These findings suggest that similar high molecular mass phosphoproteins may have associated with both PFTAIRE and CDK5, while the 58-60 kDa phosphoproteins were specifically associated with PFTAIRE. A strongly phosphorylated protein present in all samples migrated at an apparent

molecular mass of 52 kDa. This protein was phosphorylated in samples immunoprecipitated from brain (but not muscle, Figure 4.8B or cultured cells, Figure 4.10A) with each antibody, including pre-immune serum, and may represent the heavy chain of IgG that was phosphorylated nonspecifically by a kinase present in the brain lysate.

b. Is PFTAIRES kinase activity immunoprecipitated from brain due to PFTAIRES?

In order to determine whether the kinase activity in the PFTAIRES immunoprecipitations from brain cytosolic protein preparations was specifically due to PFTAIRES and not to another kinase that may have associated with PFTAIRES in the immune complex, a variety of *in vitro* experimental approaches were used. The most direct way to demonstrate PFTAIRES kinase activity *in vitro* would be to use the GST-PFTAIRES fusion protein produced in bacteria. The bacterially-produced fusion protein did not have kinase (autophosphorylation) activity, suggesting that in order to demonstrate kinase activity the recombinant protein may have required a regulatory component or substrate that was lacking in this preparation, or the fusion protein did not possess kinase activity for other technical reasons. Without knowing the identity of possible PFTAIRES regulatory components or substrates, it seemed likely that if such proteins were required, they would be present in a brain lysate. In addition, the proteins that were normally co-immunoprecipitated with and phosphorylated by PFTAIRES from brain would also be present in the lysate. Experiments designed to determine if the GST-PFTAIRES fusion protein was able to associate with and phosphorylate proteins in a brain cytosolic protein preparation are described in detail in Sections 4.5b and 4.6a.

Using a different approach to examine the kinase activity of PFTAIRES *in vitro*, immunoprecipitation-kinase assays were done with *in vitro*-translated PFTAIRES and a PFTAIRES dominant negative mutant. The dominant negative mutant of PFTAIRES was generated by a point mutation made in kinase subdomain VII to change the Asp²²⁸ residue to an asparagine (refer to Chapter 2, Section 2.4c). The aspartate residue is conserved in all protein kinases and based on structure-function studies is known to be required for the transfer of the γ -phosphate of Mg²⁺ATP (Taylor et al., 1993; Hanks and Hunter, 1995). This experiment was initially designed to examine PFTAIRES kinase activity in a kinase reaction in which *in vitro*-translated PFTAIRES was the only kinase present. However, because the PFTAIRES substrate identities were not known, the *in vitro*-translated PFTAIRES was used to assess the ability of PFTAIRES to autophosphorylate. If the D228N mutant was an inactive form of the kinase then it would not be expected to

autophosphorylate. When PFTAIRE and D228N were immunoprecipitated following translation *in vitro* with rabbit reticulocyte lysate and incubated with kinase buffer containing [$\gamma^{33}\text{-P}$]ATP, neither *in vitro*-translated protein incorporated phosphate. This observation suggested that for PFTAIRE to be active it may require regulatory proteins that were not co-immunoprecipitated with the *in vitro*-translated PFTAIRE preparation.

The alternative approach to using only *in vitro*-translated proteins was to immunoprecipitate *in vitro*-translated PFTAIRE and D228N and combine each immunoprecipitation with a brain cytosolic protein preparation (described in Chapter 2, Section 2.7g) so that "regulatory proteins" and "substrates" that might normally interact with PFTAIRE would associate with the *in vitro*-translated proteins. The difficulty with assessing PFTAIRE kinase activity by this approach was that with the addition of the brain cytosolic protein preparation, other protein kinases could associate with the *in vitro*-translated proteins, making it difficult to demonstrate definitively the kinase activity of PFTAIRE. The *in vitro*-translated PFTAIRE and D228N were each expected to interact with the same proteins in the brain cytosolic protein preparation as endogenous PFTAIRE when immunoprecipitated. If phosphorylation of the 58-60 kDa phosphoproteins in PFTAIRE immunoprecipitates was due to PFTAIRE activity then the presence of additional *in vitro*-translated PFTAIRE may increase the phosphorylation of these proteins. The association of the 58-60 kDa proteins with *in vitro*-translated D228N was expected to result in decreased phosphorylation of these proteins because the dominant negative PFTAIRE should compete with the endogenous PFTAIRE activity. Using *in vitro*-translated proteins for similar experiments, Zhou et al. (1991) demonstrated that when *in vitro*-translated *c-mos* or a *c-mos* deletion mutant was mixed with oocyte extracts both proteins were co-immunoprecipitated with β -tubulin but, β -tubulin was only phosphorylated in complexes containing the wild type *in vitro*-translated *c-mos*. This data provided evidence that the kinase activity of *c-mos* and not another kinase that may have associated with the *c-mos* immune complex was responsible for phosphorylation of β -tubulin (Zhou et al., 1991). The results of this experiment with PFTAIRE, shown in Figure 4.9B, demonstrate that in the presence of *in vitro*-translated PFTAIRE there was an increase in the phosphorylation of the 58-60 kDa proteins compared to their phosphorylation by endogenous brain PFTAIRE (compare lane 4 and lane 5). In the presence of *in vitro*-translated D228N the phosphorylation of these same proteins was reduced (compare lane 3 and lane 5) relative to phosphorylation by endogenous PFTAIRE.

Scintillation counting of the excised 58-60 kDa phosphoprotein bands indicated that there was an approximate two-fold increase in phosphorylation in the presence of *in vitro*-translated PFTAIRE, compared to phosphorylation by PFTAIRE immunoprecipitated from brain. In the presence of *in vitro*-translated D228N phosphorylation of the 58-60 kDa proteins was reduced by about one-half, compared to their phosphorylation by PFTAIRE immunoprecipitated from brain. These results demonstrate that when the 58-60 kDa proteins were associated with wild type *in vitro*-translated PFTAIRE an increase in phosphorylation occurred, while association of these proteins with *in vitro*-translated D228N inhibited the ability of endogenous PFTAIRE to phosphorylate the 58-60 kDa proteins. The results obtained with the D228N *in vitro*-translated protein do not exclude the possibility that some endogenous PFTAIRE (or another kinase) may have been associated with the 58-60 kDa proteins when they bound to the *in vitro*-translated PFTAIRE or D228N. However, the decrease in phosphorylation of the 58-60 kDa proteins that was associated with D228N, indicates that their phosphorylation was dependent on interaction with an active form of PFTAIRE.

The remaining half of each immunoprecipitated sample used for the kinase assay in Figure 4.9B was used to prepare the western blot shown in Figure 4.9C to confirm that equal amounts of the *in vitro*-translated proteins were immunoprecipitated for the assay. When *in vitro*-translated proteins were combined with PFC5 pre-adsorbed with C-terminal PFTAIRE peptide (Figure 4.9C lanes, 3 and 4) or with pre-immune serum (lane 8) for immunoprecipitations the *in vitro*-translated proteins were not detected on the western blot and the 58-60 kDa phosphoproteins were not present in the kinase assay samples (Figure 4.9B lanes 1, 2 and 6).

To more directly demonstrate the kinase activity of PFTAIRE, PFTAIRE and the PFTAIRE dominant negative mutant (D228N) were also used for a transfection assay. Attempts to overexpress PFTAIRE in several cell lines that express little or no endogenous PFTAIRE were unsuccessful as a result of poor transfection efficiency and decreased cell viability (See Chapter 5). Figure 4.10 shows the results of an experiment in which 293 cells were transfected transiently with either the pcDNA3 plasmid vector, pc3PFO_{rf} (PFTAIRE) or pc3D228N_{orf} (D228N). The 293 cells are adenovirus-transformed human embryonic kidney cells that express an endogenous PFTAIRE species (Figure 4.10B, upper arrowhead and Chapter 5 Figure 5.7). The PFC5 antibody was used to immunoprecipitate the endogenous and transfected PFTAIRE species from a cytosolic protein preparation of the lysed cultures. Half of each immunoprecipitated sample was used for the kinase assay (Figure 4.10A). The results of the kinase assay indicated that the 58-60

kDa proteins that were phosphorylated by PFTAIRE immunoprecipitated from mouse brain (Figures 4.8 and 4.9) were also present in the immunoprecipitation-kinase assays performed with vector-transfected 293 cell lysates (Figure 4.10A, lane 3). Scintillation counting of excised 58-60 kDa phosphoprotein bands demonstrated that immunoprecipitation of PFTAIRE from PFTAIRE-transfected cultures resulted in an approximately two-fold increase in the phosphorylation of the 58-60 kDa proteins compared to their phosphorylation by endogenous PFTAIRE, immunoprecipitated from vector-transfected cultures (compare lane 4 with lane 3). The phosphorylation of the 58-60 kDa proteins was reduced by about 50% when PFTAIRE was immunoprecipitated from D228N-transfected cultures compared to their phosphorylation by endogenous PFTAIRE (compare lane 5 with lane 3). When pre-immune serum was used for immunoprecipitations with PFTAIRE- and D228N-transfected cell lysates no kinase activity was detected (lanes 1 and 2). When PFC5 was pre-adsorbed with the C-terminal PFTAIRE peptide prior to immunoprecipitation, phosphorylation of the 58-60 kDa proteins was reduced (lane 6). Similar to the results obtained with the *in vitro*-translated proteins presented in Figure 4.9, these results demonstrate that when transfected wild type PFTAIRE was immunoprecipitated the phosphorylation of the associated 58-60 kDa proteins was increased, but when transfected D228N was immunoprecipitated there was a decrease in the phosphorylation of these proteins. Both sets of experiments suggest that PFTAIRE kinase activity is required for the phosphorylation of the 58-60 kDa proteins that co-immunoprecipitate with it and that their phosphorylation is not due to another protein kinase.

The remaining half of each immunoprecipitated sample used for the kinase assay in Figure 4.10A, was used to prepare the western blot shown in Figure 4.10B, to demonstrate the presence of the endogenous and transfected PFTAIRE species. The arrowheads indicate where endogenous 293 cell PFTAIRE migrated (Figure 4.10B, lanes 3, 4 and 5 upper arrowhead) and where the transfected PFTAIRE and D228N migrated (lanes 4 and 5 lower arrowhead) following immunoprecipitation. Neither of the proteins was present in samples immunoprecipitated with pre-immune serum or in the sample that included the C-terminal PFTAIRE peptide during the immunoprecipitation (lane 6). Figure 4.10B also shows that each immunoprecipitated sample had approximately equal amounts of protein and that transfected PFTAIRE and D228N were present at levels approximately equal to that of endogenous PFTAIRE.

4.5 What proteins does PFTAIRE phosphorylate?

a. Are NF-H peptides substrates for PFTAIRE kinase activity?

The 58-60 kDa phosphoproteins identified in PFTAIRE immunoprecipitation-kinase assays appeared to be the major target of PFTAIRE kinase activity. Proteins of 200-205 kDa that were phosphorylated to a lesser extent than the 58-60 kDa proteins were also associated with PFTAIRE immunoprecipitated from brain protein preparations. When the PFTAIRE C-terminal peptide was included during immunoprecipitations the detection of these phosphoproteins was decreased, suggesting that PFTAIRE may be directly involved in their phosphorylation (Figure 4.8A). Phosphoproteins of approximately 200 kDa were also present in kinase reactions that contained immunoprecipitated CDK5. The phosphorylated NF-H protein prepared from nervous tissue is detected at molecular mass of approximately 200 kDa on SDS-polyacrylamide gels due to the high content of charged amino acids and phosphates in its carboxy-terminal domain and anomalous electrophoretic mobility (Julien and Mushynski, 1982). In addition, neurofilament protein preparations contain a major endogenous kinase activity that phosphorylates histone H1 (Pant and Veeranna, 1995), like CDK5. Dephosphorylated NF-H and peptides derived from the carboxy-terminal domain of NF-H were phosphorylated, *in vitro*, by purified CDK5 in kinase assays (Lew et al., 1992b; Shetty et al., 1993). It is possible that the 200 kDa phosphoprotein that was co-immunoprecipitated with CDK5 was NF-H, and that NF-H may also be co-immunoprecipitated with PFTAIRE. When kinase reactions were performed with immunoprecipitated PFTAIRE using a GST-NFH fusion protein (C-terminal region of mouse NF-H) as a substrate, no phosphorylation of the fusion protein occurred. On the other hand, immunoprecipitated CDK5 weakly phosphorylated the GST-NFH fusion protein.

To further test whether PFTAIRE was able to phosphorylate serine residues located in the carboxy-terminal tail domain of NF-H, two synthetic peptides derived from the primary sequence of mouse NF-H (gift of Dr. Harish Pant) were tested as substrates in kinase reactions containing PFTAIRE and CDK5 immunoprecipitated from mouse brain protein extracts. NF-H peptide 1 was a 14-mer (residues 838-851) (Julien et al., 1988) containing two different KSP motifs, KSPXK and KSPEK, which each occur 4 times in the mouse NF-H sequence. The KSP motifs in this NF-H peptide were preferentially phosphorylated by purified p34^{cdc2} and CDK5 in *in vitro* kinase assays (Hisanaga et al., 1991; Shetty et al., 1993). NF-H peptide 2 was a 15-mer that contained two repeats of the KSPXE motif and corresponded to a sequence that occurs 39 times in the carboxy-terminal tail region of mouse NF-H (Julien et al., 1988). The results of kinase

reactions that included the NF-H peptides as substrates are presented in Figure 4.11. Neither NF-H peptide was phosphorylated by immunoprecipitated PFTAIRE. CDK5, immunoprecipitated from the same brain cytosolic protein preparation, strongly phosphorylated NF-H peptide 1 (containing KSPXK and KSPEK) and did not phosphorylate NF-H peptide 2 (KSPXE x 2), confirming the results of previously published experiments using these peptides in kinase assays with purified CDK5 (Shetty et al., 1993). The results of this kinase assay indicate that unlike CDK5, PFTAIRE does not phosphorylate NF-H *in vitro*, and that the 200 kDa phosphoprotein present in the PFTAIRE immune complex may not be NF-H. Thus PFTAIRE and CDK5 immune complexes may each contain a unique set of protein components. These results also suggest that the sequence recognized by PFTAIRE in target substrates may differ from the consensus for phosphorylation by p34cdc2 kinase or CDK5.

b. What are the PFTAIRE-interacting proteins in brain homogenates?

The identification of proteins associated with and phosphorylated by immunoprecipitated PFTAIRE may provide crucial information for determining the function of this cdc2-related kinase in the nervous system. The quantities of PFTAIRE and the major phosphoproteins (58-60 kDa and 200-205 kDa proteins) present in kinase reaction samples were exceedingly low, and none of these proteins were detected on protein gels stained with Coomassie blue or by silver staining. Ultimately, affinity chromatography with the PFC5 antibody will be required to obtain sufficient quantities of PFTAIRE and PFTAIRE-interacting proteins (that may co-purify with PFTAIRE) from brain to determine the identity of these proteins using peptide sequencing and cloning. In a preliminary effort to obtain larger quantities of PFTAIRE-interacting proteins from a mouse brain cytosolic protein preparation, an alternative approach was chosen, using the GST-PFTAIRE fusion protein produced in bacteria (described in Chapter 2, Section 2.7i). A similar technique was used to demonstrate the association of Ash/Grb2 with dynamin (Miki et al., 1994) and to identify a protein kinase associated with the cytoplasmic domain of the tumor necrosis factor receptor (Damay et al., 1994). Briefly, the cytosolic protein fraction from a homogenate of mouse brain was applied to glutathione-Sepharose to deplete endogenous glutathione S-transferase from the protein preparation. The flow through fraction was then mixed with glutathione-Sepharose beads adsorbed with GST-PFTAIRE to allow binding of PFTAIRE-interacting proteins. After removal of the brain cytosolic protein fraction, the beads were washed several times. Elutions with glutathione were expected to contain the fusion protein and any PFTAIRE-

interacting proteins that may have associated with the fusion protein. An identical column matrix was processed without the brain cytosolic protein fraction to compare the elution of GST-PFTAIRE alone. When proteins in each set of elutions were analyzed by SDS/PAGE and the gels were silver stained, no differences were detected in the eluants from the two columns. In a similar experiment, Darnay et al. (1994) used lysates of cultured cells labelled with ^{35}S -methionine to demonstrate that a protein in the lysates associated with a GST-TNFR fusion protein. It is possible that the sensitivity of the silver staining was insufficient for detecting new proteins that may have bound to and eluted with GST-PFTAIRE, and that by using ^{35}S -methionine labelled 293 cells, PFTAIRE-interacting proteins may have been detected and possibly isolated by this method.

4.6 PFTAIRE is phosphorylated by proteins present in the brain

a. Phosphorylation of the GST-PFTAIRE fusion protein

To determine if the GST-PFTAIRE fusion protein could associate with and phosphorylate the same proteins in the brain cytosolic protein preparation that were present in the PFTAIRE immune complex an experiment similar to the one described above (Section 4.5b) was performed. After applying the brain cytosolic protein preparation to the GST-PFTAIRE coupled glutathione-Sepharose, the fusion protein was not eluted with glutathione. The glutathione-Sepharose beads were washed extensively, kinase buffer was added to the beads and the kinase reaction was performed with proteins bound to the GST-PFTAIRE coupled glutathione-Sepharose beads. A schematic representation of the experiment is presented in Chapter 2 (Figure 2.12). As described above, an identical column matrix was processed without the brain cytosolic protein preparation in order to examine the GST-PFTAIRE autophosphorylation kinase activity. When samples were separated by SDS/PAGE the 58-60 kDa and 200-205 kDa phosphoproteins were not detected on autoradiograms. However, a highly phosphorylated protein corresponding to the GST-PFTAIRE fusion protein (approximately 74 kDa) was present in kinase reactions in which the brain cytosolic protein preparation had been applied to GST-PFTAIRE beads. In the absence of the brain cytosolic protein preparation GST-PFTAIRE was not phosphorylated. This result suggested that: 1) another kinase in the brain protein preparation associated with and phosphorylated GST-PFTAIRE or; 2) a regulatory protein in the brain preparation associated with GST-PFTAIRE and stimulated an autophosphorylation kinase activity of the fusion protein.

The experiment was repeated using the GST-PFTAIRE and GST-D228N (PFTAIRE dominant negative mutant) to determine how the GST-PFTAIRE became phosphorylated. The results of this experiment are presented in Figure 4.12. In the absence of the brain protein preparation neither fusion protein was autophosphorylated in kinase reactions (lanes 6 and 8). When the brain cytosolic protein preparation was applied to glutathione beads coupled with either GST-PFTAIRE or GST-D228N, both proteins (74 kDa) were phosphorylated in the subsequent kinase reactions (lanes 5 and 7). The phosphorylation of GST-D228N was similar to that of GST-PFTAIRE, suggesting that another brain kinase had associated with and phosphorylated both fusion proteins, rather than stimulation of GST-PFTAIRE autophosphorylation kinase activity. In a control reaction the brain cytosolic protein preparation was applied to GST coupled glutathione-Sepharose. This kinase reaction demonstrated that phosphorylation of the fusion proteins was not due phosphorylation of GST, because the 27 kDa GST was not detected as a phosphoprotein (lane 3). In addition, the phosphoproteins in the reactions containing GST-PFTAIRE and GST-D228N did not represent the phosphorylation of proteins that may have bound nonspecifically to the glutathione-Sepharose beads (compare lanes 5 and 7 to lane 1). The results of this experiment indicate that, like other CDKs and cdc2-related kinases, PFTAIRE may be phosphorylated by other protein kinases that may regulate its activity.

**b. Immunoprecipitated PFTAIRE is phosphorylated in kinase reactions:
two dimensional gel electrophoresis analysis of PFTAIRE kinase reactions**

Analysis of PFTAIRE immunoprecipitation-kinase reactions by SDS/PAGE and autoradiography demonstrated that when PFTAIRE was immunoprecipitated from brain cytosolic protein preparations, proteins that remained associated with the kinase were also phosphorylated by it. In order to better resolve the components of the PFTAIRE immune complex and demonstrate that PFTAIRE was distinct from the major phosphoproteins in the immunoprecipitated protein complex, immunoprecipitation-kinase reaction samples were analyzed by two dimensional gel electrophoresis and western blotting. Following immunoprecipitation of PFTAIRE from a brain cytosolic protein preparation, kinase assays were performed and samples were prepared for isoelectric focussing. Prior to loading samples for isoelectric focussing, 15 μ g of protein from the brain cytosolic protein preparation (used for immunoprecipitating PFTAIRE) was added samples to ensure that PFTAIRE would be detected on the western blot (refer to Chapter 2, Section 2.7j for details of the experiment).

Figure 4.13 shows the results of the two dimensional gel electrophoresis of a PFTAIRE kinase assay sample. The autoradiogram obtained when the nitrocellulose membrane was exposed to film prior to immunoblotting is shown in Figure 4.13A and it demonstrates how the different phosphoproteins in the PFTAIRE immunoprecipitation-kinase assay sample were separated. The position of the well containing the molecular mass standards during electrophoresis on the SDS-polyacrylamide gel was slightly lower than the position of the tube gel containing the PFTAIRE sample, so the molecular masses of the proteins appear slightly larger than what was detected previously on SDS/PAGE. The highest molecular mass spots (60-62 kDa range) are likely to represent the 58-60 kDa phosphoproteins that were consistently detected in PFTAIRE kinase assay samples. The two dimensional resolution of the PFTAIRE complex demonstrates that these phosphoproteins may actually correspond to a single protein that was present in multiple phosphorylation states due to the occurrence of multiple spots of the same apparent molecular mass along the pH gradient from approximately pH 8.0-pH 7.2. The 200-205 kDa phosphoproteins were not detected in this analysis. From this autoradiogram, it was not possible to determine if any of the phosphorylated proteins corresponded to PFTAIRE.

When the same nitrocellulose membrane was immunoblotted with the PFC5 antibody a single protein, that migrated close to the molecular mass of PFTAIRE (52 kDa), was detected at approximately pH 8.4 (Figure 4.13B). The identification of PFTAIRE at pH 8.4 on the immunoblot is in good agreement with the pI value of 8.95 calculated from the PFTAIRE sequence using the DNA Star sequence analysis program. To confirm that this technique yielded appropriate results with another protein, a CDK5 immunoprecipitation-kinase assay sample was subjected to the same analysis. A single protein of approximately 32 kDa was detected at pH 7.4-7.5 with the CDK5 antibody, corresponding well with the calculated pI value for CDK5 of 7.6 (not shown).

When the two autoradiograms shown in Figure 4.13 were aligned, the faint phosphorylation spot indicated in Figure 4.13A by a small arrowhead corresponded exactly to the PFTAIRE spot detected by the PFC5 antibody on the immunoblot in Figure 4.13B. When the PFC5 antibody was pre-adsorbed with PFTAIRE C-terminal peptide and the immunoprecipitation-kinase assay sample was subjected to the same two dimension gel electrophoresis analysis, neither the faint PFTAIRE spot nor the 58-60 kDa spots were detected (not shown). Thus the two dimensional analysis of PFTAIRE immunoprecipitation-kinase assay samples demonstrated that the major phosphoproteins in the PFTAIRE immune complex were not PFTAIRE but were other

proteins that co-immunoprecipitated. The alignment of the PFTAIRE spots also indicated that following immunoprecipitation, PFTAIRE was weakly phosphorylated in the kinase reaction. From this data it is not known whether the weak phosphorylation of PFTAIRE was the result of autophosphorylation or whether another kinase was present in the PFTAIRE protein complex to phosphorylate PFTAIRE. The data presented in Section 4.6a (Figure 4.12) suggested that GST-PFTAIRE and GST-D228N were phosphorylated by another brain kinase that associated with these fusion proteins. However, one cannot exclude the possibility that PFTAIRE immunoprecipitated from brain may be associated with a regulatory protein capable of stimulating PFTAIRE autophosphorylation activity.

4.7 Discussion

a. The PFC5 antibody recognizes a protein in the adult mouse nervous system encoded by the PFTAIRE cDNA

To begin to examine the function of PFTAIRE in the nervous system it was essential to generate antibodies that could be used to characterize the distribution of PFTAIRE protein and its kinase activity. The results presented in Sections 4.2-4.4 of this chapter demonstrated that the PFC5 antibody, raised against a PFTAIRE C-terminal synthetic peptide, recognized a specific protein. The antibody reacted with the PFTAIRE *in vitro* translated protein by immunoprecipitation, transfected PFTAIRE (Figure 4.1, Figure 4.4, Figure 4.10), and the GST-PFTAIRE fusion protein in bacteria. The antibody reacted specifically with a 52 kDa protein present in tissues from the central and peripheral nervous systems but did not detect this protein in lung, which was the only other tissue that PFTAIRE mRNA was identified in (Figure 4.2 and 4.3). When the PFC5 antibody was pre-incubated with C-terminal peptide before immunoblotting the ability of the antibody to react with the 52 kDa protein was reduced, suggesting that the protein recognized by this antibody was the endogenous mouse PFTAIRE (Figure 4.3). Although the PFTAIRE protein detected on immunoblots of mouse nervous tissue was 52 kDa, the PFTAIRE *in vitro* translation product was detected on SDS-polyacrylamide gels at the size predicted from the PFTAIRE amino acid sequence (47.5 kDa). However, the presence of three in frame stop codons located upstream of the PFTAIRE initiation codon is consistent with the PFTAIRE cDNA clone encoding the full length protein. The anomalous migration of PFTAIRE protein from nervous tissue on SDS-polyacrylamide gels, compared to the PFTAIRE *in vitro*

translation product, may be due to post-translational modification of PFTAIRE in nervous tissue. The NF-H protein, prepared from nervous tissue, similarly shows decreased electrophoretic mobility on SDS-polyacrylamide gels due to phosphorylation (Julien and Mushynski, 1982) that does not occur when the full length NF-H cDNA is translated *in vitro* or expressed in mouse fibroblasts (Chin and Liem, 1990).

When the PFC5 antibody was used for indirect immunofluorescence to examine the localization of PFTAIRE in tissues from the central nervous system, specifically in adult mouse spinal cord and cerebellum, the protein showed the same distribution as PFTAIRE mRNA. In the spinal cord PFTAIRE was identified in neurons throughout the gray matter in the posterior and anterior horns in large neurons that contained neurofilaments, such as motor neurons, and in populations of smaller neurons that lack neurofilaments (Figure 4.5). The PFTAIRE protein was also localized in the spinal cord white matter, like PFTAIRE mRNA, but not in axons or in astrocytes. Although the identity of the PFC5-immunoreactive cells in the white matter has not been confirmed with antibodies, the morphology of these cells suggests that they could be oligodendrocytes. In the cerebellum, PFTAIRE was identified in neurons from the three layers of the cerebellar cortex, and was not detected in the white matter.

PFTAIRE, p34^{cdc2} and CDK5 are the only members of the cdc2 kinase family to be characterized in the developing and adult nervous system to date. In contrast to PFTAIRE, cdc2 mRNA and p34^{cdc2} are highly expressed in proliferating neuronal precursors prior to neurogenesis, particularly in the ventricular zone (Hayes et al., 1991; Zhao et al., 1995). In the postnatal and adult nervous system cdc2 mRNA and p34^{cdc2} occur at low levels in a few populations of postmitotic neurons (Hayes et al., 1991; Wood and Zinsmeister, 1993; also refer to Chapter 3) compared to the widespread and abundant expression of PFTAIRE in postmitotic neurons. Wood and Zinsmeister (1993) demonstrated expression of p34^{cdc2} kinase or a cdc2-related kinase with an anti-PSTAIR antibody in the nuclei of many of the neurons that express PFTAIRE mRNA and protein. However, no further characterization of the protein detected in their analysis has been reported. PFTAIRE and CDK5 also show differences in their temporal and spatial expression patterns. Low levels of the CDK5 mRNA and protein are detected in nervous tissue from mouse embryos at 11-12 days gestation in cells that also express cdc2 mRNA. During embryonic development and postnatal development CDK5 mRNA, protein, and kinase activity

progressively increase (Tsai et al., 1993; Iino et al., 1994). Although the distribution of PFTAIRE mRNA and protein in the adult mouse spinal cord and cerebellum shows some similarities to the distribution of CDK5 mRNA described by Iino et al. (1994), the spatial distribution of the two cdc2-related kinases is not identical. In the CNS both PFTAIRE and CDK5 were expressed in the spinal cord gray matter in most populations of neurons but unlike PFTAIRE, CDK5 was not detected in the white matter (Iino et al., 1994). The CDK5 mRNA was expressed strongly in the Purkinje cell layer and weakly in the granule cell layer of the cerebellum (Iino et al., 1994), in contrast to the moderate expression of PFTAIRE mRNA and protein in all three layers of the cerebellum and in deep cerebellar nuclei.

Although PFTAIRE and CDK5 are co-expressed in some neuronal populations, the subcellular localizations of the two proteins differ markedly. In CNS neurons PFTAIRE was localized predominantly in the cytoplasm and nucleus, although PFTAIRE was also detected in the axon hillock of some motor neurons and in axons located in the granule cell layer of the cerebellum. Indirect immunofluorescence labelling of sciatic nerve sections demonstrated that in the peripheral nervous system PFTAIRE was localized in axons and around axons, probably in Schwann cells (Figure 4.7). In contrast to the predominantly nuclear and cytoplasmic localization of PFTAIRE in CNS neurons, the localization of CDK5 was initially reported to be axonal, predominantly in mature fasciculated axonal systems of the CNS and PNS (Tsai et al., 1993). The reported localization of CDK5 in mature axons, rather than in immature axons and growth cones led to the suggestion that its function may be related to maintaining axonal integrity (Tsai et al., 1993). The CDK5 localization data and data demonstrating that neurofilament proteins (NF-M and NF-H), peptides derived from the carboxy-terminal tail region of NF-H and bovine tau are substrates *in vitro* for purified CDK5 have contributed to the hypothesis that CDK5 may function *in vivo* as a protein kinase that phosphorylates major components of the mature neuronal cytoskeleton, in particular neurofilament proteins and tau (Tsai et al., 1993; Lew et al., 1992b; Shetty et al., 1993; Paudel et al., 1993; Pant and Veeranna, 1995; Lew et al., 1995). The more recent demonstration that CDK5 is localized in neuronal cell bodies, nuclei, neurites and growth cones of cultured primary neurons indicates that CDK5 may also function early in neuronal differentiation, during axonal outgrowth (Nikolic et al., 1996). The localization of PFTAIRE in various compartments of neurons and its expression in neurons and in specific populations of glia suggest that PFTAIRE may also have more than one function in the mature nervous system.

b. The kinase activity of PFTAIRE

The PFC5 antibody recognized specifically the PFTAIRE protein expressed from CMV-PFTAIRE plasmids in transfected cells on immunoblots, by immunoprecipitation and by indirect immunofluorescence. The antibody reacted with a specific protein in immunoblotted samples of nervous tissue. The protein was also detected in the same populations of cells where PFTAIRE mRNA was expressed. The kinase activities of many CDKs isolated either by biochemical purification or by immunoprecipitation has been demonstrated by *in vitro* kinase assays with potential substrate proteins or peptides in the presence of Mg^{2+} and $[\gamma^{32}\text{-P}]\text{ATP}$. Many of the CDK-cyclin complexes phosphorylate several substrate proteins *in vitro*, but for many of these kinases it remains to be determined if the proteins and peptides they phosphorylate *in vitro* are actually targets *in vivo* (Nigg, 1995).

After p34^{cdc2} was shown to phosphorylate specific sites in histone H1 as cells enter mitosis (Langan et al., 1989), histone H1 has been used as an *in vitro* substrate to assess the kinase activities of other CDKs and cdc2-related kinases. p34^{cdc2}, CDK2 and CDK5 all phosphorylate histone H1 *in vitro* (Koff et al., 1992; Lew et al., 1992a; Shetty et al., 1992) while CDK4, CDK6 and CDK7 (CAK) do not (Matsushime et al., 1992; Meyerson and Harlow, 1994; Poon et al., 1993). Although CDK5-cyclin D1 complexes were immunoprecipitated from the WI38 human fibroblast cell line (Xiong et al., 1992), these complexes were not shown to have histone H1 kinase activity. When purified or immunoprecipitated from nervous tissue or neuronal cultures CDK5 displayed strong histone H1 kinase activity *in vitro* (Lew et al., 1992a; Shetty et al., 1992; Tsai et al., 1993; Beaudette et al., 1993) and phosphorylated the same histone H1 peptide sequences as p34^{cdc2} (Beaudette et al., 1993). However, it has not been determined if histone H1 is actually a substrate *in vivo* for neuronal CDK5 kinase activity. By examining the substrate specificity of CDK5 with histone H1 peptides, Beaudette et al. (1993) demonstrated that CDK5 preferentially phosphorylated a peptide sequence that contained the consensus for phosphorylation by p34^{cdc2} kinase (S/T-P-X-K/R). The additional requirements for phosphorylation by CDK5 include a proline residue in the -2 position (the phosphoacceptor residue is numbered as 0), a basic residue in the +2 position and a cluster of basic residues located C-terminal to the consensus motif. These data suggest that other members of the cdc2 kinase family may also phosphorylate sites similar to the p34^{cdc2} kinase consensus sequence

and that proteins containing these sites (such as histone H1) may be useful for demonstrating the kinase activity of newly identified family members *in vitro*.

Based on these findings, I began to test the PFTAIRE kinase activity *in vitro* using histone H1, neurofilament protein preparations, NF-H peptides and purified bovine tau as experimental substrates. The NF-M, NF-H and tau proteins are abundant and highly phosphorylated proteins in adult nervous tissue that all contain multiple phosphoserine/threonine residues in sequence motifs that are similar to the consensus for phosphorylation by p34^{cdc2}. In addition, all of these proteins are substrates *in vitro* for p34^{cdc2} and CDK5 (Hisanaga et al., 1991; Lew et al., 1992b; Vulliamt et al., 1992; Baumann et al., 1992; Paudel et al., 1993). However, PFTAIRE immunoprecipitated from mouse brain did not demonstrate kinase activity towards any of these substrates *in vitro*. PFTAIRE also did not phosphorylate an NF-H peptide containing sites phosphorylated by p34^{cdc2} and CDK5, suggesting that phosphorylation of substrates by PFTAIRE may occur on serine or threonine residues at sites that differ significantly from the consensus for phosphorylation by p34^{cdc2}. Although the experimental substrates were not phosphorylated by PFTAIRE, PFTAIRE did phosphorylate specific proteins that were co-immunoprecipitated with it from the mouse brain cytosolic protein preparation (Figure 4.8A). Three lines of evidence suggest that PFTAIRE was the kinase activity responsible for phosphorylation of the proteins that were co-immunoprecipitated: 1) phosphorylation of these proteins was reduced when the PFC5 antibody was pre-adsorbed with PFTAIRE C-terminal peptide prior to immunoprecipitation (Figure 4.7A), 2) phosphorylation of these proteins was decreased in the presence of *in vitro*-translated D228N (mutated PFTAIRE) (Figure 4.9), and 3) transfected D228N competed with endogenous PFTAIRE when immunoprecipitated resulting in a reduced phosphorylation of the proteins that associated with D228N/PFTAIRE (Figure 4.10). All of these experiments suggested that the protein recognized by the PFC5 antibody was a protein kinase and that the major targets of PFTAIRE activity were proteins that co-immunoprecipitated with it.

A more direct demonstration of PFTAIRE kinase activity in an *in vitro* assay, using *in vitro*-translated PFTAIRE or the recombinant GST-PFTAIRE fusion protein produced in bacteria, was not possible because a PFTAIRE substrate protein was not identified. Nor was it possible to demonstrate autophosphorylation kinase activity with the *in vitro*-translated PFTAIRE or GST-

PFTAIRE. The lack of kinase activity in the *in vitro* preparations of PFTAIRE may be indicative of a need for a regulatory component that was not present in these preparations. The histone H1 kinase activity of CDK5 expressed in bacteria was shown to be dependent on association of the recombinant CDK5 with its non-cyclin regulatory component, p25 (p35) (Lew et al., 1994; Qi et al., 1995). Demonstration of PFTAIRE kinase activity *in vitro* may similarly require the identification of a regulatory protein.

c. What are the proteins co-immunoprecipitated with PFTAIRE?

The phosphorylation of the proteins that co-immunoprecipitated with PFTAIRE was dependent on association with PFTAIRE kinase activity, but whether these proteins were regulators of PFTAIRE activity or actual substrates has not been determined. Protein kinases like cAMP-dependent protein kinase and many of the CDKs have been co-purified or co-immunoprecipitated with regulatory proteins. The cAMP-dependent protein kinase was purified from brain as a complex with the RI and RII regulatory subunits and other protein substrates (Lohmann et al., 1980; Theurkauf and Vallee, 1982). The purification of starfish oocyte MPF revealed that this factor was a heterodimer of one molecule of p34^{cdc2} associated with one molecule of cyclin B (Labbé et al., 1989). In addition, p34^{cdc2} is associated with cyclin A and cyclin B when immunoprecipitated from lysates of HeLa cells (Pines and Hunter, 1990; Pines and Hunter, 1989). CDK2, CDK4 and CDK5 all associate with cyclin D1, the proliferating cell nuclear antigen (PCNA) and p21 when immunoprecipitated from lysates of the normal human fibroblasts (Xiong et al., 1992). Cyclin H associates with CDK7 in HeLa and COS cells (Fisher and Morgan, 1994; Mäkelä et al., 1994) and CDK8 co-immunoprecipitates with cyclin C from HeLa cells (Tassan et al., 1995). The kinase activity of neuronal CDK5 is regulated by its association with the neuron-specific non-cyclin proteins p35 and p67 (Lew et al., 1994; Tsai et al., 1994; Shetty et al., 1995) and both of these regulatory proteins co-purify with CDK5 (Lew et al., 1992a; Kobayashi et al., 1993; Shetty et al., 1995). Neuronal CDK5 also co-immunoprecipitates with cyclin E from lysates of adult mouse brain, although the kinase activity of this CDK5 complex has yet to be demonstrated (Miyajima et al., 1995). Many of the CDKs identified to date remain associated with regulatory proteins, such as cyclin proteins. Early experiments demonstrating the kinase activity of p34^{cdc2} showed that a 62 kDa protein that co-immunoprecipitated with p34^{cdc2} from HeLa cell lysates was also phosphorylated by the kinase (Draetta and Beach, 1988). The "substrate"

protein that associated with p34^{cdc2} was later identified as the regulatory component cyclin B (Draetta et al., 1989). The association of neuronal CDK5 with the p25 (p35) regulatory component *in vitro* also results in phosphorylation of p25 (p35), but not of the kinase itself (Lew et al., 1994, Qi et al., 1995). Thus it is certainly possible that the proteins which were co-immunoprecipitated with and phosphorylated by PFTAIRE were regulatory proteins.

A PFTAIRE substrate has not been identified by the standard *in vitro* kinase assay protocol where kinase activity is assessed by the addition of experimental substrate proteins or peptides to kinase preparations (Section 4.4). The 58-60 kDa proteins that co-immunoprecipitated with PFTAIRE were the major targets of PFTAIRE activity in the immunoprecipitation-kinase assay used to demonstrate that PFTAIRE possessed kinase activity. Although there are many examples of protein kinases remaining associated with regulatory proteins upon immunoprecipitation, the co-immunoprecipitation of kinases with substrate proteins has not been well described, possibly because of the transient interaction that occurs between enzyme and substrate. However, there are protein kinase activities that phosphorylate cytoskeletal components of cells such as intermediate filament proteins, tubulins and microtubule associated proteins that do remain associated with these proteins when they are purified or immunoprecipitated (Pant and Veeranna, 1995; Zhou et al., 1991; Lohmann et al, 1980; Theurkauf and Vallee, 1982), suggesting that interactions between kinases and cytoskeletal proteins may be more stable than enzyme-substrate interactions that occur between cytosolic proteins. Whether the 58-60 kDa proteins that were phosphorylated by PFTAIRE are substrates or regulatory components remains to be determined either by biochemical purification and cloning of these PFTAIRE-associated proteins or by interaction cloning using the yeast two-hybrid method.

Information obtained from the characterization of PFTAIRE, such as PFTAIRE subcellular localization, the apparent molecular mass of the phosphoproteins and the two dimensional gel electrophoresis analysis of the PFTAIRE immune complex can be compared to similar information about other known proteins to make logical predictions regarding the identity of the PFTAIRE-associated proteins. For example, three cyclin proteins, cyclin A, B and E have apparent molecular masses of 60 kDa (human cyclin A), 62 kDa (human cyclin B) and 56 kDa (mouse cyclin E) (Giordano et al., 1989; Pines and Hunter, 1989; Miyajima et al., 1995), similar to the molecular mass of the phosphoproteins associated with PFTAIRE. Cyclin A localizes predominantly in the

nucleus while cyclin B is present in the cytoplasm until the onset of mitosis, when it translocates to the nucleus (Pines and Hunter, 1991). Although PFTAIRE shows similar subcellular localization in the nucleus and cytoplasm of neurons, neither cyclin A nor cyclin B is expressed in postmitotic neurons (Hayes et al., 1991; Freeman et al., 1994; Zhao et al., 1995). In contrast, cyclin E mRNA and protein are expressed in adult nervous tissue, in primary cultures of neurons and glia and in PC12 cells following treatment with NGF (Damjanov et al., 1994; Miyajima et al., 1995; Dobashi et al., 1995). Cyclin E also co-immunoprecipitated with CDK5 from adult brain protein preparations (Miyajima et al., 1995) and was associated with a cdc2-like kinase in squid axoplasm (Takahashi et al., 1995). Based on these observations and the apparent molecular mass of cyclin E, this protein may be a candidate to consider when attempting to identify the proteins co-immunoprecipitated with and phosphorylated by PFTAIRE.

Another known protein, microtubule-associated tau, shares some features of the PFTAIRE-associated 58-60 kDa proteins. Although CDK5 has been shown to phosphorylate bovine tau *in vitro* (Paudel et al., 1993), neither immunoprecipitated CDK5 nor PFTAIRE phosphorylated the commercially available bovine tau when used in kinase assays described in this chapter. It is possible that this purified tau preparation was not completely dephosphorylated. The most strongly labelled proteins in PFTAIRE immunoprecipitation-kinase assay samples had an apparent molecular mass of 58-60 kDa, and their migration on SDS-polyacrylamide gels suggested that they may actually represent different phosphorylation states of the same protein. There are six isoforms of tau protein in adult brain ranging in size from approximately 55-65 kDa (Weingarten, 1975). The tau proteins are the products of an alternatively spliced gene (Kosik et al., 1989; Goedert et al., 1989; Himmler et al., 1989), and each of these isoforms is differentially phosphorylated (Watanabe, 1993). When proteins in the PFTAIRE immune complex were separated by two dimensional gel electrophoresis the 58-60 kDa phosphoproteins were resolved as multiple spots of the same apparent molecular mass along the pH gradient from approximately pH 7.2-8.0 (Figure 4.13). The two dimensional analysis of these proteins shows similarities to the two dimensional resolution of the 55-56 kDa species of porcine tau, rat tau and human tau detected with the Tau-1 antibody (Cleveland et al., 1977; Butler and Shelanski, 1986; Ksiezak-Reding et al., 1988). This observation raises the question of whether PFTAIRE and tau may be associated when PFTAIRE is immunoprecipitated from a brain cytosolic protein fraction.

Expression of specific tau isoforms and changes in tau phosphorylation are

developmentally regulated (Kosik et al., 1989; Watanabe, 1993). Using mass spectrometry and phosphopeptide sequencing, Watanabe et al.(1993) demonstrated that fetal rat tau proteins were phosphorylated at 8-10 sites while adult rat tau proteins were phosphorylated at only 5 sites. Expression of highly phosphorylated tau proteins correlates with the period of intense synaptogenesis that occurs during the first two weeks of postnatal development in the rat cortex. This is followed by a reduction in the phosphorylation of tau that coincides with stabilization of the cytoskeleton (Watanabe et al., 1993; Brion et al., 1994). In addition, the immature, more highly phosphorylated forms of tau are less efficient than adult forms of tau in promoting tubulin polymerization and microtubule assembly *in vitro* (Francon et al., 1982; Goedert et al., 1990). This has led to the suggestion that highly phosphorylated tau isoforms may be required during synapse formation and reorganization when a less stable microtubule network is required, while the less phosphorylated adult forms are required to maintain a more stable cytoskeletal network (Brion et al., 1994). Analysis of mice lacking tau protein has demonstrated that tau is required for stabilization and organization of microtubules in small caliber axons (Harada et al., 1994). It has been suggested that different proline directed kinases may phosphorylate tau at different times during development. PFTAIRE mRNA was barely detected in fetal tissue, but then showed a marked upregulation on the day of birth and a progressive increase in expression during the first two weeks of postnatal development (See Chapter 3, Section 3.5a). Thus the upregulation of PFTAIRE mRNA expression correlates with the presence of highly phosphorylated fetal tau during the period of active synaptogenesis and stabilization of the neuronal cytoskeleton in rodents.

Tau contains multiple Ser/Thr-Pro phosphorylation sites and several different proline directed kinases, including p42 MAP kinase (ERK2), GSK-3 β , p34^{cdc2} and CDK5 phosphorylate tau *in vitro* at sites that are are phosphorylated in paired helical filament tau (Drewes et al., 1992; Ledesma et al., 1992; Ishiguro, et al., 1993; Vulliet et al., 1992; Baumann et al., 1993; Paudel et al., 1993). All of these kinases, except for p34^{cdc2} are associated with tau during purification of microtubule-associated proteins from normal adult brain tissue and paired helical filaments (Baumann et al., 1993). The size and pI of the 58-60 kDa PFTAIRE-associated phosphoprotein showed similarities to some tau isoforms. However, when the Tau-1 antibody was used to determine whether the 58-60 kDa phosphoprotein was tau (immunoblots of two dimensional gels), the tau protein was not detected. Prior to isoelectric focussing 15 μ g of brain extract was

added to kinase assay samples to determine whether tau proteins co-migrated with the phosphorylated 58-60 kDa protein in the PFTAIRE immune complex. The Tau-1 antibody only detects tau protein when the Ser 199 and Ser 202 (human tau) residues within this epitope are not phosphorylated. However, the addition of brain extract to the samples should have enabled detection of some tau protein, regardless of the identity or phosphorylation state of the 58-60 kDa protein in the PFTAIRE immune complex. Thus it was not possible to conclude from this experiment whether the 58-60 kDa phosphoprotein associated with PFTAIRE was tau.

Although the 58-60 kDa protein that co-immunoprecipitated with PFTAIRE from brain showed similarities (molecular mass and pI) to isoforms of tau, a similar or identical protein was also present in the 293 cell PFTAIRE immune complex (Figure 4.10). Tau protein is generally considered a neuronal protein. However, tau was detected with the Tau-1 antibody in nuclei in cell lines such as human neuroblastoma cells, non-neuronal cells and in monkey kidney cells (Loomis et al., 1990). The function of tau in nuclei of non-neuronal cells is presently unknown. It is not known whether 293 cells express any tau isoforms but the possibility that the 58-60 kDa PFTAIRE-associated protein in brain and 293 cell immunoprecipitates could be tau cannot be excluded.

The proteins that I have presented as potential candidates for PFTAIRE substrates are examples that share some features with the 58-60 kDa phosphoprotein that is specifically associated with PFTAIRE. However, there is a plethora of identified (and presumably unidentified) proteins that have the same apparent molecular mass as these phosphoproteins or a similar subcellular localization as PFTAIRE. If this protein is a PFTAIRE substrate *in vivo*, then its presence in postmitotic neurons and in proliferating cells suggests that together PFTAIRE and this protein may perform different functions in diverse cell types.

d. Does phosphorylation regulate PFTAIRE kinase activity?

Phosphorylation of PFTAIRE was demonstrated in two different experiments, suggesting that PFTAIRE may associate with other protein kinases. When GST-PFTAIRE and GST-D228N (mutated at the aspartate residue required for phosphate transfer) were combined with a brain cytosolic protein preparation, to allow proteins to bind to the recombinant fusion proteins, both fusion proteins were phosphorylated in subsequent kinase reactions. In a control reaction GST was not phosphorylated following incubation with the brain cytosolic protein preparation, indicating that the PFTAIRE component of each fusion protein had been phosphorylated (Figure

4.11). Because GST-D228N was phosphorylated to the same extent as GST-PFTAIRE, the data suggested that phosphorylation was due to another kinase from the brain protein preparation that associated with the fusion proteins, and not to a regulatory protein activating PFTAIRE autophosphorylation. When PFTAIRE kinase reactions containing immunoprecipitated brain PFTAIRE were analyzed by two dimensional gel electrophoresis and western blotting, PFTAIRE was identified as a single spot on the immunoblot (Figure 4.13B) that corresponded to a weakly phosphorylated protein (Figure 4.13A). PFTAIRE was distinct from the other strongly phosphorylated protein in the immune complex, corresponding to the 58-60 kDa protein. This data suggested that either endogenous brain PFTAIRE possessed some autophosphorylation kinase activity or another kinase was part of the immunoprecipitated protein complex. The phosphorylation of GST-PFTAIRE and GST-D228N suggests that another kinase associated with the fusion proteins, but it is not possible to determine from the two dimensional gel analysis whether another kinase was co-immunoprecipitated with PFTAIRE from brain or whether PFTAIRE autophosphorylation occurred. It is possible that *in vivo* another kinase may phosphorylate PFTAIRE to regulate its activity. Kinase assays performed with the D228N mutant (Figures 4.9 and 4.10) indicate that if another kinase did associate with immunoprecipitated PFTAIRE, it did not appear to be responsible for the phosphorylation of the 58-60 kDa protein in the immune complex.

The kinase activity of p34^{cdc2} is regulated in part by the phosphorylation of three residues, Thr14, Tyr15 and Thr160, by Myt1, Wee1 and the CDK activating kinase (CAK) (Mueller et al., 1995; Parker et al., 1992; Solomon et al., 1993; Fesquet et al., 1993; Poon et al., 1993). These potential phosphorylation sites are conserved in most cdc2-related kinases, including PFTAIRE (Tyr99, Ser100, Ser243) and phosphorylation of these sites by Wee1, Myt1 or CAK-like protein kinases expressed in nervous tissue may regulate PFTAIRE kinase activity. CDK5 may be regulated by Wee1 kinase and a Wee1-like kinase activity has been detected in bovine brain extracts and cloned from a mouse brain library (Lew et al., 1995). Serine 243 (Ser243) of PFTAIRE is located in the position corresponding to Thr161 of p34^{cdc2}, suggesting that this site may be a potential target for phosphorylation by a CAK-like activity in nervous tissue. A CAK activity has also been detected in bovine brain extracts. This activity does not appear to be responsible for activation of CDK5 (Lew et al., 1995), which does not require phosphorylation for

activation (Qi et al., 1995). The conservation of sites that are required for regulation of p34cdc2 kinase activity and the presence of Wee1-like and CAK-like activities in nervous tissue suggest that cdc2-related kinase activities from diverse cell types may be regulated in similar ways. In addition to the conserved residues that suggest possible regulation by Wee1-like or CAK-like activities, consensus motifs for phosphorylation by cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) were identified in the PFTAIRE primary sequence (See Chapter 3, Section 3.3). Because recombinant *in vitro* preparations of PFTAIRE (*in vitro*-translated PFTAIRE and GST-PFTAIRE) did not demonstrate autophosphorylation activity, it seems likely that PFTAIRE activity may be regulated by interactions with other proteins, including other protein kinases.

e. Conclusions

The PFC5 antibody, raised against a C-terminal peptide predicted from the PFTAIRE cDNA, has been characterized to demonstrate the specificity of the antibody for immunoprecipitation, immunoblotting and immunofluorescence experiments. The antibody reacted with a single protein of an apparent molecular mass of 52 kDa, which was present in adult mouse nervous tissue from the CNS and PNS. Based on experiments demonstrating the specificity of the antibody the 52 kDa protein is considered the endogenous PFTAIRE species expressed in the nervous system. The kinase activity of PFTAIRE was demonstrated when PFTAIRE was immunoprecipitated from a cytosolic protein preparation of brain. Proteins that were co-immunoprecipitated with PFTAIRE were specifically phosphorylated. In particular, a 58-60 kDa PFTAIRE-associated protein appeared to be the major target of PFTAIRE kinase activity in this assay, though the identity of this protein has not been determined. *In vitro* translated PFTAIRE and preparations of GST-PFTAIRE from bacteria did not demonstrate autophosphorylation kinase activity, but the GST-PFTAIRE was phosphorylated by another kinase present in brain lysate, suggesting that PFTAIRE activity may be regulated by its association with other proteins, including other protein kinases. The identification of PFTAIRE-interacting proteins, substrates and regulatory proteins, will be essential to ultimately understand the function of this cdc2-related kinase in the adult nervous system. The characterization of PFTAIRE kinase has demonstrated that PFTAIRE differs in many ways from CDK5, the only other cdc2-related kinase that has been well characterized in the nervous system. The two related protein kinases are localized in different compartments in adult CNS neurons, associate with and phosphorylate distinct proteins when immunoprecipitated, may require different recognition sequences for phosphorylation of

substrate proteins and may be regulated differently. Targeted disruption of the CDK5 gene has recently demonstrated that CDK5 is important for normal brain development and establishing a normal cytoskeletal architecture in nervous tissue (Oshima et al., 1996) suggesting that CDK5 activity is required early in nervous system development. Like CDK5, PFTAIRE may also perform functions that are unrelated to the cell cycle functions of CDKs in proliferating cells. However, the characterization of these two proteins has demonstrated the distinct properties of PFTAIRE and CDK5 which suggest that the function of PFTAIRE in the nervous system may differ from that of CDK5.

Figure 4.1. Reactivity and specificity of anti-PFTAIRE polyclonal antibodies.

A) Autoradiogram showing immunoprecipitation of PFTAIRE after in vitro-translation using rabbit reticulocyte lysate. Immunoprecipitation with pre-immune serum (lane 1), C-terminal PFTAIRE antibody PFC5 (lane 2), C-terminal antibody (PFC5) pre-incubated with a molar excess of antigenic peptide (lane 3), pre-immune serum (lane 4), N-terminal PFTAIRE antibody PGN5 (lane 5), N-terminal antibody (PGN5) pre-incubated with a molar excess of antigenic GST-PFTAIRE fusion protein (lane 6). The PFTAIRE translation product prior to immunoprecipitation is shown in lane 7. **B)** Western blot of PFTAIRE-transfected (pcPF-COS7) vs. nontransfected COS7 cell lysate immunoprecipitations. Cytosolic protein was prepared from lysed culture 24 hours after transfection. Samples were immunoprecipitated with pre-immune serum or the C-terminal PFTAIRE antibody (PFC5) and used to prepare a western blot that was immunoblotted with the N-terminal antibody (PGN5). Lanes 1 and 2 are immunoprecipitations performed with pre-immune serum or PFC5 and lysates prepared from nontransfected cells. Lanes 3 and 4 are immunoprecipitations using pre-immune serum or PFC5 and lysates prepared from PFTAIRE-transfected cells. **C)** The same cytosolic protein preparations used for the immunoprecipitations shown in panel B were also used for immunoprecipitations with pre-immune serum (lanes 1 and 3) and the N-terminal PFTAIRE antibody, PGN5 (lanes 2 and 4). The western blot prepared from these samples was immunoblotted with the the C-terminal PFTAIRE antibody (PFC5).

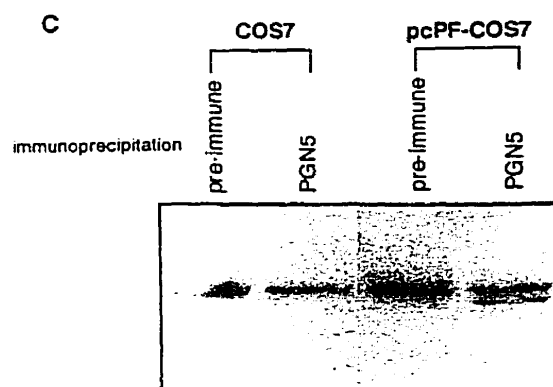
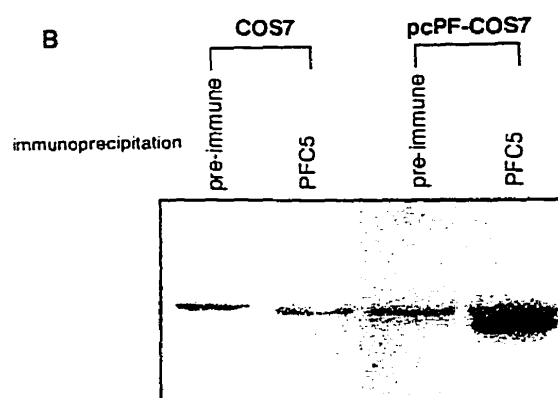
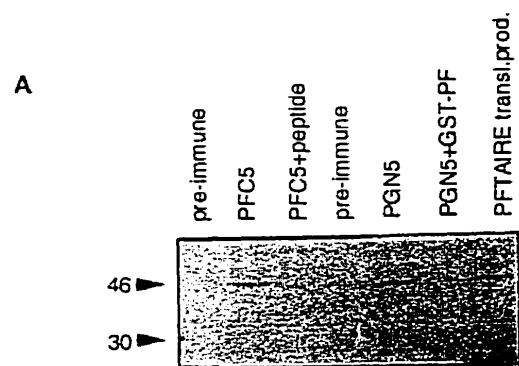


Figure 4.2. Protein expression of PFTAIRE in adult mouse tissues.

Cytosolic protein samples (15 µg) prepared from adult mouse tissues, brain (lane 1), cerebellum (lane 2), spinal cord (lane 3), sciatic nerve (lane 4), lung (lane 5), skeletal muscle (lane 6), kidney (lane 7) and liver (lane 8) were separated by electrophoresis on a 10% SDS-polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane. The C-terminal PFTAIRE antibody, PFC5, (1:2500) horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:1000) and ECL were used to detect PFTAIRE. The specific protein recognized by PFC5 in nervous tissue migrated at an apparent molecular mass of 52 kDa.

1 2 3 4 5 6 7 8

67 ▶

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— — — — —

43 ▶

30 ▶

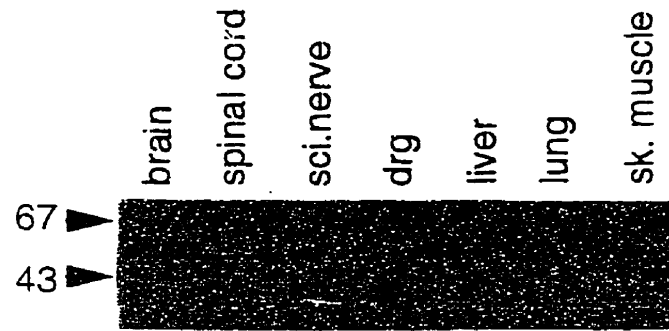
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Figure 4.3 Specificity of the PFC5 antibody for the 52 kDa PFTAIRE protein detected in mouse nervous tissue.

Cytosolic protein samples (15 µg) prepared from adult mouse tissues, brain, spinal cord, sciatic nerve, dorsal root ganglia, liver, lung, and skeletal muscle were separated by electrophoresis on a 10% SDS-polyacrylamide gel, followed by electrophoretic transfer to a nitrocellulose membrane.

A) The C-terminal PFTAIRE antibody, PFC5, (1:2500) horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:1000) and ECL were used to detect PFTAIRE. **B)** The PFC5 antibody (1:2500) was pre-incubated with an approximately 35-fold molar excess of C-terminal PFTAIRE peptide (3 µg peptide: 1 µg antibody) prior to immunoblotting. The same conditions for immunoblotting were used for the blots shown in A and B. The small arrowhead in B indicates the position of the 52 kDa PFTAIRE protein.

A



B

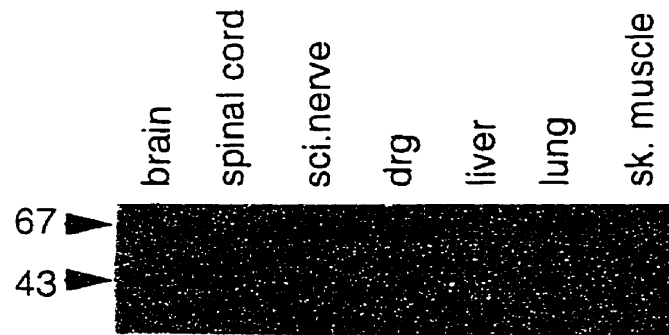


Figure 4.4 Indirect immunofluorescence detection of PFTAIRE in transfected cells with the PFC5 antibody.

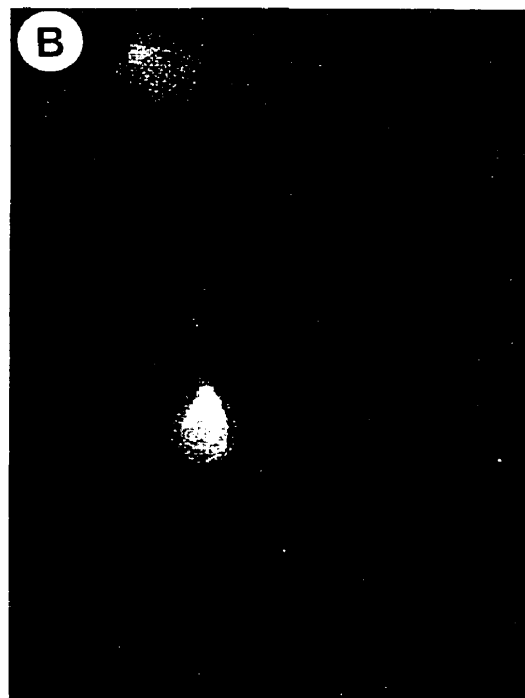
A) and B) NIH 3T3 cells were transfected transiently with the pc3PF plasmid for overexpression of PFTAIRE. Twenty-four hours after transfection cells were fixed and processed for indirect immunofluorescence with pre-immune serum (A) or the PFC5 antibody (B) and fluorescein conjugated-donkey anti-rabbit IgG. C) and D) Vero cells were transfected transiently with the pHermes-L/PF plasmid to over-express PFTAIRE, fixed after 24 hours and processed for indirect immunofluorescence with C) pre-immune serum and D) the PFC5 antibody.

PFTAIRE-TRANSFECTED CELLS

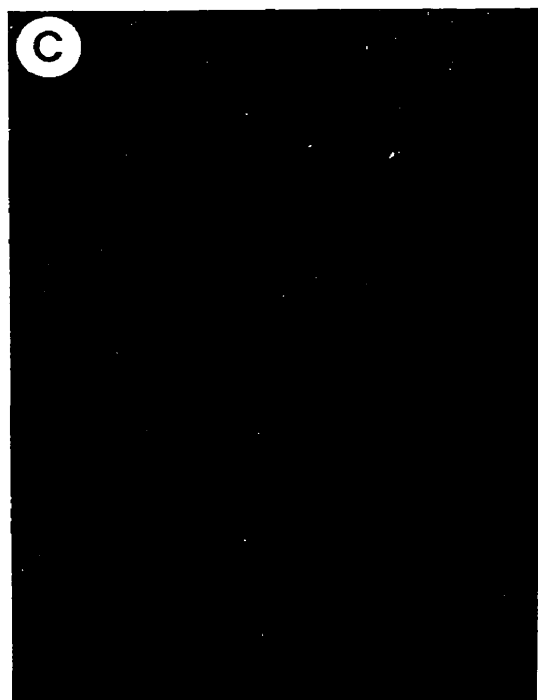
pre-immune



anti-PFTAIRE (PFC5)



pre-immune



anti-PFTAIRE (PFC5)

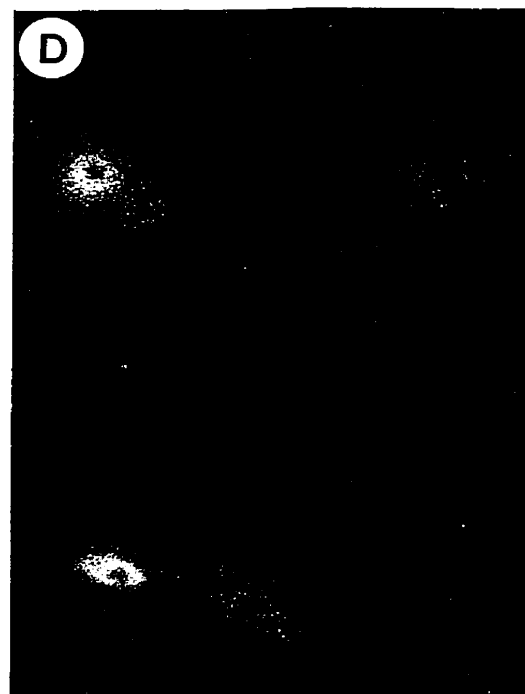


Figure 4.5. Indirect immunofluorescence labelling of mouse spinal cord with antibodies recognizing PFTAIRE and hypophosphorylated NF-H.

8 μ m sections of spinal cord were incubated with (A) pre-immune serum (1:100) and the (B) C-terminal PFTAIRE antibody, PFC5 (1:100). A section (8 μ m) of spinal cord was labelled with (C) PFC5 (1:100) and (D) SMI32 (1:1000), which recognizes a hypophosphorylated epitope of the neurofilament-heavy (NF-H) protein, to show large motor neurons of the anterior horn.

Arrowheads indicate the same cell bodies in C and D to compare the subcellular localization of PFTAIRE with that of NF-H. The small arrow in D indicates NF-H in a motor neuron axon. The small arrow in C indicates PFTAIRE in the proximal axon of a motor neuron. A and B are the same magnification. Scale bar in A, 10 μ m. C and D are the same magnification. Scale bar in C, 5 μ m.

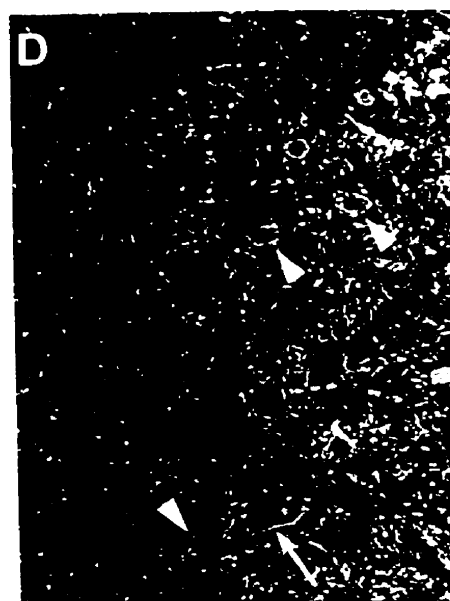
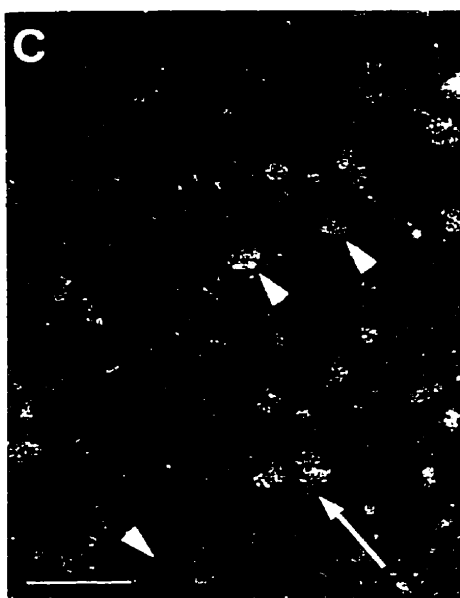
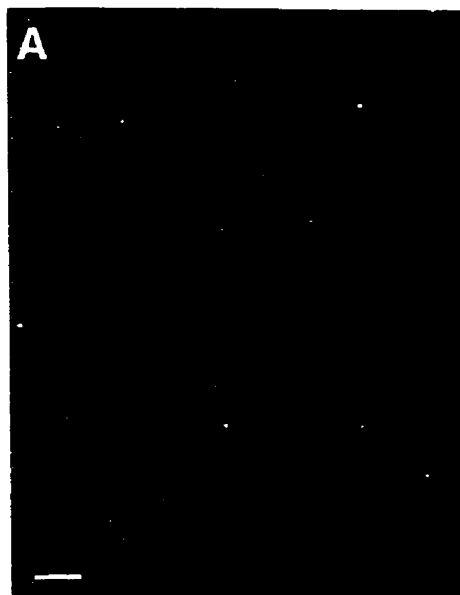


Figure 4.6 Indirect immunofluorescence labelling of mouse cerebellum with antibodies recognizing PFTAIRE and hypophosphorylated NF-H.

The same section (8 μm) of adult mouse cerebellum was labelled with (C) PFC5 (1:100) and (D) SMI32 (1:1000), which recognizes a hypophosphorylated epitope of the NF-H protein for comparison of the distribution of PFTAIRE and NF-H in the three layers of the cerebellar cortex and the subcellular localizations of the two proteins. In panel B pre-immune serum (1:100) was used as the primary antibody on another section of cerebellum. Panel A demonstrates the localization of PFTAIRE mRNA in the cerebellar cortex by in situ hybridization, using a 5' 290 bp PFTAIRE cDNA probe, (refer to Chapter 3, Section 3.5b) for comparison with the PFC5 labelling of PFTAIRE shown in panel C. Scale bar, 10 μm .

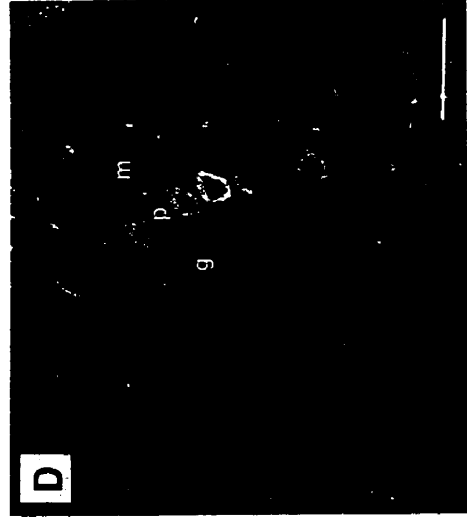
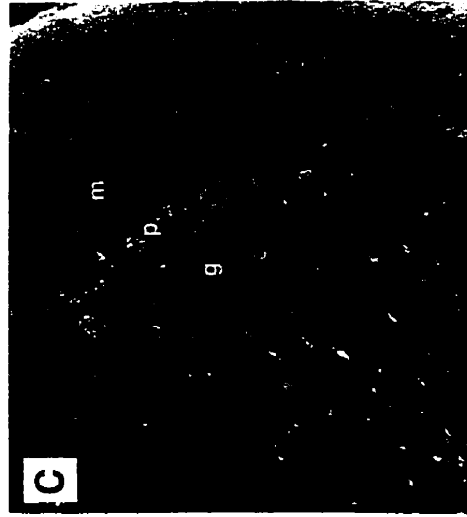
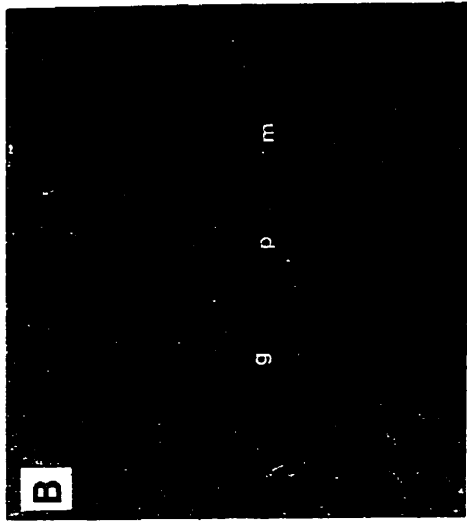


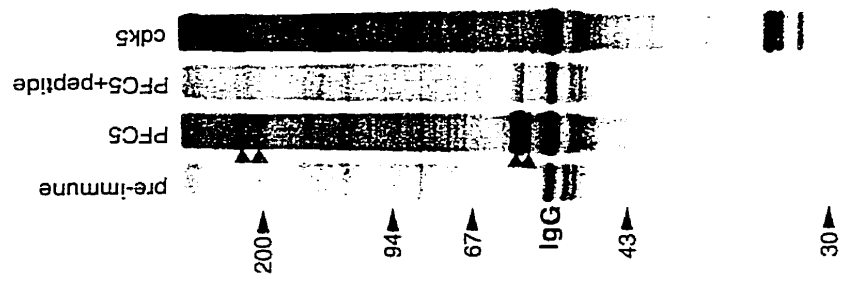
Figure 4.7 Indirect immunofluorescence labelling of mouse sciatic nerve with antibodies recognizing PFTAIRE and hyperphosphorylated NF-H.

The same section (8 μ m) of adult mouse sciatic nerve was labelled with (B) PFC5 (1:100) and (C) SMI31 (1:1000), which recognizes a hyperphosphorylated epitope of NF-H occurring predominantly in axons, to compare the distribution of the two proteins. The large arrowheads indicate axons that contain both PFTAIRE and NF-H. Smaller arrowheads in B indicate PFC5 labelling around the myelin, possibly in Schwann cells. Panel A is a different section labelled with pre-immune serum. All panels are the same magnification. Scale bar, 50 μ m.

Figure 4.8 PFTAIRE kinase activity in brain immunoprecipitates.

A) A cytosolic protein preparation from adult mouse brain was used for immunoprecipitations with either pre-immune serum, the PFTAIRE antibody (PFC5), PFC5 pre-adsorbed with an 100-fold molar excess of PFTAIRE peptide (PFC5 + peptide), or an anti-CDK5 polyclonal antibody (Pharmingen). Kinase buffer containing 5 μ Ci [γ ³³-P]ATP was added to the immunoprecipitates and samples were incubated for one hour at 30°C. The samples were electrophoresed on a 10% SDS-polyacrylamide gel that was processed for autoradiography. Arrowheads indicate the specific phosphoproteins associated with PFTAIRE, migrating at approximately 58-60 kDa and 200-205 kDa. **B)** Cytosolic protein preparations from adult mouse brain and skeletal muscle were used for immunoprecipitations with PFC5 or PFC5 pre-adsorbed with an 100-fold molar excess of PFTAIRE peptide (PFC5 + peptide). The kinase assay and analysis of samples were performed as described for panel A.

A



B

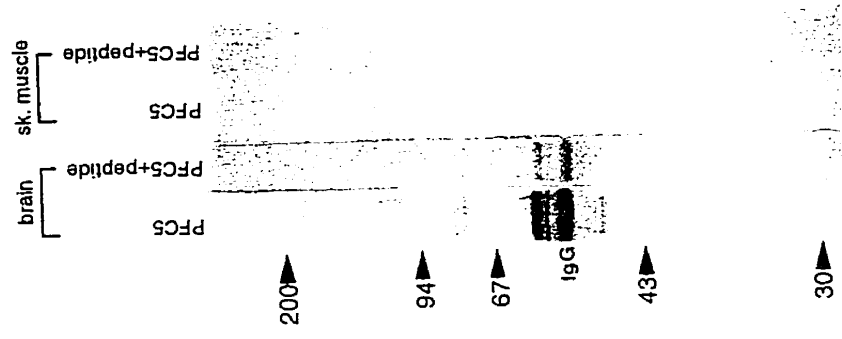
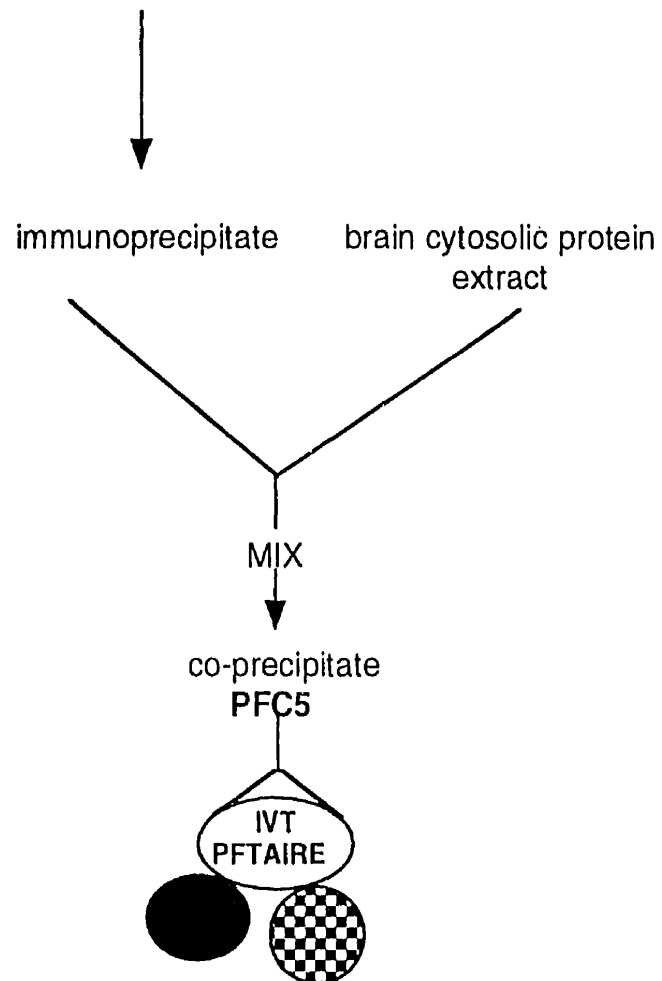


Figure 4.9 Phosphorylation of 58-60 kDa proteins from brain following co-precipitation with *in vitro* translated PFTAIRE and D228N.

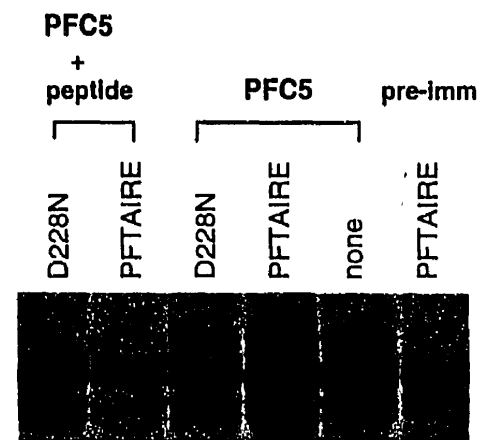
A) Scheme for binding, co-precipitating and phosphorylating 58-60 kDa proteins from brain, using *in vitro*-translated PFTAIRE and the PFTAIRE mutant, D228N. *In vitro*-translated proteins were immunoprecipitated and then mixed with a cytosolic protein preparation from adult mouse brain to bind PFTAIRE-interacting proteins. Protein complexes were washed several times, resuspended and divided into two equal samples. Kinase buffer containing 5 μ Ci [γ^{33} -P]ATP was added to one set of samples which was incubated for 1 hour at 30°C for kinase assays. **B)** Kinase assay samples were analyzed by electrophoresis on a 10% SDS-polyacrylamide gel and autoradiography. Kinase assays containing the *in vitro*-translated proteins D228N and PFTAIRE immunoprecipitated with PFC5 pre-adsorbed with the C-terminal PFTAIRE peptide (PFC5 + peptide) are shown in lanes 1 and 2, respectively. Kinase assays of PFC5-immunoprecipitated samples are shown in lanes 3 (*in vitro*-translated D228N), 4 (*in vitro*-translated PFTAIRE) and 5 (endogenous brain PFTAIRE, no *in vitro*-translated protein). A kinase assay performed with *in vitro*-translated PFTAIRE, immunoprecipitated with pre-immune serum is shown in lane 6. **C)** The remaining half of each sample used in the kinase assays shown in panel B was used to prepare a western blot. The nitrocellulose membrane was incubated with the PFC5 antibody (1:2500) to detect *in vitro*-translated PFTAIRE and D228N (arrowhead) in the protein complexes (lanes 5 and 6). Lanes 1 and 2 are the D228N and PFTAIRE *in vitro*-translated proteins prior to immunoprecipitation.

A

in vitro translated PFTAIRE or D228N



B



C

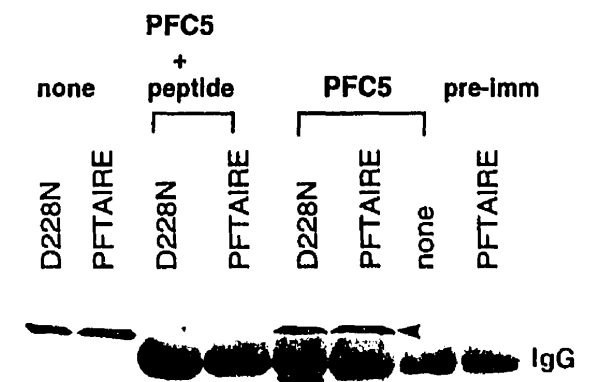


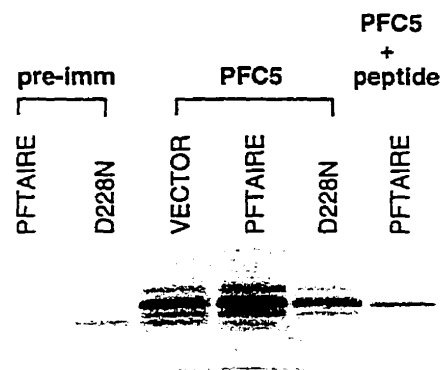
Figure 4.10 Transfection of 293 cells with PFTAIRE and the PFTAIRE mutant D228N.

293 cells (adenovirus transformed human embryonic kidney cells) were transfected with the pcDNA3 plasmid vector, pcDNA3-PFTAIRE (pc3PFT_{orf}) or pcDNA3-D228N (pc3D228N_{orf}). Following transfection cells were lysed and the cytosolic protein fraction was used for immunoprecipitations. Each immunoprecipitation was divided into two samples of equal volume. **A)** Following several washes one set of samples was processed for kinase assays, performed on proteins bound to the protein A-Sepharose. Samples were analyzed by electrophoresis on a 10% SDS-polyacrylamide gel that was processed for autoradiography. Lanes 1 and 2 are kinase assays performed with pre-immune immunoprecipitated samples prepared from PFTAIRE- and D228N-transfected cells. Lanes 3, 4 and 5 are kinase assays of PFC5-immunoprecipitated samples from vector-, PFTAIRE- and D228N-transfected cell lysates. An immunoprecipitation-kinase assay performed using PFC5 pre-adsorbed with the C-terminal PFTAIRE peptide (PFC5 + peptide) and PFTAIRE-transfected cell lysate is shown in lane 6. **B)** The remaining half of each sample shown in panel A was used to prepare a western blot that was immunoblotted with the PFC5 antibody (1:2500) to detect an endogenous PFTAIRE species from 293 cells (upper arrowhead, lanes 3, 4 and 5) and transfected PFTAIRE and D228N (lower arrowhead, lanes 4 and 5).

A

KINASE ASSAY

precipitating antibody



B

WESTERN BLOT

precipitating antibody

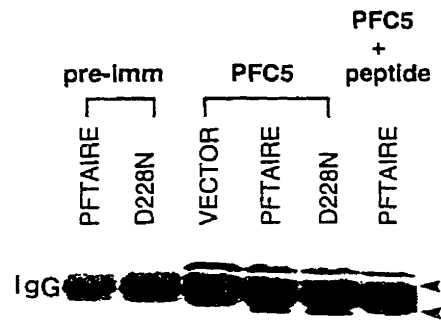
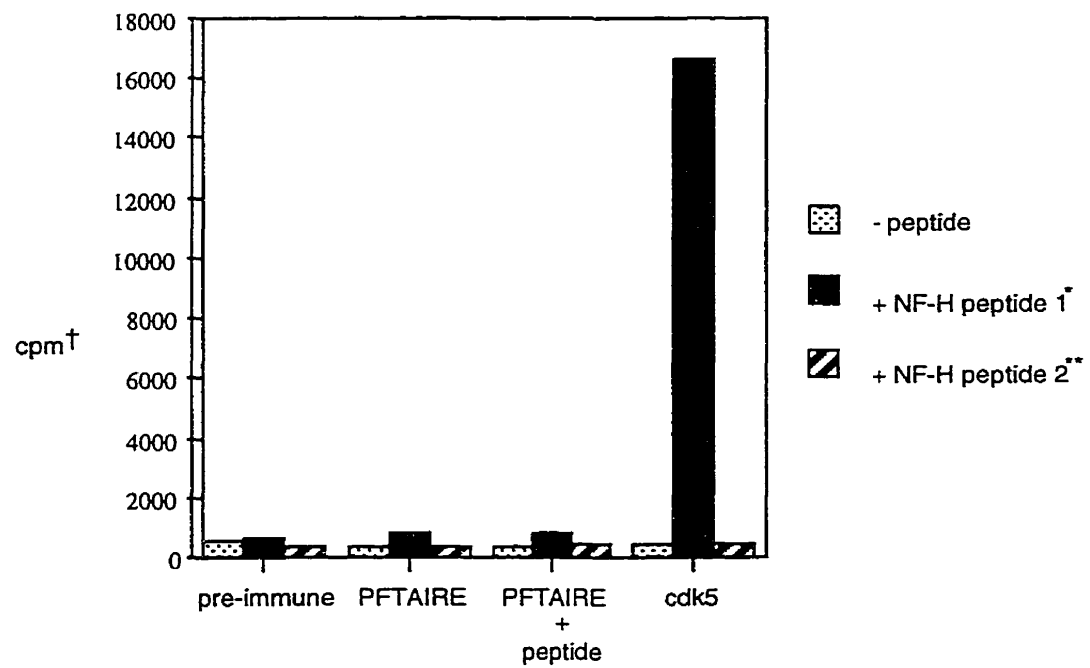


Figure 4.11 Kinase assays with synthetic peptide substrates derived from mouse NF-H.

A brain cytosolic protein preparation was immunoprecipitated with pre-immune serum, the PFC5 antibody, the PFC5 antibody + C-terminal PFTAIRE peptide or an anti-CDK5 antibody. Immune complexes were washed several times and kinase assays were performed in the absence of exogenous substrate (speckled bars) or in the presence of NF-H peptide 1 (filled bars) or NF-H peptide 2 (striped bars). One-fifth of each kinase reaction was spotted on to phosphocellulose membranes. Membranes were washed several times in 75 mM H_3PO_4 , dried in 95% ethanol and counted in scintillation fluid to determine the amount of ^{33}P incorporated by peptides. Each point on the graph represents the average of counts from duplicate kinase assay samples. Error bars were too small to be drawn on the graph.



†cpm represents the average of duplicate samples

*NF-H peptide 1 VKSPAKEKAKSPEK (KSPXK, KSPEK)

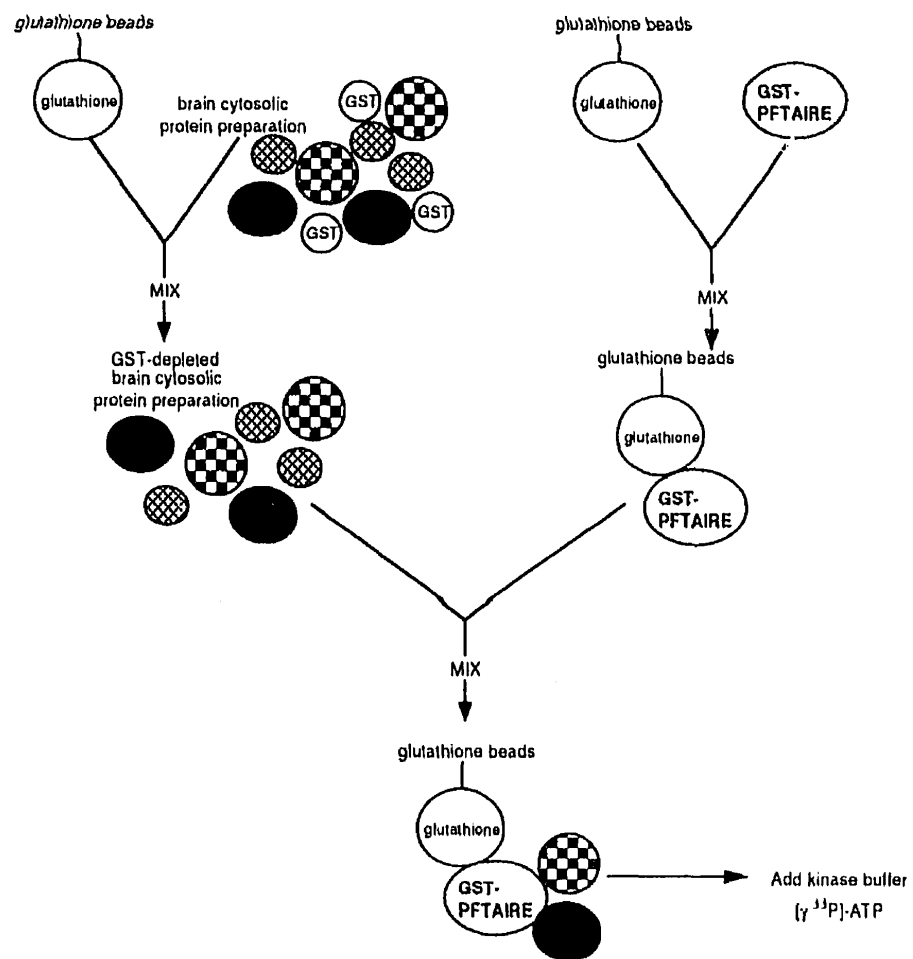
**NF-H peptide 2 EAKSPAEEKSPAEEK (KSPXE) ₂

Figure 4.12 Phosphorylation of GST-PFTAIRE and GST-D228N by proteins in a brain lysate.

A) Scheme for binding proteins in a brain cytosolic protein preparation to GST-fusion proteins. A brain cytosolic protein preparation was depleted of endogenous GST, by mixing the lysate with glutathione-Sepharose beads. The flow through brain lysate (GST depleted) was mixed with approximately equal amounts of soluble GST, GST-PFTAIRE or GST-D228N coupled to glutathione-Sepharose beads for 2 hours at 4°C. When the lysate was removed, beads were washed several times. Kinase reactions were performed with proteins bound to the glutathione beads by adding kinase buffer containing 5 μ Ci [γ -³³P]ATP to each sample and incubating 1 hour at 30°C. Samples were electrophoresed on a 10% SDS-polyacrylamide gel that was stained with Coomassie blue and processed for autoradiography.

B) An autoradiogram demonstrating phosphorylation of the GST-PFTAIRE and GST-D228N fusion proteins by a protein kinase in the brain cytosolic protein preparation. The first two lanes are control samples using uncoupled glutathione-Sepharose beads to demonstrate that in the absence of fusion proteins a few proteins from the brain lysate bound nonspecifically to the glutathione-Sepharose beads and were weakly phosphorylated. Lane 3 shows that no phosphorylation of GST (27 kDa) occurred when GST coupled beads were exposed to brain lysate. Lanes 5 and 7 show that the 74 kDa GST-PFTAIRE and GST-D228N fusion proteins were equally phosphorylated in kinase reactions following exposure to the brain lysate.

A



B

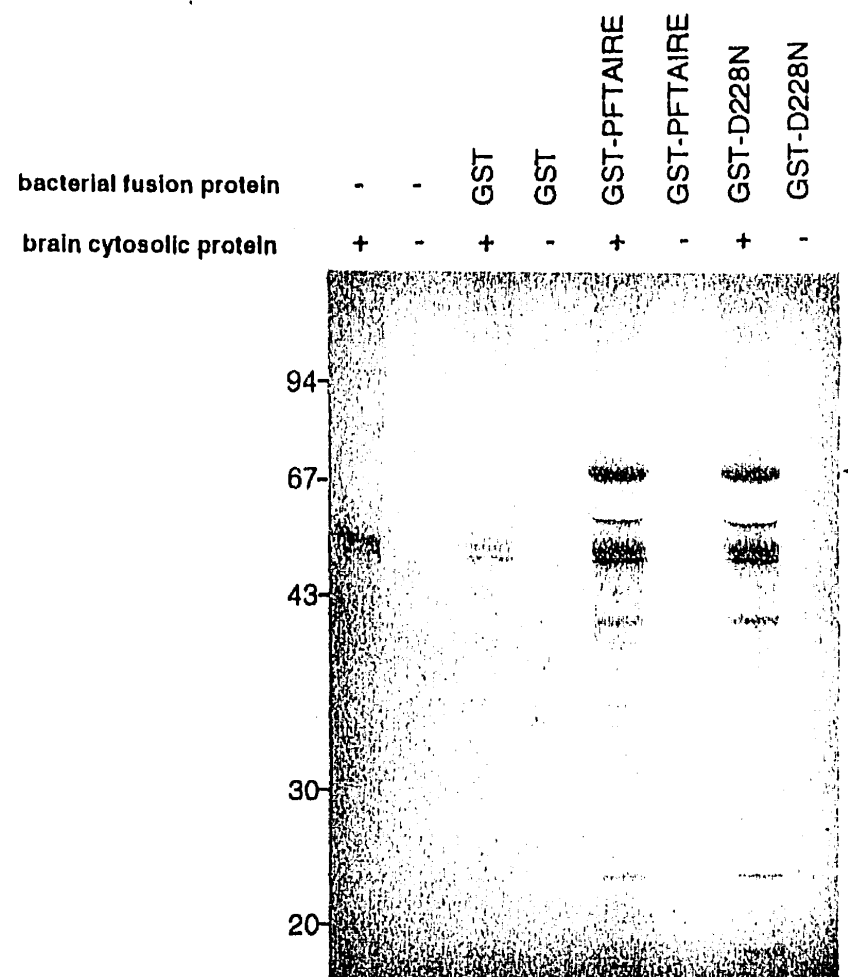
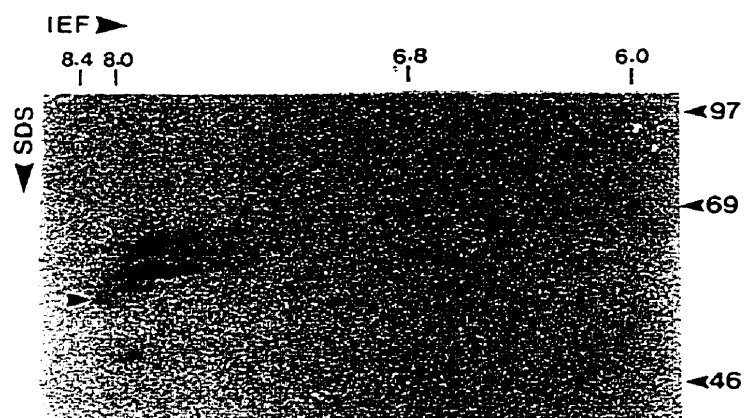


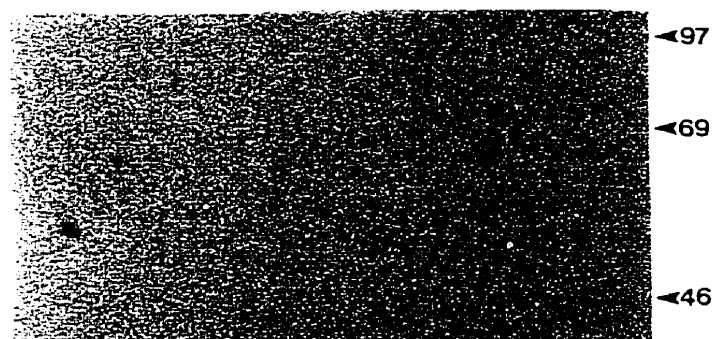
Figure 4.13 Two dimensional gel electrophoresis of a PFTAIRE kinase assay sample.

A) A PFTAIRE kinase assay sample was analyzed by isoelectric focussing on a pH 3-10 gel in the first dimension and a 10% SDS-polyacrylamide gel in the second dimension. The sample was transferred from the SDS-polyacrylamide gel to a nitrocellulose membrane. Panel A is an autoradiogram obtained after exposing the nitrocellulose membrane to film to demonstrate the separation of proteins in the PFTAIRE immune complex that were phosphorylated. The 58-60 kDa proteins that were normally present in PFTAIRE immunoprecipitation-kinase assays correspond to the phosphoproteins with the highest apparent molecular mass in this autoradiogram. **B)** The same nitrocellulose membrane was immunoblotted with the PFC5 antibody (1:2500) to determine if any of the phosphoproteins detected in panel A corresponded to PFTAIRE. The spot indicated by a small arrowhead in panel A corresponded to the PFTAIRE detected on the immunoblot (approximately pH 8.4) when the two autoradiograms were aligned.

A



B



CHAPTER 5
TRANSFECTION OF THE PFTAIRE cDNA
IN CELL LINES

5.1 Introduction

Although it was demonstrated using a PFTAIRE-specific antibody that the cloned PFTAIRE cDNA encodes a protein present in neurons and that this protein functions as a protein kinase (Chapter 4), these studies did not address directly the function of PFTAIRE in the nervous system. Data from kinase assays indicated that potential substrates such as histone H1, NF-H and tau proteins were not phosphorylated by PFTAIRE under the assay conditions used and these experiments did not provide insight into the function of the kinase. The identity of the 58-60 kDa protein that co-immunoprecipitated with PFTAIRE and appeared to be the major target of this kinase activity could not be readily determined from its apparent molecular mass, pI or the subcellular distribution of PFTAIRE.

In order to study the function of the novel PFTAIRE kinase two basic options have been considered. The gene in question can either be overexpressed or its expression can be inhibited or eliminated *in vitro* (i.e., in transfected cell lines) or *in vivo* (i.e., in transgenic animals) and the consequences of either approach may help to determine function. PFTAIRE mRNA and protein are expressed in postmitotic nervous tissue. Among the non-neuronal tissues examined for PFTAIRE expression, only lung expressed PFTAIRE mRNA, however, PFTAIRE protein was not detected in this tissue. This suggested that PFTAIRE expression may be restricted to neural cell types and that well characterized cell lines that can be differentiated to a neuronal phenotype, such as PC12 cells, may be the most appropriate cells for further characterization of PFTAIRE and its kinase activity. If endogenous PFTAIRE expression was upregulated when these cells were differentiated, then overexpression of PFTAIRE in undifferentiated cells or inhibition of PFTAIRE expression (with a dominant negative mutant or antisense plasmid) in differentiated cells might provide insight into the function of PFTAIRE. In addition, "neuronal" cell lines that express PFTAIRE may also express the proteins that PFTAIRE normally interacts with *in vivo*, i.e., regulatory or substrate proteins, providing a system in which these proteins could be identified and characterized. On the other hand, overexpression in non-neuronal cells that express little or no endogenous PFTAIRE may also provide information about why PFTAIRE expression *in vivo* is restricted predominantly to the nervous system.

In the experiments described in this chapter, I have transfected cultured cells with the PFTAIRE cDNA under the control of the human cytomegalovirus (CMV) promoter to obtain a high level of PFTAIRE expression. Four different plasmid constructs were made for this purpose: 1)

pcPF, 2) pc3PF, 3) pc3PF_{off} and 4) pHermes-L/PF. All of these plasmids are described in Chapter 2 (Section 2.4c). This chapter presents preliminary data obtained when these plasmids were used either to derive permanent cell lines that overexpressed PFTAIRE or to transiently overexpress PFTAIRE.

5.2 Identifying cell lines that express PFTAIRE

Prior to transfecting cells, lysates were prepared from several cell lines and the cytosolic fractions were analyzed by immunoblotting with the anti-mouse PFTAIRE antibody (PFC5) to determine which cell lines expressed endogenous PFTAIRE. Endogenous PFTAIRE was not detected on immunoblots of extracts (15 µg) from NIH 3T3 mouse fibroblasts, Ltk⁻ mouse fibroblasts or C2C12 mouse myoblasts (Figure 5.1A). An endogenous protein of approximately 58 kDa, which is larger than mouse brain PFTAIRE (52 kDa), was detected in Vero cells and COS-7 cells. A much lower level of a PFTAIRE species that has the same molecular mass as mouse brain PFTAIRE was also detected in COS-7 cells. Both of these cell lines are fibroblast-like cells derived from the kidney of African green monkey but Vero cells are from normal monkeys, while COS-7 cells are SV40-transformed and express large T antigen. Higher molecular weight PFTAIRE protein species were also detected in extracts from another fibroblast-like kidney cell line, 293, (adenovirus-transformed human embryonic kidney cells) (see Figure 5.6). It is possible that the higher molecular weight protein species detected with the PFC5 antibody in these cells was a closely related and more abundant protein in primate cells. An extremely low level of endogenous PFTAIRE was detected in the mouse neuroblastoma cell line N18TG2, and this protein has the same molecular mass (52 kDa) as mouse brain PFTAIRE. The immunoblot in panel B of Figure 5.1 shows expression of PFTAIRE in the rat adrenal pheochromocytoma cell line, PC12, which differentiates to a sympathetic neuronal phenotype when grown in medium supplemented with nerve growth factor (NGF), (Green and Tischler, 1976). The PFTAIRE species detected in PC12 cells was slightly larger (56 kDa) than mouse brain PFTAIRE. PFTAIRE expression in PC12 cells was low in cultures of undifferentiated, proliferating cells and in cultures that were differentiated (non-proliferating) for 7 days in medium containing NGF. In lysates prepared from the differentiated PC12 cells, low levels of two additional proteins of slightly higher molecular mass were detected. These proteins may represent post-translationally modified isoforms of PFTAIRE in the differentiated cells.

Although PFTAIRE mRNA and protein were not detected in mouse kidney (see Chapter 3, Figure 3.6 and Chapter 4, Figure 4.2) three proliferating primate kidney cell lines have been identified that express a PFTAIRE species (or a closely related protein) at a level comparable to what is found in mouse nervous tissue. Proliferating N18TG2 and PC12 cells which may be similar to undifferentiated cells in the embryonic nervous system demonstrated low levels of PFTAIRE expression that did not increase upon differentiation to a neuronal phenotype (PC12). Although PFTAIRE was not detected on immunoblots from NIH 3T3 cells, a very low level of endogenous protein could be detected in these cells when the PFC5 antibody was used for indirect immunofluorescence (see Chapter 4, Figure 4.4). Thus several cell lines that express very low levels of PFTAIRE were identified that could serve as hosts to overexpress PFTAIRE.

5.3 Generating permanent cell lines that overexpress the PFTAIRE cDNA

Based on the results presented in Section 5.1 several attempts were made to transfect PFTAIRE into cell lines that expressed little or no endogenous PFTAIRE in order to generate cell lines that would permanently overexpress PFTAIRE. NIH 3T3 cells were either co-transfected with the pcPF plasmid (CMV-PFTAIRE) and a plasmid in which expression of the neomycin resistance gene was controlled by the Rous sarcoma virus (RSV) promoter (RSV-Neo) or they were transfected with the pc3PF plasmid (CMV-PFTAIRE), containing the neomycin resistance gene expressed from the SV40 promoter. NIH 3T3 cells were transfected using either a liposome-mediated transfection procedure or DNA-calcium phosphate precipitates. Transfected cells were selected in growth medium containing the antibiotic geneticin (G418) as described in Chapter 2 (Section 2.8b) and individual clones were isolated for expansion once mock transfected cells (no plasmid DNA) were completely killed (approximately 10-14 days post-transfection). In order to determine which clones overexpressed the transfected PFTAIRE cDNA, approximately 20-30 clones per experiment were expanded and used to prepare total RNA for Northern blot analysis with the 5' 290 bp PFTAIRE cDNA probe. Although all of the isolated clones were G418-resistant, none expressed the PFTAIRE cDNA. When Ltk⁻ cells were transfected with either plasmid to generate PFTAIRE expressing cell lines similar results were obtained. Little or no endogenous PFTAIRE was detected in immunoblotted samples of nontransfected NIH 3T3 or Ltk⁻ cell lysates but, each of these cell lines expressed the same endogenous PFTAIRE mRNA transcript that was present in adult mouse nervous tissue and lung.

Similar to NIH 3T3 and Ltk⁻ cells, lung tissue also expresses PFTAIRE mRNA but PFTAIRE was not detected in lung protein samples on immunoblots (see Chapter 4, Figure 4.2). These observations raise the possibility that although endogenous PFTAIRE mRNA is present, the protein may be unstable and rapidly degraded in certain cells. The results of the transfection experiments with NIH 3T3 and Ltk⁻ cells suggest that the G418-resistant clones that were selected may have initially expressed the transfected cDNA but during the course of selection transcription of the transfected PFTAIRE gene was silenced or the plasmid was lost. PC12 cells were also transfected with either the pcPF/RSV-Neo plasmids or the pc3PF plasmid using the liposome-mediated procedure or an electroporation procedure, established in Dr. Julien's laboratory by Dr. Q. Zhu for the purpose of transfecting embryonic stem (ES) cells. Neither transfection procedure yielded neomycin resistant PC12 clones. The inability to produce cell lines that would permanently express elevated levels of the PFTAIRE cDNA using cells that normally express little or no PFTAIRE indicated that it may not be possible to study the function of PFTAIRE in this experimental paradigm. These results also suggested the possibility that overexpression of PFTAIRE may not be tolerated by proliferating cells.

5.4 Using the pHermes-L/PF amplicon construct to produce PFTAIRE herpes viruses for expression of the PFTAIRE cDNA in cell lines

When various cell lines were transfected with the PFTAIRE cDNA and the neomycin resistance gene, antibiotic-resistant clones that were isolated lacked the PFTAIRE cDNA or no clones survived the selection. An alternative approach, using a herpes simplex virus-1 mediated DNA transfer procedure, was tested for overexpression of PFTAIRE. The advantage of using this procedure over calcium phosphate precipitate and liposome-mediated transfection procedures is that a plasmid-derived vector or amplicon containing an HSV-1 origin of replication, an HSV-1 packaging signal and the gene of interest can be packaged within herpes virions as a concatenate. The virions contain multiple copies of the gene of interest, enabling high efficiency delivery of the gene to infected proliferating cells, as well as gene delivery to primary neuronal cultures and postmitotic neurons, which are difficult to transfect by traditional transfection methods. Infected cells can express the gene of interest for several days to weeks, depending on how long they survive the infection. Thus expression mediated by a herpes virus infection may provide a longer period and higher efficiency of gene expression than a transient transfection

experiment (Breakefield and DeLuca, 1991). The decision to produce the PFTAIRE viral stocks was aimed at ultimately using the viruses to overexpress PFTAIRE in PC12 cells or to infect primary neuronal cultures with a PFTAIRE antisense amplicon construct.

The construction of the pHermes-L/PF amplicon and a brief summary of the procedure for making herpes virus stocks is described in Chapter 2 (Section 2.4c). The pHermes-L/PF amplicon contained two promoters in opposite orientations. The CMV promoter was used for expression of the PFTAIRE cDNA and the SV40 promoter controlled expression of the lac Z gene. The pHermes-L amplicon also contained the CMV and SV40 promoters but, only the SV40 promoter was used in this amplicon for driving expression of the lac Z gene. After transfections, cells were fixed and stained with X-gal, the chromogenic substrate for β -galactosidase, to ensure that a high percentage of cells were transfected before attempting to produce herpes viruses. Because both promoters were expected to be transcriptionally active in transfected cells, the number of cells stained by X-gal in the pHermes-L/PF transfected cultures was considered representative of the number of cells that would express PFTAIRE. All experiments were done using Lipofectamine reagent (method described in Chapter 2, Section 2.8b) and in each experiment cells were transfected with either the pHermes-L/PF or the pHermes-L amplicon, which served as a positive control and a provided a direct comparison for transfection efficiencies.

Figure 5.2 demonstrates that when Vero cells were transfected with each amplicon, transfections with the pHermes-L construct resulted in approximately 20-50% of cells expressing β -galactosidase (Figure 5.2A) while, on average, less than 5% of the cells transfected with pHermes-L/PF expressed β -galactosidase (Figure 5.2B). The extremely low percentage of cells transfected with the pHermes-L/PF amplicon was not optimal for proceeding to make herpes virus stocks. Variations in the ratio of amplicon DNA to Lipofectamine reagent, and in the length of time cells were maintained after transfection did not result in improved transfection efficiencies with the pHermes-L/PF amplicon. These tests were also done with other cell lines, including COS-7, NIH 3T3 and Ltk⁻, to determine whether this result was particular to the Vero cell line. However, in other cell lines that were tested expression of β -galactosidase was consistently several-fold higher in pHermes-L transfected cells than in pHermes-L/PF-transfected cells (Figure 5.3).

In addition to the discrepancy in transfection efficiency between the two plasmids, morphological differences were observed following transfections. The cells expressing β -galactosidase in the pHermes-L-transfected cultures consistently occurred in groups of cells

intensely stained by the X-gal reaction. In the pHermes-L/PF-transfected cultures the β -galactosidase expressing cells were either isolated pairs or single cells that were poorly stained. This result may have been the effect of a combination of events: 1) more cells may have been transfected with pHermes-L than with pHermes-L/PF and, 2) the cells that were transfected with pHermes-L/PF may have divided more slowly than those transfected with pHermes-L. The pHermes-L/PF-transfected cells were less flattened or spread out than the pHermes-L transfected cells, which made the pHermes-L/PF-transfected cells appear smaller or condensed (Figure 5.2). In addition, the nuclei of pHermes-L/PF-transfected cells appeared to be less well-defined than those of the pHermes-L transfected cells. These observations suggested that overexpression of PFTAIRE following transfection of the pHermes-L/PF amplicon may not be well tolerated, even in a transient assay, by several proliferating cell lines. The results obtained with the transient transfections precluded use of pHermes-L/PF to produce herpes virus stocks for expression of PFTAIRE.

5.5 Transient transfections with PFTAIRE plasmid constructs

a. Changes in cell morphology detected by indirect immunofluorescence

The transient transfections performed with the pHermes-L/PF amplicon demonstrated that forced overexpression of the PFTAIRE cDNA with this vector, in various cell lines, resulted in low transfection efficiency and changes in cell morphology. In order to determine whether these effects were related to the pHermes-L/PF vector Vero, COS-7 and NIH 3T3 cells were also transfected transiently with the pcPF and pc3PF plasmids, using the liposome-mediated transfection procedure. Twenty-four hours after transfection, cells were fixed and processed for indirect immunofluorescence with the PFC5 antibody. The results of these transfections were similar to the results obtained with the pHermes-L/PF plasmid. When the PFTAIRE cDNA was transfected, few cells were strongly labelled by the PFC5 antibody, indicating that on average approximately 5% (or less) of the cells expressed the transfected cDNA (Figure 5.4).

One of the observations about the morphology of cells transfected with the pHermes-L/PF amplicon was that the nuclei of X-gal stained cells appeared to be less well-defined than those of nontransfected cells or cells transfected with pHermes-L. To determine whether the nuclei of PFTAIRE-transfected cells differed from those of nontransfected cells, following transient transfection (approximately 20-24 hours) with either the pcPF or pHermes-L/PF

plasmids, COS-7 and Vero cells were fixed with cold methanol/acetone (50%/50%) and processed for indirect immunofluorescence labelling with the PFC5 antibody (described in Chapter 2, Section 2.8c) and the monoclonal antibody 136D6 (gift of Dr. Y. Raymond) which recognizes lamins A and C, two intermediate filament protein components of the nuclear lamina. All transfections were done in duplicate.

During eukaryotic cell division the nuclear lamina is disassembled beginning in prophase and then reconstructed in late anaphase (Gerace and Blobel, 1980). Thus lamin proteins undergo redistribution during the cell cycle. The nuclei of cells in G₁ show heterogeneous lamin protein distribution when stained with lamin antibodies (lamins A, B and C). Lamin proteins are detected: 1) as a continuous rim at the periphery of the nucleus in the nuclear lamina 2) distributed diffusely in nucleoplasm and 3) in foci within the nucleoplasm (Chaly et al., 1984; Bridger et al., 1993; Moir et al., 1994). Only lamins A and C have been detected as foci in the nucleoplasm in G₁ (Bridger et al., 1993). In S phase lamins A and C disappear from the internal foci and are found mainly in the lamina but, lamin B occurs in large foci in the nucleoplasm that are associated with sites of DNA replication (Moir et al., 1994). During mitosis lamins A and C are completely dissociated from nuclear envelope fragments and dispersed in the mitotic cytoplasm while lamin B remains associated with nuclear envelope fragments (Gerace and Blobel, 1980). Beginning in anaphase lamin proteins reassemble to form the nuclear lamina around decondensing chromatin.

In Figures 5.5 and 5.6, COS-7 (Figure 5.5) and Vero (Figure 5.6) cells transfected with PFTAIRE plasmids were stained with PFC5 and the anti-lamin A/C antibody (136D6). The immunofluorescence labelling with the lamin antibody demonstrates that the nuclei of the PFTAIRE immunoreactive cells differed from those of most nontransfected cells in a given field. The nuclei of PFTAIRE-transfected cells were somewhat smaller than nuclei of surrounding nontransfected cells and were often irregularly shaped. In addition, the perinuclear distribution of lamins in the PFTAIRE-transfected cells was frequently uneven or discontinuous and the lamins appeared punctate in some areas (Figures 5.5 and 5.6B and D, arrowheads). In cells that expressed very high levels of PFTAIRE, the lamin proteins were barely detectable. In the majority of the nontransfected cells the distribution of the lamin proteins was uniform perinuclear and diffuse in the nucleoplasm, though some cells occasionally displayed punctate areas of staining. Fluorescein conjugated to donkey anti-rabbit IgG was used to detect PFTAIRE immunoreactive

cells and Texas Red conjugated to goat anti-mouse IgG was used for detecting lamin immunoreactivity. No overlap of the signal from each fluorophor was detected, suggesting that the punctate lamin distribution of the PFTAIRE immunoreactive cells was not a fluorescence labelling artifact. In addition, some nontransfected cells, that were not labelled by the PFTAIRE antibody, showed similar punctate lamin staining. Labelling with the PFC5 antibody demonstrated that PFTAIRE-transfected cells contained multiple nucleoli and frequently occurred in pairs. These observations suggested that the cells could be daughter cells in the G₁ phase of the cell cycle. The discontinuous and punctate distribution of the lamin proteins in the PFTAIRE-transfected cells was also suggestive of these cells being in the G₁ phase of the cell cycle (Paddy et al., 1990; Bridger et al., 1993). Bridger et al. (1993) demonstrated that when nuclei of proliferating human epidermal fibroblasts in G₁ were labelled by indirect immunofluorescence with antibodies that react with lamins A and C, two populations of cells were identified. One population of cells displayed the continuous perinuclear lamin distribution, while the other showed discontinuous perinuclear staining with internal (nucleoplasm) lamin foci. The foci were observed predominantly in cells that were in early G₁. Bridger et al. (1993) postulated that the foci may be formed by lamins that are associated with decondensing chromatin and accumulated in the nucleoplasm following assembly of the new nuclear membrane. Lamins A and C bind to mitotic chromosomes *in vitro* (Glass and Gerace, 1990), which suggests that the lamin proteins may be associated with some chromatin in newly formed daughter cells. It has been suggested that the lamins in the internal foci may relocate more slowly to the nuclear envelope as new daughter cells progress through G₁ (Bridger et al., 1993).

The distribution of lamin proteins in PFTAIRE-transfected cells has been compared with what has been reported in the literature for the purpose of determining what stage of the cell cycle the PFTAIRE-transfected cells may be in. Most PFTAIRE-transfected cells showed changes in nucleus size, shape and lamin distribution and consistently appeared different from the majority of nontransfected cells in a given field, suggesting that many of the transfected cells may be arrested in a particular phase of the cell cycle. The punctate lamin distribution detected in the PFTAIRE-transfected cells most closely resembles the lamin distribution of cells in the early part of G₁. The lamin foci described by Bridger et al. (1993) were internally located in the nucleoplasm, but the areas of punctate lamin staining in the PFTAIRE-transfected cells appeared to be localized

at the nuclear periphery. One of the features of apoptotic cell death is the clumping of dense chromatin near the nuclear periphery (Kerr et al., 1972; Wyllie et al., 1984). It is possible that the punctate lamin staining observed in PFTAIRE-transfected cells corresponds to lamin that is associated with clumped chromatin. Cell counts from 15 fields (similar to Figures 5.5 and 5.6) revealed that in an average field of 67 cells, 9 cells (13.4% of total) displayed the discontinuous perinuclear and punctate lamin staining. The majority of cells, in the 15 fields of counted cells, had uniform lamin staining, indicating that these cells may have been in mid-G₁. On average, 2.3 cells per field (3.4% of total) were PFTAIRE-immunoreactive. Of the PFTAIRE-immunoreactive cells, 87% (or 2 PFTAIRE-immunoreactive cells per field) showed punctate lamin staining. In the remainder of PFTAIRE-immunoreactive cells lamin proteins were barely detectable. The correlation between an early G₁ or possibly apoptotic state and PFTAIRE-transfected cells was high and suggested that PFTAIRE overexpression may have contributed to this phenotype.

b. Demonstrating PFTAIRE kinase activity with transfected cells

After demonstrating that the PFTAIRE immunoprecipitated from mouse brain possessed kinase activity it was necessary to demonstrate that this activity was due to PFTAIRE and not to another kinase activity that may have co-immunoprecipitated with PFTAIRE. The most direct way to demonstrate this was to overexpress PFTAIRE and show that increased expression of PFTAIRE resulted in increased phosphorylation of PFTAIRE-associated proteins, while overexpression of a dominant negative mutant form of PFTAIRE could inhibit the activity of endogenous PFTAIRE by competing for association with the proteins that endogenous PFTAIRE interacts with. When cells were transfected transiently with the pcPF, pc3PF and pHermes-L/PF plasmids, PFTAIRE was overexpressed in a very low percentage of cells, less than 5% on average as determined by indirect immunofluorescence. In the majority of experiments the transfection efficiencies were too low to detect the transfected PFTAIRE in cell lysates by western blotting, suggesting that increases in PFTAIRE kinase activity would not be readily detectable.

All of the PFTAIRE plasmids used for transfections contained the entire PFTAIRE cDNA, including 5' and 3' untranslated sequences. For many mRNAs, stability is related to translatability. Most mRNAs need to be translated in order to be degraded, so factors that inhibit translation tend to increase mRNA stability (Sachs, 1993). Many of the features of 5' UTR (untranslated regions) that have been examined tend to decrease translation efficiency, hence stabilizing mRNA molecules. These features include the formation of secondary stem-loop structures and the

presence of upstream AUG codons (Sachs, 1993; Pain, 1996). Many unstable mRNAs contain in their 3' UTR one or several AU-rich elements (ARE consensus, AUUUA) that can mediate mRNA decay by stimulating deadenylation. The length of the poly (A) tail of an mRNA molecule has also been shown to influence stability. A poly(A) tail of 10 or fewer nucleotides makes an mRNA molecule susceptible to rapid degradation (Sachs, 1993). The PFTAIRE nucleotide sequence contains two upstream AUG codons (positions 52-54, 106-108) and five ARE sequences in the 3' UTR (positions 1592-1596, 1596-1600, 2001-2005, 2066-2070, 2440-2444). To eliminate the possibility that elements within the 5' or 3' UTR interfered with expression of PFTAIRE in transfected cells, by contributing to mRNA instability, only the coding sequence of PFTAIRE was used to generate the PFTAIRE eukaryotic expression plasmids, pc3PF~~orf~~ and pc3D228N~~orf~~ (described in Chapter 2, Section 2.4c). Three cell lines, including COS-7, NIH 3T3 and 293 cells, were transfected transiently with the pc3PF~~orf~~ plasmid to determine if removal of the 5' and 3' sequences enhanced the transfection efficiency. All transfections were done using calcium phosphate precipitates and 24 hours after transfection, cells were lysed and cell lysates from pcDNA3-transfected and pc3PF~~orf~~-transfected were analyzed for PFTAIRE expression by immunoblotting with the PFC5 antibody. Figure 5.7 shows that transfected PFTAIRE was not detected in either COS-7 or NIH 3T3 cells. However, the transfected PFTAIRE (47 kDa) was detected in 293 cells along with two endogenous PFTAIRE protein species that were slightly larger than mouse brain PFTAIRE, but similar to the higher molecular weight species detected in COS-7 and Vero cells (see Figure 5.1A). Although this experiment suggested that removal of the 5' and 3' sequences from the PFTAIRE cDNA did not improve transfection efficiencies in COS-7 and NIH 3T3 cells, it did identify a cell line that could be used to transfect and express PFTAIRE at a level that was detectable on immunoblots. Prior to this experiment COS-7 and NIH 3T3 cells were transfected with pcPF or pc3PF using Lipofectamine and calcium phosphate precipitates and essentially no differences in transfection efficiency were observed with the two methods. Thus the lack of PFTAIRE expression in these cell lines after transfection with the pc3PF~~orf~~ plasmid was not considered the result of using the calcium phosphate method. The result of this experiment suggested that transfected PFTAIRE was probably expressed in a higher percentage of 293 cells than in all other cell lines that had been transfected previously and that this cell line could be used to study PFTAIRE kinase activity with the dominant negative PFTAIRE mutant D228N.

Transient transfection of 293 cells with the pc3PFTAIRF plasmid resulted in detectable levels of PFTAIRE expression. In order to use transfected 293 cells for kinase assays that would directly compare the consequences of expression of the pc3PFTAIRF and pc3D228NORF plasmids on phosphorylation of proteins co-immunoprecipitated with PFTAIRE it was necessary to obtain transfected cultures expressing similar amounts of the two proteins. Following transfection with pcDNA3, pc3PFTAIRF and pc3D228NORF plasmids, cells were treated with G418 to select clones that would permanently express these plasmids. The rapid growth rate of 293 cells prevented selection of individual clones, but after approximately 10 days in selection medium, large populations of cells had been selected that were derived from several clones, i.e., stable pools of transfected cells were obtained. All transfections were started with equal numbers of plated cells and equivalent amounts of plasmid DNA. When cells were counted at the end of the selection period, in two independent experiments, the transfections with the pc3PFTAIRF plasmid resulted in half the number of neomycin-resistant cells that were obtained from transfections with either pcDNA3 or pc3D228NORF. This observation suggests that during the selection period the pc3PFTAIRF plasmid was eliminated from cells, causing them to lose neomycin resistance, or that overexpression of PFTAIRE resulted in decreased cell viability (Section 5.5a). The neomycin-resistant cells were not examined by indirect immunofluorescence to determine if there were morphological differences between pc3PFTAIRF and vector (pcDNA3)-transfected or pc3D228NORF-transfected cells. The stable pools of neomycin-resistant cells were used for the kinase assays presented in Chapter 4 (Section 4.4b) to demonstrate that increased expression of PFTAIRE resulted in an increase in the phosphorylation of the 58-60 kDa protein that co-immunoprecipitated with PFTAIRE, while a decrease in phosphorylation of the PFTAIRE-associated protein occurred when cells were transfected with pc3D228NORF. This result indicated that the phosphorylation of the 58-60 kDa PFTAIRE-associated protein was likely due to PFTAIRE kinase activity and not to the activity of another kinase in the PFTAIRE immune complex, providing additional biochemical evidence that the PFTAIRE cDNA encodes a functional protein kinase.

5.6 Discussion

a. Cell lines that constitutively overexpress PFTAIRE could not be derived

The results of several experiments in which PFTAIRE plasmids were transfected to derive permanent cell lines for constitutive overexpression of PFTAIRE were described in this chapter. It

was not possible to obtain cell lines that constitutively overexpressed PFTAIRE using a variety of cell lines, three PFTAIRE plasmid constructs and different transfection procedures. When transfected cells were selected for neomycin resistance, either the resistant clones did not express transfected PFTAIRE or no clones survived the selection. The most successful experiment was with 293 cells, which survived selection as a mass population derived from several clones. There are three possible explanations for how neomycin-resistant clones that did not express PFTAIRE were obtained in experiments with NIH 3T3 and Ltk⁻ cells. When the pcPF plasmid was transfected, co-transfection of the RSV-Neo plasmid was required to select antibiotic-resistant clones. It is possible that in these experiments 1) the two plasmids did not co-transfect, 2) the PFTAIRE plasmid was eliminated from cells following co-transfection or 3) cells that expressed the PFTAIRE plasmid were eliminated during the neomycin selection. None of these explanations would be applicable to results obtained with the pc3PF plasmid, which contained the PFTAIRE cDNA and the neomycin-resistance gene but, yielded neomycin-resistant clones that did not express transfected PFTAIRE. Based on these results, alternative explanations for the lack of PFTAIRE expression in neomycin-resistant clones might be: 1) PFTAIRE mRNA was unstable and degraded; or 2) transcription of the transfected gene was inactivated during selection.

Each of the PFTAIRE plasmids (except pc3PF_{orf}) used for transfections contained the 5' and 3' untranslated regions of the PFTAIRE cDNA. Sequences in the untranslated regions of mRNAs influence the translatability and stability of these molecules (Sachs, 1993). Two upstream AUG codons and multiple AU-rich elements (ARE) were identified in the nucleotide sequences of the PFTAIRE 5' and 3' UTR. The presence of upstream AUG codons can decrease the efficiency of translation of mRNA molecules, thereby increasing their stability. The AU-rich elements located in the 3' UTR can destabilize endogenous mRNA (Sachs, 1993; Pain, 1996). The PFTAIRE mRNA and protein are well expressed in nervous tissue, suggesting that these elements do not interfere with mRNA transcription and translation in nervous tissue. The mRNA is also present in lung and in cell lines, such as NIH 3T3 and Ltk⁻, even though very little endogenous protein was detected on immunoblots of extracts prepared from lung, NIH 3T3 and Ltk⁻ cells. The presence of PFTAIRE mRNA in these tissues and cell lines suggests that the elements identified in the 5' and 3' UTR have little influence on endogenous PFTAIRE mRNA stability. Thus it seems unlikely that PFTAIRE mRNA was not detected in G418-resistant clones because of instability of mRNA

transcribed from the transfected plasmids. It is possible that the upstream AUG codons do influence translation in cells expressing endogenous PFTAIRE mRNA and very low levels of protein, because upstream AUG codons can influence translation in a tissue-specific manner (Pain, 1996).

When plasmids are transfected they are often integrated as tandem repeats (Wigler et al., 1977) and frequently these repeats are not transcribed. It has been suggested that the repeated arrangements of transgenes contributes to the formation of heterochromatin, which results in transcriptional silencing of repeated gene sequences (Dorer and Henikoff, 1994). The genomic DNA from neomycin-resistant clones obtained following transfection of PFTAIRE plasmids was not analyzed to determine whether or not the PFTAIRE gene was present in clones that did not express the mRNA, so it is not known whether the lack of PFTAIRE expression can be explained by gene silencing. Decreased cell viability was associated with transfected pc3PForf compared to transfections with pc3D228Norf and pcDNA3 during the selection of stable populations of 293 cells (Chapter 5, Section 5.5b). In addition, the few cells that overexpressed PFTAIRE following transient transfections with various PFTAIRE plasmids showed morphological changes that resembled the morphology of cells that were arrested in early G₁ or undergoing apoptotic cell death. In summary, these observations suggest that the difficulties associated with deriving permanent cell lines may be related to elimination of the PFTAIRE cDNA from transfected cells or silencing of transcription, since forced transient overexpression in proliferating cells correlated with morphological changes indicating compromised cell viability.

b. Does transient overexpression of PFTAIRE in proliferating cells lead to G₁ arrest or cell death?

Fibroblast cell lines, expressing little or no endogenous PFTAIRE protein, failed to yield stable neomycin-resistant clones that constitutively expressed PFTAIRE. A number of experiments were then conducted to transiently transfect PFTAIRE plasmid constructs to enable overexpression over a short period of time in these cell lines. These experiments are summarized in Table 5.1. Regardless of which PFTAIRE plasmid or cell line was used for these experiments, transfections consistently resulted in a very low percentage of cells (on average, 5% or less) expressing PFTAIRE. Transfections with the pHermes-L and pHermes-L/PF plasmids, which could be monitored by staining cells for expression of β -galactosidase activity, demonstrated the insertion of the PFTAIRE cDNA in the pHermes-L plasmid resulted in a major decrease in the

numbers of cells that were transfected and expressed β -galactosidase activity. These transfections also demonstrated that the morphology of cells transfected with the pHermes-L/PF plasmid differed from those transfected with the pHermes-L plasmid (Section 5.4, Figures 5.2 and 5.3). The PFTAIRE-transfected cells generally appeared less flattened, smaller and rounder than the lac Z (pHermes-L)-transfected cells. Other differences included less clearly defined nuclei and cytoplasmic vacuoles and protrusions in pHermes-L/PF-transfected cells that were not observed in pHermes-L transfected cells. In general, the morphology of the PFTAIRE-transfected cells did not resemble the "normal" fibroblast morphology of the surrounding nontransfected cells or lac Z-transfected cells.

The apparent change in nuclear morphology that was observed following transient transfection of pHermes-L/PF was examined in a preliminary experiment by indirect immunofluorescence using an antibody recognizing lamins A and C. This experiment demonstrated that there were morphological differences between the nuclei of cells transfected with PFTAIRE and the nuclei of surrounding nontransfected cells. The nuclei of PFTAIRE transfected cells were frequently irregularly shaped compared to the predominantly round nuclei observed in nontransfected cells. The distribution of the lamin proteins at the nuclear periphery in PFTAIRE-transfected cells was often discontinuous and punctate in comparison to the uniform distribution of these proteins in the majority of nontransfected cells. The punctate distribution of lamins observed in the PFTAIRE-transfected cells did not appear to be an artifact caused by the immunofluorescence labelling with two antibodies because occasionally nontransfected cells also displayed punctate lamin staining. The correlation between the punctate nuclear lamin staining and PFTAIRE immunoreactivity was high, with an average of 87% of the PFTAIRE-transfected cells demonstrating the punctate lamin distribution. Only 10% of nontransfected cells showed a similar staining pattern. This data suggested that overexpression of PFTAIRE was related to the changes in lamin distribution that were observed.

The localization of lamin proteins during various stages of the cell cycle has been described by others (Gerace and Blobel, 1980; Chaly et al., 1984; Bridger et al., 1993; Paddy et al., 1993; Moir et al., 1994) and the distribution of lamins in PFTAIRE-transfected cells most closely resembled that of cells in early G₁. The lamina in these cells usually appeared to be discontinuous and there were concentrated regions or foci of lamin immunoreactivity similar to the foci described by Paddy et al., 1990 and Bridger et al., 1993. Bridger and co-workers (1993)

demonstrated that lamin A and C immunoreactive foci were detected in the nucleoplasm predominantly during early G₁, associated with dense areas of chromatin. The observation that the PFTAIRE-transfected cells often occurred as isolated pairs or single cells and frequently contained fragmented or dissociated nuclei also suggested that these could be daughter cells in G₁. Based on the PFTAIRE and lamin immunofluorescence labelling most of these observations are consistent with the idea that these cells could be daughter cells arrested in G₁, except that the lamin foci that were in PFTAIRE-transfected cells detected did not appear to be internally localized. The punctate regions of lamin staining appeared to be located at the nuclear periphery. Glass and Gerace (1990) have demonstrated that lamins A and C bind to and begin to assemble on mitotic chromosomes, *in vitro*, which could explain how these proteins may accumulate within nuclei, as foci associated with chromatin in early G₁ daughter cells (Bridger et al., 1993). On the other hand, only a small fraction of the chromatin in the nuclear periphery may be associated with lamins in G₁ cells (Paddy et al., 1990). If a punctate lamin distribution is due to association of lamins with chromatin, then according to the results of Paddy et al. (1990) the presence of lamin foci at the nuclear periphery in PFTAIRE-transfected cells would be inconsistent with the idea that these cells are in the G₁ state.

One of the morphological features of cells undergoing apoptosis is the condensation or aggregation of chromatin in dense masses near the nuclear envelope that subsequently protrude from the cytoplasm and form some of the apoptotic bodies which eventually dissociate from cells as they die (Kerr et al., 1972; Wyllie et al., 1984; Dini et al., 1996). The lamin foci detected in PFTAIRE-transfected cells appeared to be located at the nuclear periphery and it is possible that these foci were lamin proteins associated with dense chromatin in cells that were dying. Occasionally the lamins were detected as a strongly labelled bleb protruding from the nucleus (Figure 5.5D). Using indirect immunofluorescence to monitor changes in lamin A and B protein distribution during the course of mitosis and apoptosis, Oberhammer et al. (1994) demonstrated that the nuclei of apoptotic rat fibroblasts were frequently irregularly shaped and smaller than normal nuclei. In addition, the lamin proteins had a punctate distribution at the nuclear periphery because of their localization around condensed chromatin (Oberhammer et al., 1994). After nuclear fragmentation, during apoptosis, the lamin proteins were degraded and barely detected by immunofluorescence labelling. The distribution of lamins in PFTAIRE-transfected cells more

closely resembles lamin protein distribution during apoptosis demonstrated by Oberhammer et al. (1994) than the localization of lamins in G₁ cells. Other features of cells undergoing apoptosis, such as a more rounded and shrivelled appearance, were initially observed in the pHermes-L/PF-transfected cells stained with X-gal. Dissociation of the nucleolus, which was frequently detected in PFTAIRE-transfected cells labelled with the PFC5 antibody, is a feature of cells in the G₁ state of the cell cycle but also occurs during apoptosis (Kerr et al., 1972). In conclusion, the morphological changes that occurred when various cell lines were transfected transiently with PFTAIRE plasmids showed similarities to the morphology of cells in the early part of G₁ and features that characterize apoptotic cell death. Examination of transfected cells by electron microscopy or examination of the state of the chromatin in these cells by the TUNEL reaction, staining with Hoechst dye or bromo-deoxyuridine (Br dU) labelling would clarify whether these cells were undergoing apoptosis. These preliminary results suggest that PFTAIRE-transfected cells may have been daughter cells in G₁ or cells undergoing cell death and that this phenotype was a consequence of PFTAIRE overexpression.

c. How might PFTAIRE overexpression in proliferating cells result in cell death?

The transitions between different cell cycle states in proliferating eukaryotic cells are subject to regulation at checkpoints, such as the “restriction point” in late G₁ when a cell commits itself to another round of DNA replication (G₁-S transition) or at the start and completion of mitosis (Hunter and Pines, 1994). Positive and negative external signals, as well as internal controls, influence what occurs at these checkpoints and essentially determine whether cells progress to the next cell cycle state. The internal controls are there to ensure that cell cycle progression is stopped in response to irregularities such as unrepaired, damaged DNA or faulty spindle assembly (Hunter and Pines, 1994; Nigg, 1995). The consequences of deregulated or loss of internal controls are frequently oncogenesis or apoptotic cell death.

Transient transfections with PFTAIRE plasmids demonstrated that overexpression of the PFTAIRE cDNA in proliferating cell lines that expressed low to moderate levels of endogenous PFTAIRE proteins resulted in very few cells overexpressing transfected PFTAIRE. Within the 24 hour transfection period, the cells that overexpressed PFTAIRE appeared to be arrested in the G₁ phase of the cell cycle or undergoing apoptotic cell death. These results suggest that forced expression of PFTAIRE had disrupted the normal cell cycle progression, possibly by interfering

with the activities of proteins that regulate progression through G₁. The coordinated activities of many different proteins including, the kinase activities of CDK-cyclin complexes, CDK inhibitory proteins (CKIs), other protein kinases and phosphatases that positively and negatively regulate CDK activities, tumor suppressor proteins, transcription factors and proteins involved in DNA replication are required to ensure progression through each phase of the cell cycle. (Refer to Figure 1.3 in Chapter 1 for a summary of the proteins involved in regulating cell cycle progression.) Deregulation of pRB (retinoblastoma protein), p53, *c-myc*, E2F and cyclin D1 (Lee et al., 1992; Jacks et al., 1992; Donehower et al., 1992; Evans et al., 1992; Field et al., 1996; Yamasaki et al., 1996; Wang et al., 1994; Sicinski et al., 1995; Freeman, et al., 1994; Yan and Ziff, 1995; Kranenberg et al., 1996) contribute to oncogenesis and apoptosis, while inappropriate expression of p34^{cdc2} has been shown to trigger “mitotic catastrophes” or apoptosis (Heald et al., 1993; Shi et al., 1994). Because many of PFTAIRE-transfected cells that appeared to be undergoing apoptosis may have been in the G₁ phase of the cell cycle, it is possible that overexpression of PFTAIRE resulted in deregulation of proteins that function during G₁, as cells prepare to start DNA replication and cell division again or become quiescent.

The activities of cyclin D1 and pRB during G₁ appear to be interdependent (Hunter and Pines, 1994). During G₁ cyclin D1 overexpression in fibroblasts causes: 1) pRB to be phosphorylated earlier than normal, 2) a reduction in cell size, 3) a reduction in requirements for growth factors, 4) accelerates the G₁ phase of the cell cycle and 5) cell death (Jiang et al., 1993; Quelle et al., 1993; Kranenberg et al., 1996). Some of the changes that occur in response to cyclin D1 overexpression, in particular the reduced requirement for growth factors for proliferation, are similar to the characteristics of transformed cells (Hunter and Pines, 1994). The retinoblastoma tumor suppressor protein, pRB, is considered the major *in vivo* target of the CDK4-cyclin D1 complex. Most of the sites on pRB that are phosphorylated *in vivo*, when pRB begins to be hyperphosphorylated and inactive in late G₁, are phosphorylated *in vitro* by CDK4-cyclin D1. It has been suggested that the role of CDK4-cyclin D1 is to inactivate pRB so that cells can progress through the G₁/S phase boundary to replicate their DNA. It has also been suggested that hypophosphorylated pRB (active) is involved in stimulation of cyclin D1 transcription because in cells that lack functional pRB there is a downregulation of cyclin D1 and CDK4-cyclin D1 complexes (Müller et al., 1994).

In addition to induction of a transformed phenotype, upregulation or overexpression of cyclin D1 is associated with cell cycle arrest in PC12 cells, apoptotic cell death in primary neuronal cultures, neuroblastoma and fibroblast cell lines (Yan and Ziff, 1995; Quelle et al., 1993; Freeman et al., 1994; Kranenberg et al., 1996). Overexpression of cyclin D1 following transient transfection of a CMV-cyclin D1 plasmid in NIH 3T3 and CV-1 (monkey kidney) cells resulted in increased cyclin D1 expression, increased CDK4-cyclin D1 activity and apoptotic cell death (Kranenberg et al., 1996). In primary neuronal cultures and in neuroblastoma cells, cyclin D1 expression is upregulated upon withdrawal of neurotrophic support which correlates with apoptosis (Freeman et al., 1994; Kranenberg et al., 1996). Based on these findings, Kranenberg and co-workers (1996) suggested that the absolute level of cyclin D1 expression may determine whether cells respond to overexpression by showing increases in proliferation (moderate overexpression) or apoptosis (high overexpression). The demonstration by Quelle et al. (1993) that permanent cell lines overexpressing cyclin D1 could only be obtained when cyclin D1 was moderately overexpressed with weak promoter is in agreement with this hypothesis. Whether pRB is involved in cyclin D1-induced apoptosis is unclear, however, lack of pRB expression or activity has been correlated with apoptosis in neuronal cell types (Jacks et al., 1992; Lee et al., 1992; Freeman et al., 1994; Slack et al., 1995) while transfection of pRB into osteosarcoma cells lacking pRB provides protection from radiation-induced apoptosis (Haas-Kogan et al., 1995). It is possible that, similar to cyclin D1, the absolute level of PFTAIRE expression may determine the fate of cells transiently overexpressing the PFTAIRE cDNA. A high level of PFTAIRE expression may result in aberrant cell cycles and apoptosis by interfering with the activities of cyclin D1 or pRB in G₁.

In summary, preliminary data from transfection experiments indicate forced expression of the PFTAIRE cDNA in several proliferating fibroblast cell lines was not well tolerated by these cells. Attempts to derive stable cell lines that constitutively overexpressed PFTAIRE resulted in the selection of antibiotic-resistant clones that did not express the PFTAIRE mRNA or no cells survived the selection. The lack of PFTAIRE expression in antibiotic-resistant clones suggests the possibility that the transfected cDNA was eliminated from cells, that transcription was silenced if the cDNA was integrated or that cells which integrated and overexpressed the transgene died. Transient transfections of fibroblasts with CMV-PFTAIRE plasmids resulted in very few cells overexpressing PFTAIRE. Cells that did overexpress PFTAIRE frequently occurred as single

cells or isolated pairs of cells that demonstrated some features of daughter cells that have completed cell division. Many of these cells also displayed features of cells undergoing apoptosis such as a rounded and shrivelled appearance, smaller, irregularly shaped nuclei and a punctate distribution of lamin proteins at the nuclear periphery. These findings suggest that ectopic overexpression of PFTAIRE may correlate with an apoptotic phenotype. It is possible that there is a threshold level of PFTAIRE expression that is tolerated by proliferating cells which express low levels of endogenous PFTAIRE, and that overexpression of this protein kinase may compromise cell viability by interfering with normal cell cycle activities.

Table 5.1 Summary of transfection experiments

	Cell line	COS-7	NIH 3T3	Vero	Ltk-	293	PC12	NIH 3T3	Ltk-	293
Untransfect.	western	58+52 kDa	-	58 kDa	-	58+54 kDa	56 kDa	-	-	58+54 kDa
	immunofl.	+	+	+	ND	ND	++	+	ND	ND
	IP/western	-	ND	ND	ND	58 kDa	ND	ND	ND	58 kDa
	IP/kinase	+	+	+	+	++	++	+	+	++
pHermL/PF	TRANSIENT TRANSFECTIONS						STABLE TRANSFECTIONS			
	western	ND	ND	ND	ND	ND	Not transfected			
	immunofl.	low%, +++	low%, +++	low%, +++	ND	ND				
	β-gal	low%	low%	low%	low%	ND				
pcPF	IP/western	ND	ND	ND	ND	ND				
	IP/kinase	ND	ND	ND	ND	ND				
pc3PF	western	ND	ND	ND	ND	ND	No stable clones isolated		Not trans-fected	
	immunofl.	low%, +++	low%, +++	low%, +++	ND	ND				
	IP/western	transf. (47 kDa)	ND	ND	ND	ND				
pc3PF _{orf}	IP/kinase	ND	ND	ND	ND	ND	No stable clones isolated		Not trans-fected	
	western	endog. (58 kDa)	-	ND	-	ND				
	immunofl.	low%, +++	low%, +++	low%, +++	ND	ND				
pc3PF _{orf}	IP/western	ND	ND	ND	ND	ND	Not transfected		ND	
	IP/kinase	ND	ND	ND	ND	ND				
	western	endog. (58 kDa)	-	ND	ND	58+54kDa + 47 kDa				
	immunofl.	ND	ND	ND	ND	ND				
pc3PF _{orf}	IP/western	ND	ND	ND	ND	ND	Not transfected		ND	
	IP/kinase	ND	ND	ND	ND	ND				
	western	endog. (58 kDa)	-	ND	ND	58+54kDa + 47 kDa				
pc3PF _{orf}	immunofl.	ND	ND	ND	ND	ND	Not transfected		ND	
	IP/western	ND	ND	ND	ND	ND				
	IP/kinase	ND	ND	ND	ND	ND				

ABBREVIATIONS

western = immunoblot with PFC5 antibody
immunofl. = indirect immunofluorescence with PFC5 antibody
IP/western = immunoblot of immunoprecipitated proteins
IP/kinase = immunoprecipitation kinase assay

- = not detected
+ = weak
++ = moderate
+++ = strong

low % = <5% cells transfected
ND = not done

Figure 5.1 Immunoblot analysis of PFTAIRE expression in cell lines.

Cytosolic protein extracts (15 µg) prepared from various cell lines were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred electrophoretically to nitrocellulose for immunoblotting with the PFTAIRE antibody, PFC5. A) Upper panel, immunoblot of protein extracts from NIH 3T3 (mouse fibroblasts), Vero (monkey kidney fibroblasts), Ltk⁻ (mouse fibroblasts), N18TG2 (mouse neuroblastoma), C2C12 (mouse myoblasts), COS-7 (SV40-transformed monkey kidney fibroblasts) and mouse brain (15 µg). Lower panel, Coomassie stained gel of the same extracts used for the immunoblot in the upper panel. B) Left panel, immunoblot of protein extracts from mouse brain (15 µg), proliferating PC12 cells (rat pheochromocytoma) and PC12 cells differentiated for 7 days in medium containing NGF. Right panel, Coomassie stained gel of the same extracts used for the immunoblot in the left panel.

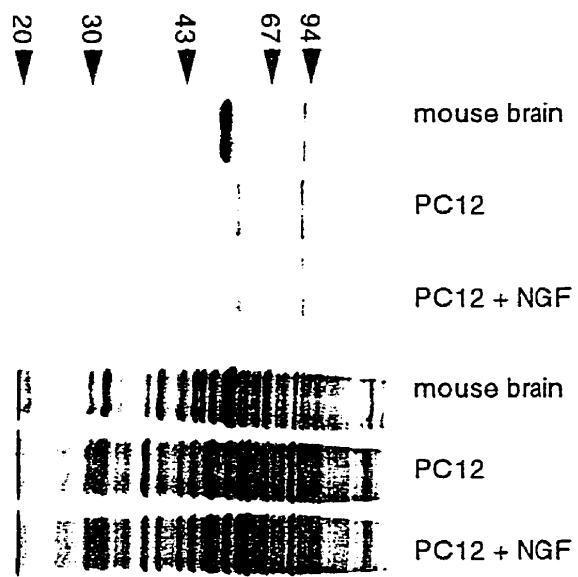
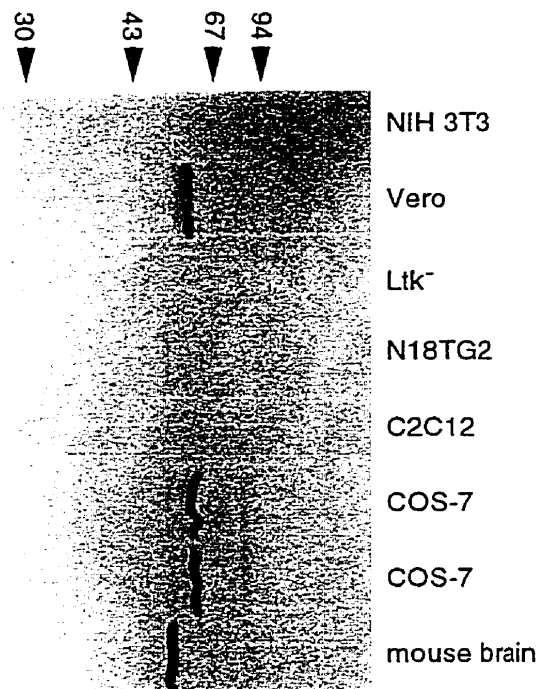
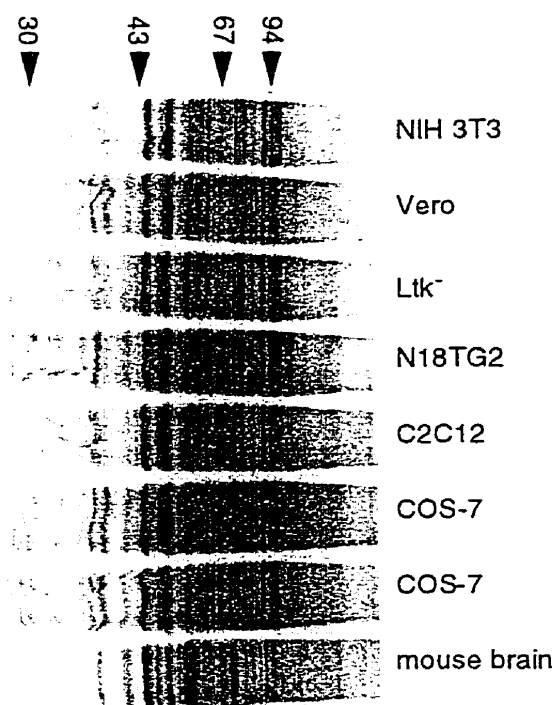


Figure 5.2 Vero cells transfected with the pHermes-L and pHermes-L/PF plasmids.

Vero cells were transfected transiently with the pHermes-L plasmid (A and C) to overexpress lac Z or with the pHermes-L/PF plasmid (B and D) to overexpress PFTAIRE and lac Z`promoter.

Twenty-four hours after transfection cells were fixed and stained with an X-gal solution to monitor the efficiency of transfections. The X-gal stained cells in panels B and D (pHermes-L/PF transfections) are considered cells expressing the PFTAIRE cDNA. Panels C and D are higher power magnifications of cells shown in panels A and B. Magnification in A and B is 50 x.

Magnification in C and D is 100 x.

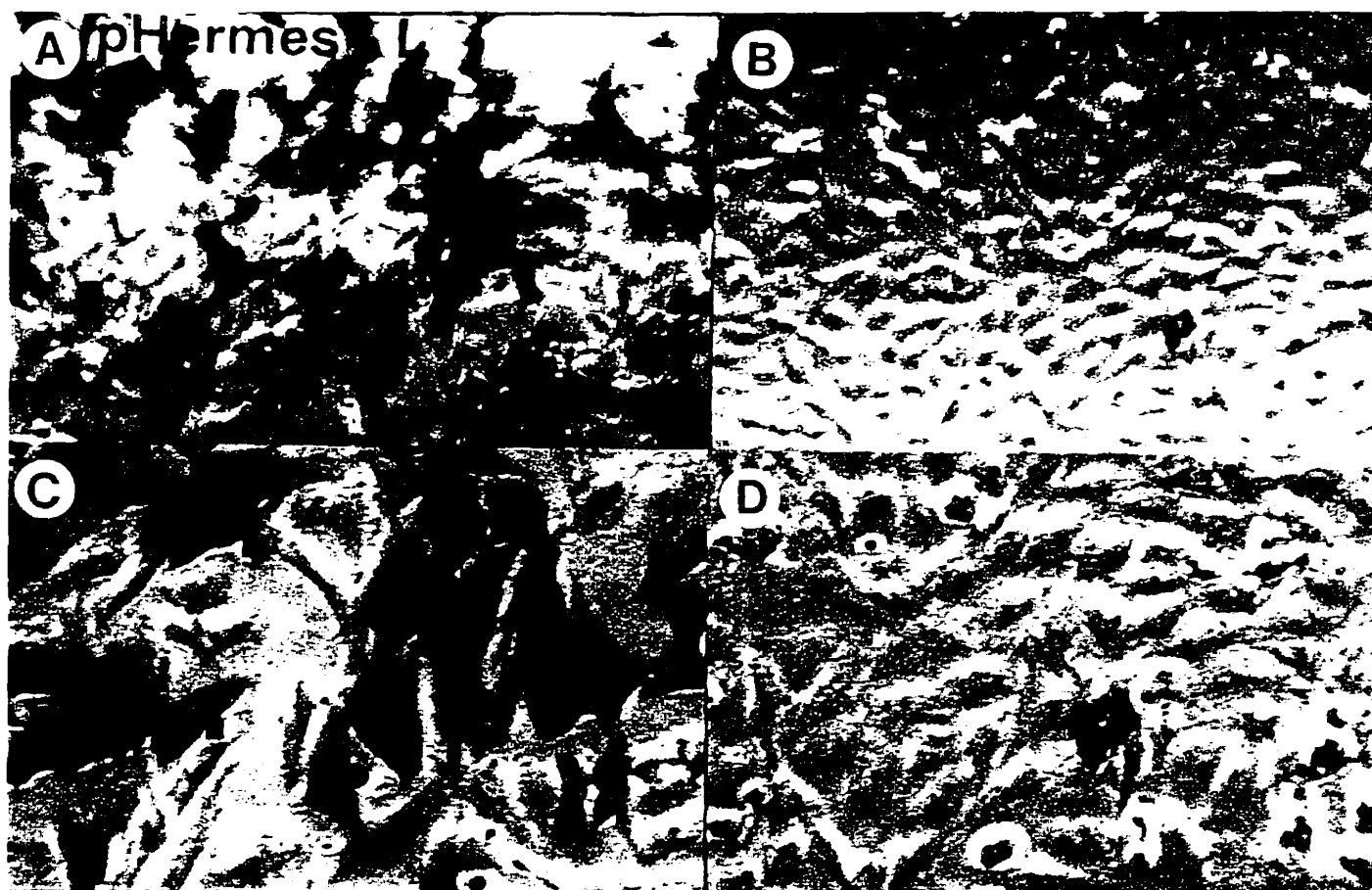


Figure 5.3 COS-7 and NIH 3T3 cells transfected with the pHermes-L and pHermes-L/PF plasmids.

COS-7 and NIH 3T3 cells were transfected transiently with the pHermes-L plasmid (A and C) for overexpression of lac Z or with the pHermes-L/PF plasmid (B and D) to overexpress PFTAIRE and lac Z. Twenty-four hours after transfection cells were fixed and stained with an X-gal solution to monitor the efficiency of transfections. The X-gal stained cells in panels B and D (pHermes-L/PF transfections) are considered cells expressing the PFTAIRE cDNA. The magnification in all panels is 50 x.

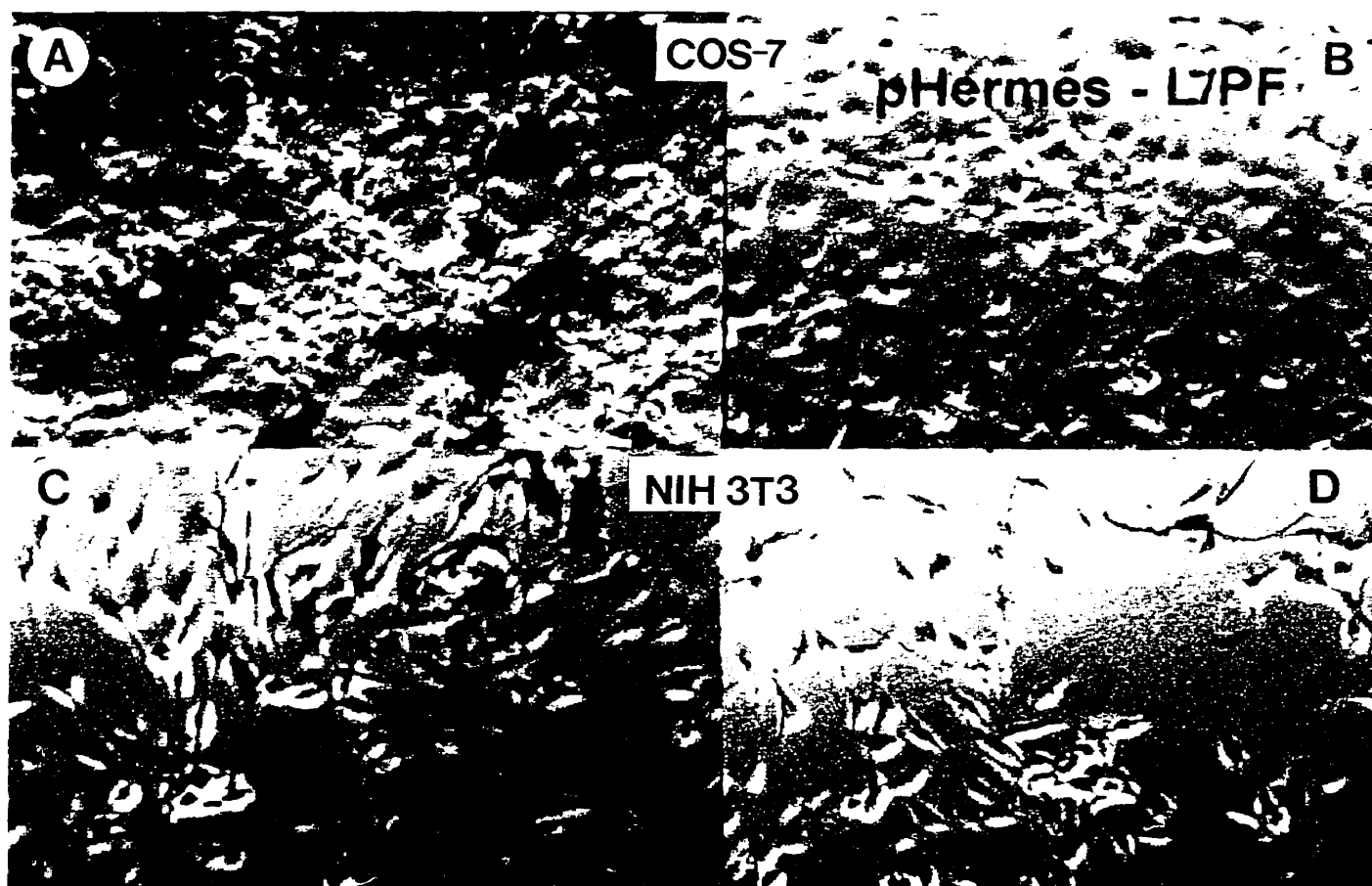


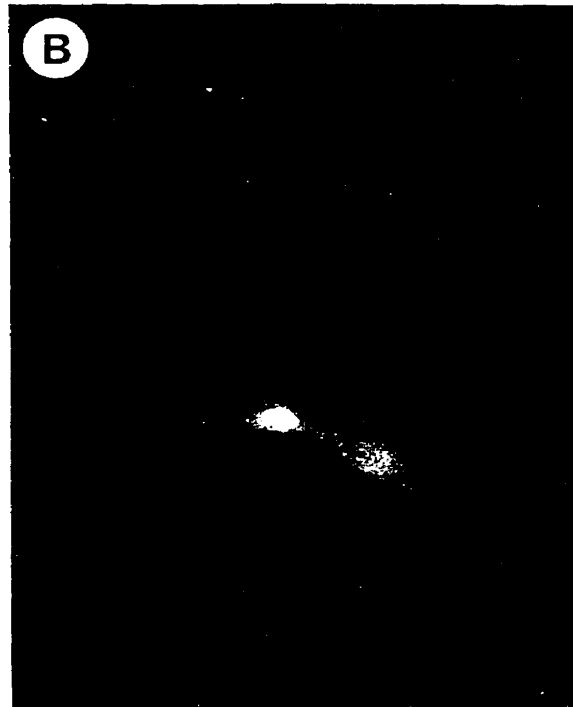
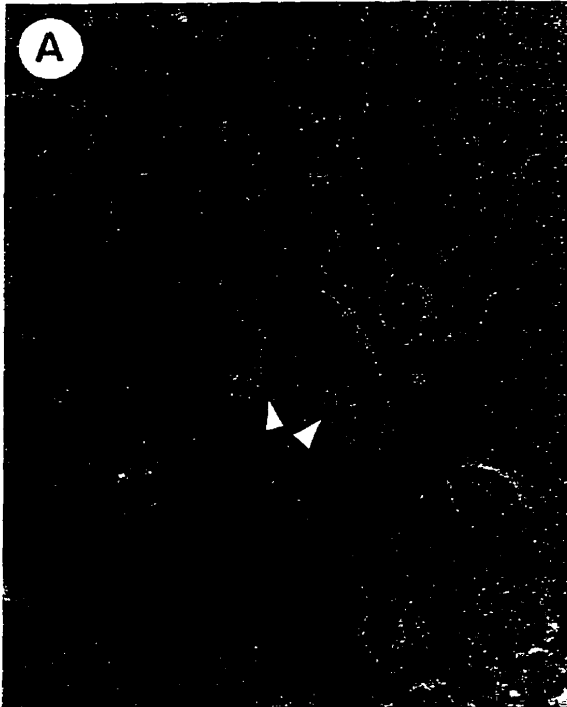
Figure 5.4 Indirect immunofluorescence labelling of NIH 3T3 and Vero cells transfected transiently with the PFTAIRE plasmid, pc3PF.

NIH 3T3 (A and B) and Vero (C and D) were transfected with the pc3PF plasmid to overexpress PFTAIRE. Twenty-four hours after transfection cells were fixed and processed for indirect immunofluorescence with the PFC5 antibody (B and D). Panels A and C are phase contrast micrographs of the same cells shown in B and D to demonstrated the number of cells in each field. Arrowheads in A and C indicate the PFC5 labelled cells in B and D. Scale bar, 30 μ m.

pc3PF TRANSFECTED CELLS

NIH 3T3

anti-PFTAIRE (PFC5)



Vero

anti-PFTAIRE (PFC5)

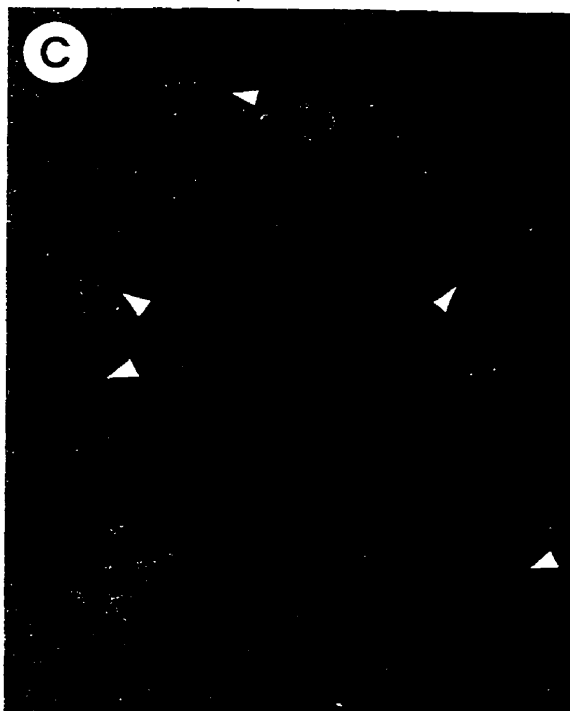


Figure 5.5 Anti-PFTAIRE and anti-lamin A/C labelling of COS-7 cells transfected transiently with the PFTAIRE cDNA.

COS-7 cells were transfected transiently with either the pcPF plasmid (A and B) or the pHermes-L/PF plasmid (C and D). Cells were fixed 24 hours after transfection and processed for indirect immunofluorescence with PFC5 and the anti-lamin A/C monoclonal antibody, 136D6. Each pair of micrographs represents the same field of cells. Arrowheads in B and D indicate the same cells labelled by PFC5 shown in panels A and C. Scale bar, 20 μ m.

PFTAIRE TRANSFECTED COS-7 CELLS

anti-PFTAIRE (PFC5)

anti-LAMIN A/C (136D6)

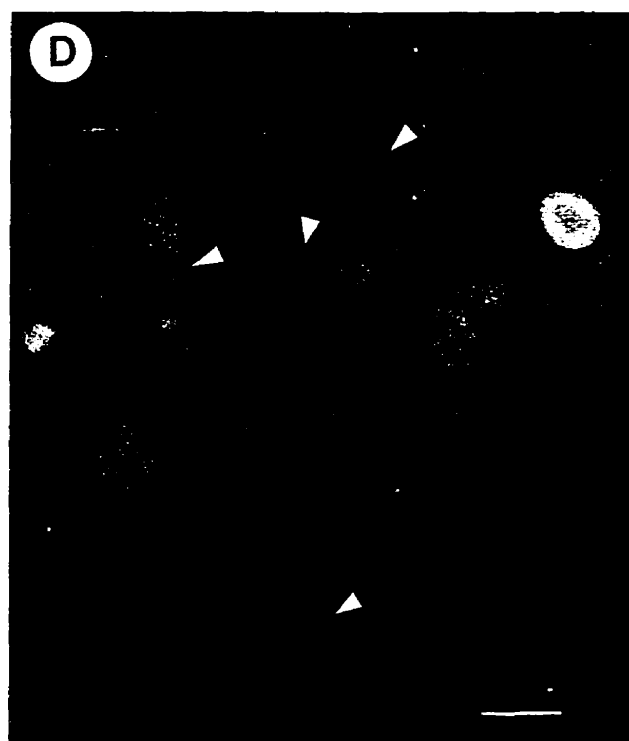
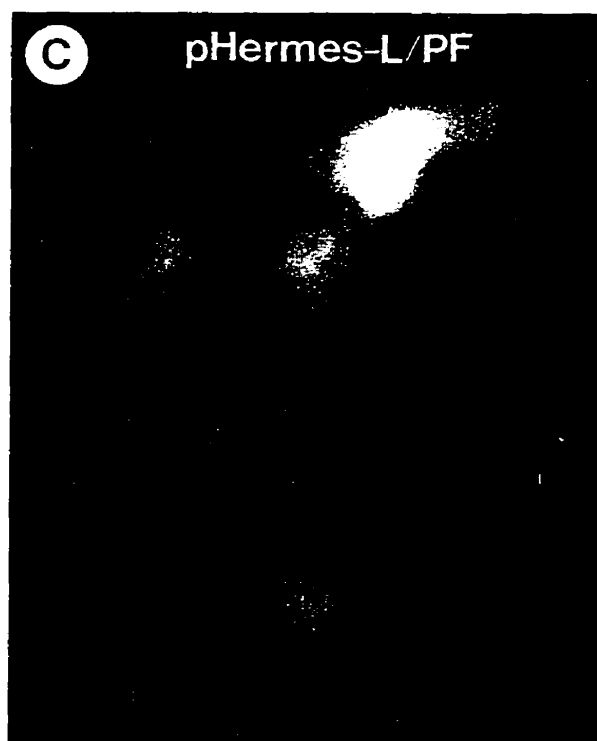
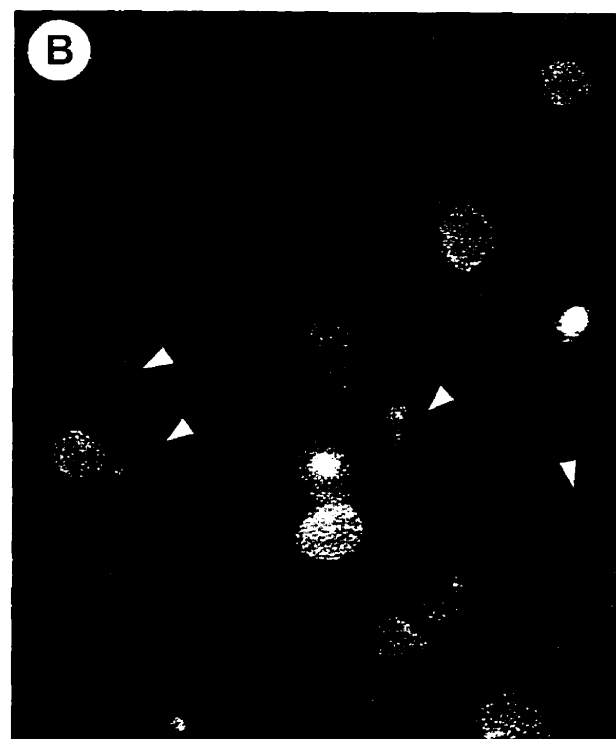
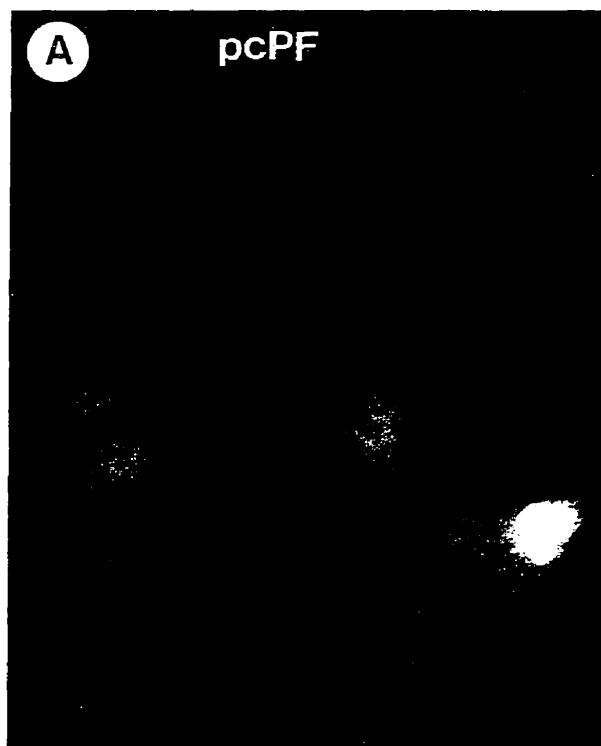


Figure 5.6 Anti-PFTAIRE and anti-lamin A/C labelling of Vero cells transfected transiently with the PFTAIRE cDNA.

Vero cells were transfected transiently with either the pcPF plasmid (A and B) or the pHermes-L/PF plasmid (C and D). Cells were fixed 24 hours after transfection and processed for indirect immunofluorescence with PFC5 and the anti-lamin A/C monoclonal antibody, 136D6. Each pair of micrographs represents the same field of cells. Arrowheads in B and D indicate the same cells labelled by PFC5 shown in panels A and C. Scale bar, 20 μ m.

PFTAIRE TRANSFECTED Vero CELLS

anti-PFTAIRE (PFC5)

anti-LAMIN A/C (136D6)

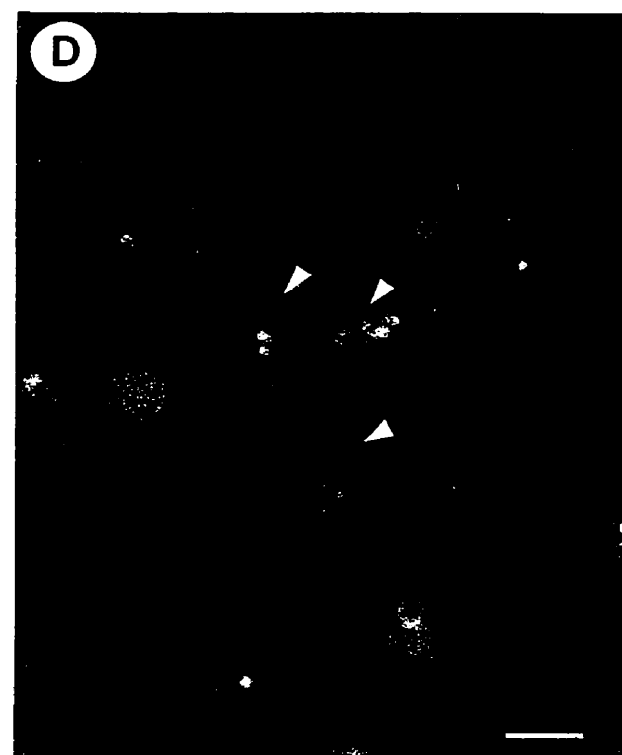
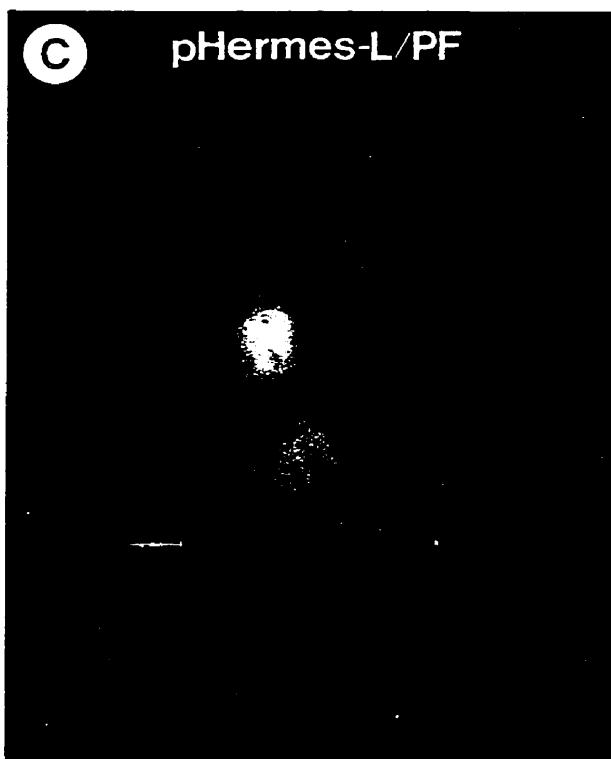
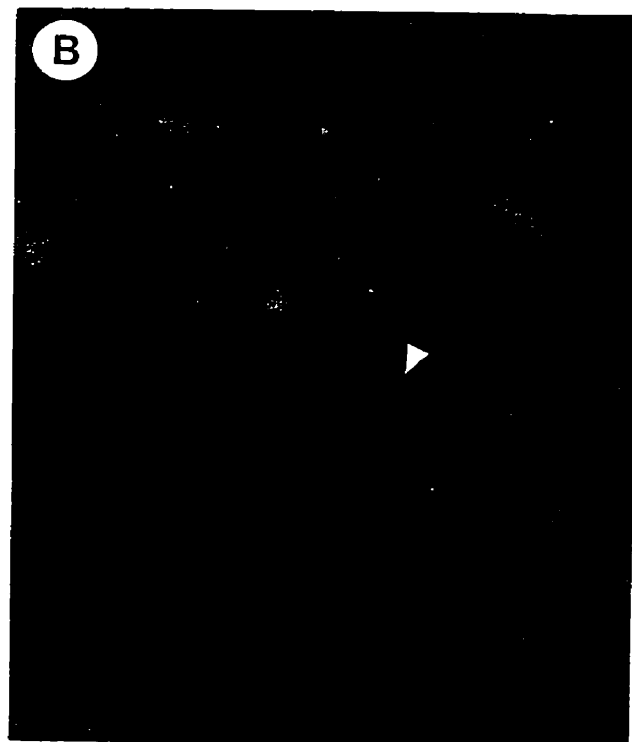
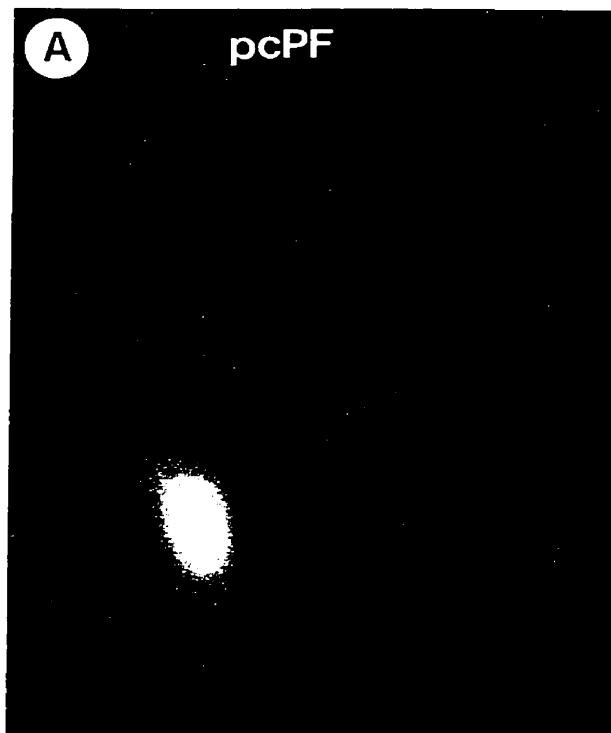
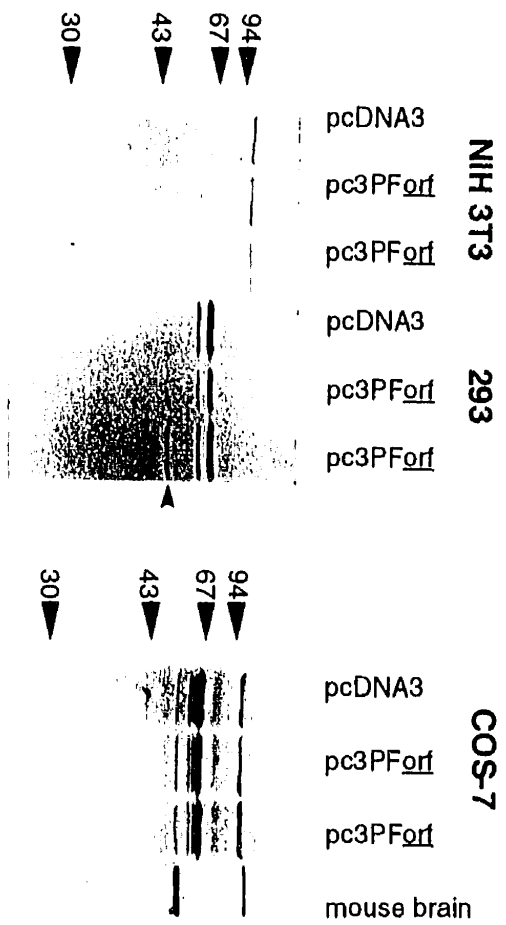


Figure 5.7 Immunoblot analysis of cells transfected with pcDNA3 and pc3PF_{orf} plasmids. Cytosolic protein extracts (15 µg) prepared from NIH 3T3, 293 and COS-7 cells transfected transiently (24 hours) with either pcDNA3 or pc3PF_{orf} were electrophoresed on 10% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose. The nitrocellulose membranes were immunoblotted with the PFC5 antibody to detect endogenous and transfected PFTAIRE protein species in the cell lines. PFTAIRE expressed after transfection of the pc3PF_{orf} plasmid in 293 cells is indicated by an arrowhead.



CHAPTER 6
GENERAL DISCUSSION

In an effort to identify a *cdc2*-related protein kinase that may function as a neurofilament kinase, I have cloned and characterized a cDNA encoding a novel *cdc2*-related protein kinase named PFTAIRE, that is expressed predominantly in the maturing and adult nervous system. Several serine/threonine protein kinases related to *p34^{cdc2}*, including the CDKs and *cdc2*-related kinases, have been identified in recent years primarily by using a variety of cDNA cloning strategies and in some cases by protein purification. Unlike *p34^{cdc2}*, only a few of these protein kinases are evolutionarily conserved from yeast to humans (Bunnell et al., 1990; Buck et al., 1995; Damagnez, 1995), though for many of these kinases homologues have been identified in diverse eukaryotic species. Thus the majority of CDKs and *cdc2*-related kinases are expressed only in multicellular eukaryotes. When the CDKs and *cdc2*-related kinases were first identified in mammals it was suggested that the presence of this multigene family in mammals may regulate the more complex signalling pathways that control cell growth and differentiation in multicellular organisms (Meyerson et al., 1992). The characterization of several CDKs has confirmed that in higher eukaryotes multiple protein kinases regulate transitions through the various stages of the cell cycle, which in yeast are regulated almost exclusively by *p34^{cdc2}* (reviewed in Hunter and Pines, 1994; Nigg, 1995). Depending on the phenotype of the cell in which they are expressed CDKs and *cdc2*-related kinases may regulate processes other than progression through the cell cycle such as exit from cell cycle, differentiation or processes related to the maturation of maintenance of morphology and function in neurons. The characterization of CDK5 has confirmed that protein kinases related to *p34^{cdc2}* do perform specialized functions, unrelated to cell cycle control, in the nervous system.

6.1 The homology between PFTAIRE and other CDKs

In general, the homology that the CDKs and *cdc2*-related kinases share with *p34^{cdc2}* is extensive (~50% or more) in the region corresponding to *p34^{cdc2}* (i.e., in the region corresponding to the highly conserved kinase subdomains defined by Hanks et al., 1988). The predicted amino acid sequence of PFTAIRE shares 48% amino acid identity with *p34^{cdc2}*. Overall, PFTAIRE shows the greatest similarity to the PCTAIRE group of *cdc2*-related kinases (Meyerson et al., 1992; Okuda et al., 1992). The predicted amino acid sequences of PFTAIRE and the human PCTAIRE kinases share 54-55% amino acid identity. In addition, all of these

proteins have extended amino- and carboxy-terminal regions relative to p34^{cdc2}, but the homology in these regions was limited. As in other CDKs and cdc2-related kinases, the three phosphorylation sites corresponding to Thr14, Tyr15 and Thr161 of human p34^{cdc2}, are conserved in the PFTAIRE sequence. The conservation of these three sites (Ser99, Tyr100 and Ser243) suggests that PFTAIRE may be regulated by protein kinases similar to Myt1, Wee1 and CAK, which regulate the kinase activities of p34^{cdc2} and other CDKs by phosphorylating these sites (Mueller et al., 1995; Parker et al., 1992; Parker and Piwnica-Worms, 1992; Fesquet et al., 1993; Poon et al., 1993; Solomon et al., 1993; Kato et al., 1994; Aprelikova, 1995).

Recently, a 3.5 kb PFTAIRE cDNA was identified in *Drosophila* (Sauer et al., 1996) and the authors indicated that this novel cDNA probably encoded a *Drosophila* PCTAIRE homologue, because the predicted amino acid sequence of *Drosophila* PFTAIRE (*Dm.* PFTAIRE) shared highest homology with the PCTAIRE kinases identified in mammals (Meyerson et al., 1992; Okuda et al., 1992). Alignment of the amino acid sequences predicted from the *Dm.* PFTAIRE and mouse PFTAIRE nucleotide sequences reveals that these two proteins share 69% amino acid identity and the majority of this homology occurs in the highly-conserved kinase subdomains (Figure 6.1). The three potential regulatory phosphorylation sites corresponding to Ser99, Tyr100 and Ser243 of murine PFTAIRE are identical in the *Dm.* PFTAIRE sequence (Ser202, Tyr203, and Ser346). *Dm.* PFTAIRE has extended amino- and carboxy-terminal regions relative to p34^{cdc2}, like mammalian the PFTAIRE and PCTAIRE kinases, but the homology between the sequences in these regions is limited. Although the proteins predicted from the *Drosophila* and murine PFTAIRE sequences differ in molecular mass, (*Dm.* PFTAIRE, 58.3 kDa; murine PFTAIRE, 47.5 kDa), the difference in size may be related to the diverse species from which the two genes have been isolated. The predicted amino acid sequence of *Drosophila* PITSLRE kinase has also been reported in the paper by Sauer et al. (1996), and it also encodes a protein that is larger than any of the human PITSLRE isoforms that have been reported previously (Bunnell et al. 1990; Xiang et al. 1994). Similar to the PFTAIRE sequences, the PITSLRE sequences from human and *Drosophila* are reported to share 65% amino acid identity in the kinase domain, and there appears to be little homology outside this region. Considering the evolutionary divergence between mammalian species and *Drosophila* it is not entirely surprising that proteins such as PFTAIRE and PITSLRE may show differences in molecular mass and primary structure, outside domains that are

required for conservation of function. Although the homology between *Dm.* and mammalian PFTAIRE is low outside the kinase domain, *Dm.* PFTAIRE is the first cdc2-related protein kinase to show greater than 55% homology to murine PFTAIRE. The 69% amino acid identity between the two PFTAIRE sequences suggests that they are evolutionarily conserved homologues. The conservation of the PFTAIRE gene from *Drosophila* to mammals indicates that the functions of PFTAIRE were required early in the evolution of multicellular organisms.

6.2 Developmentally regulated expression of PFTAIRE in the nervous system

a. Neurofilament protein phosphorylation during neuronal development

The development and maintenance of neuronal form and polarity is essential for neuronal function. The structural polarity of a neuron is in part established by the polarities of axonal and dendritic outgrowth (Jacobson, 1991). Axonal growth during development requires remodeling of the neuronal cytoskeleton. During the various stages of maturation, different intermediate filament proteins are components of the neuronal cytoskeleton. Neuroepithelial precursor cells express vimentin and nestin. In immature postmitotic neurons, nestin expression is downregulated and α -internexin is expressed. Neurofilaments are considered markers of maturing and adult neurons and are the most abundant cytoskeletal elements in neurons with large caliber axons (Hoffman et al., 1987). The neurofilament light and medium proteins, NF-L and NF-M, have been detected in immature rat neurons by 12 days gestation during axonal outgrowth, and appear to reach adult levels of expression by E15 (Carden et al., 1987). Expression of the neurofilament heavy protein, NF-H, is delayed compared to that of NF-L and NF-M and occurs only in neurons with "well-established" axons (Carden et al., 1987). NF-H has been detected in rat neurons by 15 days gestation but its expression remains lower than that of NF-L and NF-M throughout embryonic and early postnatal development (Shaw and Weber, 1982; Carden et al., 1987). When all three neurofilament proteins are expressed, the neurofilaments they form are considered mature. The maturation of neurofilaments (i.e., the increased expression of NF-H) coincides with a decrease in the velocity of axonal cytoskeletal transport (Hoffman et al., 1983; Willard and Simon, 1983), and to an increase in the radial growth of axons.

Mature neurofilaments are also highly phosphorylated and their phosphorylation state is thought to be an important determinant of axonal caliber, the rate of axonal transport, and stability of the neuronal cytoskeleton in mature neurons (reviewed in Pant and Veeranna, 1995). All three

neurofilament proteins contain potential sites for phosphorylation by cAMP-dependent protein kinase, protein kinase C and Ca^{2+} /CAM kinase in their amino-terminal head domains, and in the glutamic acid rich regions of all three proteins there are potential sites for phosphorylation by casein kinase I (refer to Chapter 1, Figure 1.5). However, the multiple KSP repeat sequences characteristic of the carboxy-terminal tail domains of NF-M and NF-H represent the majority of phosphorylation sites on neurofilaments (Julien and Mushynski, 1983; Geisler et al., 1987; Lee et al., 1988) and their phosphorylation is thought to have the greatest influence on establishing axonal caliber and stabilizing the neuronal cytoskeleton (Nixon and Sihag, 1991). Highly phosphorylated forms of NF-M are present in axons of E13 rat embryos, but NF-H phosphorylation begins late in embryonic development and continues throughout several weeks of postnatal development (Carden et al., 1987). Thus the temporal patterns of NF-H expression and phosphorylation appear to be most closely associated with increased axonal caliber, decreased axonal transport and increased stability of the cytoskeleton (Carden et al., 1987).

The identification of protein kinases that phosphorylate the carboxy-terminal KSP sites of NF-M and NF-H has remained elusive. When phosphorylation of NF-H *in vitro* by p34^{cdc2} kinase was shown to produce the same anomalous electrophoretic mobility of NF-H on SDS-polyacrylamide gels that occurs when NF-H is posttranslationally modified *in vivo* (Hisanaga et al., 1991), we considered that other related protein kinases may be more abundant than p34^{cdc2}, to perform this function in the mature nervous system. At the time of this publication by Hisanaga et al. (1991), it was known that p34^{cdc2} kinase activity was a key factor for triggering mitosis in proliferating cells and that it was not well expressed in the nervous system. It is now well established that p34^{cdc2} and a family of related protein kinases, cyclin-dependent kinases (CDKs), are the key components of pathways controlling the transitions between successive phases of the eukaryotic cell cycle (Hunter and Pines, 1994; Nigg, 1995; Morgan, 1995). As described previously (Chapter 1), the family of protein kinases related to p34^{cdc2} is extensive and the activities of many of these kinases are regulated through their interactions with cyclin proteins, other protein kinases, phosphatases and inhibitor proteins. Neurogenesis is clearly associated with a downregulation of the proteins directly involved in triggering S phase and mitosis, such as CDK2, p34^{cdc2}, cyclin A and cyclin B (Hayes et al., 1991; Freeman et al., 1994; Zhao et al., 1995; Yan and Ziff, 1995; Dobashi et al., 1995). However, several CDKs, cdc2-related kinases and

cyclins which are expressed in a variety of mitotic tissues have also been identified in the nervous system at developmental stages when the majority of cells have permanently exited from the cell cycle.

The identification in differentiated neurons of CDKs and cyclins that are required for progression through G₁, such as CDK4, cyclin D1, cyclin D2 and cyclin E has raised questions about whether these proteins may also be important for the switch from the proliferative to differentiated state (Tamaru et al., 1993; Tamaru et al., 1994; Freeman et al., 1994; Ross and Risken, 1994; Zhao et al., 1995; Yan and Ziff, 1995; Miyajima et al., 1995; Kranenberg et al., 1996). The characterization of CDK5 has indicated that its activity is restricted to the nervous system, where it may be important for development and differentiation of neurons and the organization of the neuronal cytoskeleton (Tsai et al., 1993; Nikolic et al., 1996; Oshima et al., 1996). Analysis of the expression of the cdc2-related kinase KIALRE has demonstrated that this protein kinase is expressed primarily in astrocytes in human adult brain tissue (Yen et al., 1995). Although other cdc2-related protein kinases, such as PCTAIRE-1, PCTAIRE-2, PCTAIRE-3, PITSLRE, PISSLRE and PITALRE, are expressed in adult brain tissue (Meyerson et al., 1992; Xiang et al., 1994; Graña et al., 1994a; Graña et al., 1994b; Brambilla and Draetta, 1994) no characterization of these proteins has been undertaken to determine their cellular localization or how they may function in the nervous system.

b. Developmental regulation of the expression of PFTAIRE and related kinases

PFTAIRE

The mRNA transcripts for the majority of CDKs and cdc2-related kinases that have been identified do not localize in specific tissue systems in adult animals (Meyerson et al., 1992; Hellmich et al., 1992; Okuda et al., 1992; Graña et al., 1994a; Graña et al., 1994b; Brambilla and Draetta, 1994; Xiang et al., 1994). Thus many of these protein kinases are expressed in tissues where cells maintain the capacity to divide and in tissues where the majority of the cells are postmitotic, such as the brain. The ubiquitous expression patterns of many of these protein kinase mRNA transcripts raises questions about: 1) whether the proteins and their associated kinase activities are present in all of the tissues expressing the mRNA transcripts and 2) whether the proteins perform similar or distinct functions in different cell types. The PFTAIRE cDNA was isolated from an 18 day postnatal mouse brain cDNA library. The analysis of PFTAIRE mRNA tissue distribution in adult mice has shown that a single 5.0 kb PFTAIRE mRNA transcript is

expressed predominantly in the nervous system and in the lung. Based on this observation, PFTAIRE appears to be the first member of the *cdc2* kinase family identified to date to demonstrate such a restricted pattern of mRNA expression in adult animals. In the adult central nervous system, PFTAIRE mRNA was moderately to abundantly expressed in several populations of postmitotic neurons. The predominantly neuronal expression of PFTAIRE mRNA, suggests that in adult animals PFTAIRE may function primarily in neurons and that its function is likely to be unrelated to control of cell cycle.

Analysis of the mRNA expression of *Drosophila* PFTAIRE has demonstrated that the *Dm.* PFTAIRE cDNA corresponds to a 3.5 kb mRNA transcript (Sauer et al., 1996). In embryos *Dm.* PFTAIRE mRNA is expressed in cells 'actively rearranging cell-to-cell contacts', such as in anterior and posterior midgut cells during gastrulation, in tracheal precursor cells during invagination, in myoblasts during fusion and possibly in migrating glial cells in the CNS (Sauer et al., 1996). Thus in *Drosophila* embryos PFTAIRE mRNA expression does not appear to be restricted to specific tissue systems or cell types. Although expression of the murine PFTAIRE mRNA transcript is restricted predominantly to the nervous system in adults, it is not known whether the transcript is more widely distributed in mouse embryos. The presence of an endogenous PFTAIRE-like immunoreactive species and of associated immunoprecipitable kinase activity in the 293 cell line (Chapter 4, Figure 4.10) which is derived from human embryonic kidney cells, suggests that PFTAIRE expression in mammalian embryos may not be restricted to neural cells. PFTAIRE null mice have been generated in Dr. Julien's laboratory by the targeted disruption of the PFTAIRE gene in embryonic stem cells. If PFTAIRE was more widely expressed in developing embryos, then the targeted disruption of this gene might lead to a high rate of embryonic lethality. Disruption of the PFTAIRE gene has not affected embryonic survival, suggesting either 1) PFTAIRE is not expressed in many different tissue systems or cell types in developing embryos or 2) other related genes can compensate for PFTAIRE if it functions in several different systems during mammalian development.

Although the majority of CDK and *cdc2*-related kinase mRNA transcripts are ubiquitously expressed in adult animals, some do show developmentally regulated expression in specific tissue systems. Examples of developmentally regulated expression of CDKs or *cdc2*-related kinases in specific tissue systems include: 1) the downregulation of *cdc2* mRNA in the nervous system as neuronal precursors exit from the cell cycle and become terminally differentiated

neurons (Hayes et al., 1991; Okano et al., 1993; Zhao et al., 1995); 2) the upregulation of CDK5 mRNA expression in the nervous system during fetal and postnatal development (Ino et al., 1994); 3) the downregulation of fetal PCTAIRE-1 mRNA transcripts in adult mouse liver and intestine (Okuda et al., 1993); and 4) the upregulation of fetal PISSLRE mRNA transcripts in several tissues in adult animals, including heart, brain, liver, lung and kidney (Graña et al., 1994 b). In the mouse nervous system, expression of PFTAIRE mRNA is also developmentally regulated. Extremely low levels of PFTAIRE mRNA were detected in embryonic brain tissue between E12.5-18.5, but on the day of birth PFTAIRE mRNA expression increased dramatically. During the first two weeks of postnatal development, the expression of PFTAIRE mRNA increased progressively and reached the expression level detected in adult brain by approximately 10.5 days after birth (Chapter 3, Figure 3.8). The expression of PFTAIRE mRNA is also developmentally regulated in *Drosophila* embryos, which show a transient increase in PFTAIRE mRNA between 2 and 6 hours of development that is followed by a decrease in expression from 6 hours until the third instar larval stage. By the third instar larval stage, expression of *Dm. PFTAIRE* mRNA is equivalent to the expression detected in pupae and adult flies (Sauer et al., 1996).

CDK5

Detailed analyses of mRNA expression in the developing nervous system have not been done for most of the CDKs or cdc2-related kinases, other than cdc2 (Hayes et al., 1991; Zhao et al., 1995) and CDK5 (Ino et al., 1994), so it is difficult to speculate whether any of the other related protein kinases may be required for terminal differentiation or nervous system development. Expression of CDK5 mRNA in rats begins by E12 (Ino et al., 1994). At E12 and throughout embryonic development, CDK5 mRNA is much more abundant than PFTAIRE mRNA, suggesting that CDK5 be more likely to have a role in neurogenesis than PFTAIRE. Sixty-four percent of CDK5 null mice die *in utero* after E16.5 and analysis of E18.5 and newborn CDK5 null mice revealed that development of the nervous system was abnormal (Oshima et al., 1996). Although neurogenesis in the mouse spinal cord is completed between E10-14 (Normes and Carry, 1978), at E16.5 immature neurons are migrating to the superficial layers of the mouse cortex (Angevine and Sidman, 1961) and neurogenesis in the cerebellum is incomplete (Miale and Sidman, 1961). Thus the abnormal development that occurs throughout the nervous system in CDK5 null mice strongly suggests that CDK5 expression and activity is required during neurogenesis. The inhibition of neurite outgrowth associated with the transfection of CDK5 dominant negative

mutants in primary cultures of cortical neurons also suggests that CDK5 activity is necessary for neuronal differentiation. In addition, the co-localization of CDK5 with actin microfilaments in growth cones of cultured cortical neurons, suggests a role for CDK5 in neurite outgrowth (Nikolic et al., 1996). Although CDK5 has been shown to phosphorylate NF-H *in vitro*, the expression and phosphorylation of NF-H is associated with maturation of neurofilaments and axons (Carden et al., 1987). CDK5 mRNA, protein and kinase activity are detected in neurons much earlier than phosphorylated NF-H (Tsai et al., 1993; Ino et al., 1994; Carden et al., 1987). These observations, taken together with data demonstrating a requirement for CDK5 activity for neuronal differentiation and neurite outgrowth (Nikolic et al., 1996) and the preliminary analysis of CDK5 null mice (Oshima et al., 1996), suggest that during development the principle role of CDK5 may not be to phosphorylate NF-H.

During cortical development, p35, which is required for positive regulation of CDK5 activity, reaches peak expression levels. After birth p35 expression levels in the maturing brain decline and remain high only in specific regions, such as the hippocampus, olfactory cortex and pyramidal cell layers of the neocortex (Nikolic et al., 1996). The recent analysis of mice homozygously null for the p35 gene has demonstrated that p35 and CDK5/p35 kinase activity are required for the proper formation of the cortical laminae (Chae et al., 1997). This is in agreement with the abnormal development of the cortex found in CDK5 null mice (Oshima et al., 1996). Detailed analysis of the p35 null mice revealed that the abnormal corticogenesis was due to defects in neuronal migration. Because neurons born on E15 or later failed to migrate past pre-existing neurons in the cortical plate of p35 null mice, the neurons were packed from "outside-in" as opposed to the normal "inside-out" distribution of cortical neurons. Unlike the CDK5 null mice, which show embryonic lethality, 15% of the p35 null mice were prone to spontaneous seizures and death from 3 weeks of age onward, possibly due to the abnormal formation of the cortex (Chae et al., 1997). Thus the histological and neuronal birth dating analysis of the p35 null mice is consistent with other findings demonstrating that CDK5/p35 kinase activity may be required for the early development and organization of the brain (Oshima et al., 1996), during a period when NF-H is not highly phosphorylated.

Because CDK5 expression and kinase activity are maintained in adult nervous tissue, the possibility that CDK5 functions as a protein kinase phosphorylating NF-H or tau *in vivo*, as the nervous system matures, cannot be excluded. The restricted expression of p35 in the mature

brain raises the possibility that as the nervous system matures CDK5 may associate with other regulatory proteins. Phosphorylated NF-H and another regulator of CDK5 kinase activity, p67, are co-localized in axons and cell bodies (Shetty et al., 1995; Pant and Veeranna, 1995), which suggests that CDK5/p67 complexes could be involved in neurofilament phosphorylation *in vivo*. The upregulation of PFTAIRe mRNA expression during the first two weeks of postnatal development that coincides with the progressive increase in phosphorylation of NF-H as neurofilaments and axons mature, suggested that PFTAIRe should be tested as a potential NF-H KSP kinase.

c. Functional implications of the developmentally regulated expression of PFTAIRe and future directions

The very low level of PFTAIRe mRNA detected in embryos and its marked upregulation in newborn mice suggest that, in contrast to CDK5, PFTAIRe would not be required for neurogenesis and early development of the nervous system. The targeted disruption of the PFTAIRe gene has not affected survival of PFTAIRe null mice, which also suggests that PFTAIRe may not be required for development of the nervous system in embryos. If PFTAIRe was required for development of the nervous system, then the lack of an effect on survival may indicate that another protein compensates for PFTAIRe in the PFTAIRe null mice. The upregulation of PFTAIRe expression at birth and during the first few weeks of postnatal development coincides with the upregulation of NF-H expression and its increased phosphorylation (Shaw and Weber, 1982; Carden et al., 1987). Thus on the basis of mRNA localization in neurons of adult nervous tissue and the temporal expression pattern of PFTAIRe mRNA in the brain, PFTAIRe would be a reasonable candidate for a neurofilament kinase.

As discussed previously in Chapter 3, the upregulation of PFTAIRe mRNA expression during early postnatal development also coincides with the period of reorganization in the nervous system, when synapses are being formed, strengthened and eliminated. Although activity-dependent plasticity may be important for the maturation and stability of synapses during development and during reorganization of synapses in adult animals in response to injury (sprouting from uninjured axons) (reviewed in Shatz, 1990; Jacobson, 1991; Bliss and Collingridge, 1993) the molecular mechanisms associated with activity-dependent strengthening and reorganization of synapses have not been well characterized. Numerous candidate plasticity-related genes (CPGs) that are induced in the adult rat dentate gyrus by the activation of glutamate

receptors have been cloned by differential cDNA cloning. The CPGs include immediate early genes that encode transcription factors such as *c-fos*, *c-jun* and *zif268*, other previously identified genes, and novel genes (Nedivi et al., 1993). Expression in the adult rat cortex of a subset of novel CPGs was shown to be modulated by changes in neural activity in response to light. These CPGs are also transiently expressed during the first few weeks of postnatal development, but it is not known whether they are induced in an activity-dependent manner at that time. The activation of the same set of CPGs during development and in the adult, when synapse maturation or reorganization occurs, suggests that similar molecular mechanisms may underlie these processes in maturing and adult animals (Nedivi et al., 1996). The molecular mechanisms underlying synapse strengthening, maturation and elimination in an activity-dependent manner or in response to other environmental cues during development are likely to involve signal transduction pathways that include protein kinase activities. Given the temporal expression pattern of PFTAIRE mRNA it is relevant to ask whether PFTAIRE may be involved in such processes. In addition, the observation by Sauer et al. (1996) that PFTAIRE mRNA is expressed *Drosophila* embryos in cells that are re-arranging cell contacts may be suggestive of a role in the formation of synaptic contacts in the mammalian nervous system. By analyzing wild type and PFTAIRE null mice it may be difficult to determine whether the normal upregulation of PFTAIRE expression during postnatal development is important for synapse maturation in the postnatal nervous system. A more manageable approach for determining whether PFTAIRE is required for the formation or reorganization of synapses may be to lesion specific regions of the nervous system in adult PFTAIRE null mice, such as the hippocampus or dentate gyrus, where PFTAIRE is normally highly expressed in wild type mice. If the analysis of these animals demonstrated that PFTAIRE expression and kinase activity is necessary for reorganization of synapses in response to injury, then determining whether PFTAIRE is also involved in the maturation of synapses in postnatal animals may be a relevant avenue to pursue.

The failure of the mammalian CNS to support axon regeneration after transection is a property that is acquired as the nervous system matures (Jacobson, 1991). In general, the regenerative capacity of the CNS begins to diminish early in postnatal development. It has been demonstrated that the extraneuronal milieu through which axons must grow influences their ability to regenerate and given an appropriate substrate some CNS axons can regrow into peripheral nerve grafts or into areas where glial cell inhibitory proteins have been neutralized with antibodies

(Richardson et al., 1980; David and Aguayo, 1981; Caroni and Schwab, 1988). Thus the prevailing hypothesis regarding regeneration of CNS axons is that the success or failure of axon regrowth depends primarily on environmental factors.

Recently, this hypothesis has been challenged by experiments demonstrating that the reduced ability of postnatal hamster retinal axons to reinnervate explants of midbrain tectum was due primarily to developmentally regulated changes in the retinal axons rather than cues from the target tissue (Chen et al., 1995). When retinae were dissected from hamsters between E14 and P0, there was extensive fiber growth into E14 tectal explants. When retinae were dissected from animals on postnatal day 2 or later, a 10-fold reduction in fiber growth of retinal axons into E14 tectum was observed (relative to the fiber growth observed in co-cultures of E14 retina and E14 tectum), indicating that the presence of an embryonic target did not reverse the regenerative failure of postnatal retinal cells. Similar results were obtained from experiments in which retinae were co-cultured with slices of inferior colliculus or visual cortex, indicating that the failure of postnatal retinal axons to regenerate was also not due changes in the target environment (Chen et al., 1995). A small number of axons from adult retinae were shown to grow into embryonic tectal explants, but no adult retinal axons grew into adult tectal explants. These observations confirmed previous work demonstrating that only a minority of adult rat retinal axons maintain the capacity to regenerate in a permissive environment (Vidal-Sanz et al., 1987; Villegas-Perez et al., 1988). Thus the recent observations by Chen et al. (1995) indicate that the failure of retinal axons to regenerate may be due primarily to intrinsic changes in retinal neurons that occur early in postnatal development. Analysis of proteins synthesized in developing hamster retinal ganglion cells and transported to the superior colliculus in the rapid phase of axonal transport has demonstrated that the transport of specific proteins changes during the first two weeks of postnatal development (Moya et al., 1988). These observations indicate that molecular changes that occur in this pathway during early postnatal development coincide with the failure of retinal axons to regenerate. Will the data from experiments suggesting that the loss of regenerative capacity of retinal ganglion cell axons is due primarily to intrinsic changes be generally applicable to the CNS? Does the upregulation of PFTAIRE during early postnatal development influence this property of CNS neurons? This question may be addressed in future experiments by determining whether PFTAIRE expression in wild type mice is altered in response to axotomy and whether there are differences in the abilities of CNS axons to regenerate in wild type, PFTAIRE null mice and

transgenic mice that overexpress PFTAIRE.

d. Conclusions

To conclude, I have identified a cDNA clone from a postnatal mouse brain cDNA library that encodes a novel cdc2-related protein kinase named PFTAIRE. The cDNA corresponds to a 5.0 kb mRNA transcript that is abundantly expressed in the nervous system and lung of postnatal and adult mice. PFTAIRE mRNA was localized predominantly in neuronal populations throughout the adult mouse CNS. The upregulation of PFTAIRE mRNA expression in postnatal animals suggests this protein kinase may be important for neuronal maturation or maintenance of properties associated with a mature neuronal phenotype.

6.3 PFTAIRE kinase activity

a. Substrates of PFTAIRE kinase and their potential function

On the basis of the spatial and temporal expression patterns of PFTAIRE mRNA, I have speculated that PFTAIRE is not likely to be involved in regulation of cell cycle, neurogenesis or early neuronal differentiation, but that it may be involved in maturation of neurons and the synapses they form. The characterization of the PFTAIRE gene product presented in Chapter 4 has provided additional information about the distribution of PFTAIRE in the nervous system and the subcellular localization of PFTAIRE in neurons. I have also demonstrated that the protein kinase activity of PFTAIRE immunoprecipitated from adult mouse brain protein extracts phosphorylates specific proteins that remain associated with PFTAIRE immune complexes and these substrates differ from those associated with CDK5 immune complexes. In addition, these experiments have indicated that activation of PFTAIRE may require association of PFTAIRE with regulatory proteins and that PFTAIRE is phosphorylated by another protein kinase(s) in brain. All of the data obtained from the characterization of the PFTAIRE gene product have given some insight into the enzymatic function of PFTAIRE as a protein kinase.

The PFTAIRE C-terminal antibody PFC5 was used to demonstrate by indirect immunofluorescence, that PFTAIRE protein was localized in the same populations of neuronal and glial cells in adult mouse spinal cord and cerebellum as the PFTAIRE mRNA. The subcellular localization of PFTAIRE in neurons in the CNS is predominantly nuclear and cytoplasmic. Although PFTAIRE was also detected in axons in the sciatic nerve it was not detected in all axonal

pathways. The localization of PFTAIRE in various compartments of neurons suggests that PFTAIRE may perform distinct functions in different populations of neurons or that it may be inactive in one compartment and translocated to another compartment when activated. CDK5 was initially described as being localized predominantly in axonal pathways and absent from immature axons, growth cones dendrites and neuronal cell bodies in nervous tissue from embryonic and adult mice (Tsai et al., 1993). This observation provided additional support for the hypothesis that CDK5 may function as a protein kinase phosphorylating the major components of the axonal cytoskeleton, particularly NF-M, NF-H and tau. More recently it has been demonstrated that CDK5 is localized in the cell bodies, nuclei, neurites and growth cones of cultured primary neurons and that CDK5 activity is required for neurite outgrowth in these cultured neurons (Nikolic et al., 1996) and normal development of the brain (Oshima et al., 1996). As discussed in the previous section, these data suggest a role for CDK5 early in neuronal differentiation and nervous system development, but do not exclude the possibility that CDK5 may function in the mature nervous system as a neurofilament kinase. Similarly, the localization of PFTAIRE in different compartments of neurons also raises the possibility that it may have multiple functions. The substrates that co-immunoprecipitated with PFTAIRE represent cytosolic substrates, and the nature of nuclear PFTAIRE substrates remains to be determined. The localization of PFTAIRE in the nuclei and cytoplasm of neurons suggested that it may be part of a signalling pathway, while the co-localization of PFTAIRE and NF-H in sciatic nerve axons indicated that NF-M, NF-H or tau could be considered potential substrates for PFTAIRE in axons. The presence of PFTAIRE in specific populations of glial cells in the spinal cord and sciatic nerve that have the morphological appearance of oligodendrocytes and Schwann cells further suggests that PFTAIRE may perform multiple functions in the nervous system.

The upregulation of PFTAIRE mRNA that coincides with increases in NF-H phosphorylation during postnatal development and the co-localization of PFTAIRE and NF-H in adult sciatic nerve axons suggested that NF-H was a good candidate substrate for PFTAIRE kinase activity. A protein of approximately 200 kDa, which corresponds to the apparent molecular mass of phosphorylated NF-H, was associated with PFTAIRE immune complexes and phosphorylated by the PFTAIRE kinase activity in this complex. However, a GST-NFH fusion protein was not phosphorylated by PFTAIRE that was immunoprecipitated from adult mouse brain. Immunoprecipitated PFTAIRE also did not phosphorylate NF-H synthetic peptides

containing KSP motifs in the context of KSPXK, KXPEK or KSPXE, which represent the majority of KSP motifs in mouse NF-H. These results indicate that NF-H is not likely to be a substrate for PFTAIRE.

Although p34^{cdc2} and CDK5 have been shown to phosphorylate dephosphorylated NF-H *in vitro*, data obtained from kinase assays with NF-H peptides similar or identical to those that I have used, demonstrated that the NF-H KSP motifs phosphorylated by p34^{cdc2} and CDK5 (KSPXK or KSPEK) represent a minority (8 in mouse) of the NF-H KSP sites that are phosphorylated *in vivo* (Hisanaga et al., 1991; Shetty et al., 1993; Hisanaga et al., 1993). In mouse NF-H, the majority (39) of KSP sites are represented by the motif KSPXE (Julien et al., 1988). Recently, it has been demonstrated that there is a strong correlation between the extent of NF-H phosphorylation and the level of activation of the proline-directed kinase SAPK- γ (stress-activated protein kinase) in PC12 cells (Giasson and Mushynski 1996). Treatment of PC12 cell cultures with agents that stimulated strongly the *c-jun* kinase activity of SAPK- γ resulted in hyperphosphorylation of NF-H. The increased phosphorylation of NF-H was related specifically to the activation of SAPK- γ , because stimulation of ERK1 or ERK2 kinase activity following treatment with NGF did not alter the phosphorylation state of NF-H. SAPK- γ immunoprecipitated from PC12 cells phosphorylated *in vitro*, the GST-NFH fusion protein and preferentially phosphorylated the peptide derived from mouse NF-H containing KSPXE motifs. Thus unlike p34^{cdc2} and CDK5, SAPK- γ phosphorylates *in vitro* KSP motifs that correspond to the majority of the KSP phosphorylation sites in NF-H. In addition, hyperphosphorylated NF-H was localized in the cell bodies of cultured dorsal root ganglia neurons after cells were treated to stimulate SAPK- γ activity (Giasson and Mushynski, 1996). These findings may have implications for neuropathological conditions, such as amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease and chemical intoxication by neurotoxic substances, in which neurofilaments accumulate in neuronal cell bodies in a state of aberrant phosphorylation. Hyperphosphorylated NF-H was detected in neuronal cell bodies in the brainstems of E18.5 CDK5 null mice (Oshima et al., 1996), suggesting that the activity of a protein kinase other than that of CDK5 is important for changes in neurofilament phosphorylation that are characteristic of several neuropathological conditions. In light of the findings by Giasson and Mushynski (1996), it is possible that the CDK5 null mice may show increased expression and activity of SAPK- γ . Although SAPK- γ may be an

important kinase activity phosphorylating NF-H KSP repeats in response to neuronal damage or stress, it remains to be determined in normal nervous tissue which kinase activities maintain phosphorylation of the majority of NF-H KSP repeats *in vivo*.

In addition to NF-H, histone H1 and the microtubule associated protein tau were tested as substrates for brain PFTAIRE kinase activity, *in vitro*. p34^{cdc2} phosphorylates sites in histone H1 that occur in the context of K-S/T-P-X-K (Langan et al., 1989) and histone H1 has been used as an experimental substrate to demonstrate the kinase activities of other CDKs and cdc2-related kinases, including CDK2, CDK5, and KIALRE (Fang and Newport, 1991; Lew et al., 1992a; Shetty et al., 1993; Yen et al., 1995). Although the *in vitro* phosphorylation of histone H1 by some of these kinases, such as CDK5 and KIALRE from nervous tissue, may not reflect their functions *in vivo*, it may provide some information about the sequences recognized by these kinases (Beaudette et al., 1993) in other more biologically relevant substrates. PFTAIRE did not phosphorylate histone H1 *in vitro*. This observation and the inability of immunoprecipitated PFTAIRE to phosphorylate NF-H peptides containing KSP motifs similar to the consensus for phosphorylation by p34^{cdc2} suggest that the sites phosphorylated in PFTAIRE substrate proteins may be quite different from the p34^{cdc2} consensus or that appropriate PFTAIRE activators were missing from the immune complex. CDK4/cyclin D1 and CDK6/cyclin D1 complexes also do not phosphorylate histone H1, but do phosphorylate pRB *in vitro* on many of the sites that are phosphorylated *in vivo* (Matsushime et al., 1992; Kato et al., 1993; Meyerson and Harlow, 1994). Recently, it has been demonstrated that CDK4/cyclin D1 preferentially phosphorylates serine or threonine residues in sequence motifs corresponding to P-L-S/T-P-X-K/R-H and P-L-S/T-P-I-P-K/R-H (Kitagawa et al., 1996). This analysis demonstrated that for CDK4/cyclin D1 phosphorylation there is a similar requirement for the phosphoacceptor serine or threonine residue to be followed immediately by a proline residue, but the residues flanking the S/T-P differ from those flanking the sites phosphorylated in histone H1 by other CDKs. A proline residue at the -2 position (the phosphoacceptor serine or threonine is the 0 position) and lysine or arginine residues at the +2 and +3 greatly increased the capacity of synthetic peptides to serve as substrates for CDK4/cyclin D1 (Kitagawa et al., 1996). These additional requirements are similar to those identified from the analysis of the substrate specificity of CDK5 (Beaudette et al. 1993). A similar type of analysis may also be required to determine the substrate specificity of PFTAIRE.

Tau protein contains several sites for phosphorylation by proline-directed protein kinases (Trojanowski et al., 1993), but neither PFTAIRE nor CDK5 immunoprecipitated from mouse brain protein extracts phosphorylated bovine tau under the assay conditions used (Chapter 4, Section 4.4a). Purified CDK5 and proline-directed kinases, such as p34^{cdc2}, MAP kinase (ERK2) and GSK-3 β have been shown by others to phosphorylate tau *in vitro*, on sites that are phosphorylated *in vivo* in paired helical filament tau preparations from Alzheimer's brain samples (Baumann et al., 1993; Paudel et al., 1993; Vulliet et al., 1992; Drewes et al., 1992; Ledesma et al., 1992; Ishiguro et al., 1993). Because the bovine tau was not phosphorylated by immunoprecipitated CDK5 it is possible that the preparation contained contaminating phosphatases.

The characterization of PFTAIRE kinase activity has demonstrated that although PFTAIRE immunoprecipitated from mouse brain protein extracts did not phosphorylate *in vitro* any of the experimental substrates that were tested in kinase reactions, it did phosphorylate specifically proteins that associated with it in immune complexes. By using a PFTAIRE mutant (D228N) that was predicted to be kinase inactive and function as a dominant negative inhibitor of endogenous PFTAIRE kinase activity (Chapter 4, Section 4.4b), I have demonstrated that phosphorylation of the proteins associated with PFTAIRE is likely due to the kinase activity of PFTAIRE and not that of another protein kinase in the PFTAIRE immune complex. The PFTAIRE substrate proteins in the 293 cell immune complexes were the same molecular mass as those which were co-immunoprecipitated with PFTAIRE from brain protein extracts. This observation suggests that these substrate proteins may be the same in brain and in 293 cells, but it is not clear whether their phosphorylation by PFTAIRE would serve the same purpose in postmitotic neurons and proliferating cells in culture.

The two dimensional gel electrophoresis analysis of the components of the PFTAIRE immune complex from brain protein extracts (Chapter 4, Figure 4.13) demonstrated that the strongly phosphorylated 58-60 kDa proteins specifically phosphorylated by PFTAIRE (Chapter 4, Figure 4.8) showed similarities to the two dimensional analyses of the 55-56 kDa species of porcine and human tau (Cleveland et al., 1977; Ksiezak-Reding et al., 1988). As discussed previously in Chapter 4, the upregulation of PFTAIRE mRNA expression during the first two weeks of postnatal development, coincides with developmentally regulated changes in the phosphorylation state of tau that may be important for synapse formation and stabilization of the

neuronal cytoskeleton (Watanabe et al., 1993; Brion et al., 1994). Changes in the expression and phosphorylation of tau result in the decreased expression of immature fetal tau, which is highly phosphorylated until the second or third week of postnatal development (in rats) (Brion et al., 1993) and increased expression of the less phosphorylated mature adult form of tau, which begins in the second week of postnatal development (Brion et al., 1994). A developmentally regulated increase in the number of microtubule-binding repeats and changes in the phosphorylation state of tau are associated with changes in its ability to promote microtubule assembly. Fetal tau, which contains only three microtubule-binding repeats and is highly phosphorylated, is less efficient in promoting microtubule assembly and stabilizing microtubules than adult tau, which contains four microtubule-binding repeats and is less phosphorylated (Lindwall and Cole, 1984; Francon et al., 1982; Goedert and Jakes, 1990). Fetal tau expression coincides with the period of synapse formation and reorganization whereas the increased expression of adult tau during postnatal development correlates with maturation and stabilization of synapses (Brion et al., 1994). Tau antibodies that react strongly with fetal tau and mass spectrometry combined with phosphopeptide sequencing have been used to monitor changes in the phosphorylation of tau during rat brain development (Brion et al., 1994; Mawal-Dewan et al., 1994; Watanabe et al., 1993). Highly phosphorylated fetal tau has been detected in the rat brain between E18 and approximately postnatal day 20, but the strongest immunoreactivity was observed during the early postnatal period, until approximately postnatal day 10-11 (Brion et al., 1994; Mawal-Dewan et al., 1994). This peak in fetal tau immunoreactivity coincides with the period during development when PFTAIRE expression is upregulated. Although mature forms of tau are phosphorylated on fewer sites (Watanabe et al., 1993) this change in the phosphorylation state of tau may reflect increased expression and activity of phosphatases in the maturing brain (Mawal-Dewan et al., 1994), rather than decreased expression and activity of kinases. In fibroblast and neuroblastoma cells transfected with tau, inhibition of PP2A leads to hyperphosphorylation of tau, dissociation of tau from microtubules and disassembly of microtubules (Sontag et al., 1996). The 58-60 kDa proteins associated with the PFTAIRE immune complex remain to be identified, but it may be significant to note that other proline-directed kinases (MAP kinase, GSK-3 and CDK5) that phosphorylate tau *in vitro*, have been purified as proteins associated with microtubules (Baumann et al., 1993). I have speculated that the 58-60 kDa PFTAIRE substrates associated with PFTAIRE immune complexes from brain protein extracts may be tau protein, but it is not known whether tau

is also expressed in 293 cells. As discussed previously in Chapter 4, tau protein has been detected in non-neuronal cell lines. Thus the possibility that 293 cells may express tau cannot be excluded. The possibility that the 58-60 kDa PFTAIRE substrate is an isoform of tau, is an observation that may merit further investigation. In addition, there may be several other substrate proteins, including neuronal proteins, that do not remain associated with PFTAIRE during immunoprecipitation.

The characterization of PFTAIRE kinase activity has also raised questions about associated factors that may be required for PFTAIRE to function as a protein kinase. The *in vitro* preparation of PFTAIRE, including a PFTAIRE *in vitro* translation product from rabbit reticulocyte lysate and a GST-PFTAIRE fusion protein were not autophosphorylated and without an identified PFTAIRE substrate it was not possible to test whether these preparations possessed kinase activity. When the *in vitro* translated PFTAIRE was combined with brain protein extract, the phosphorylation of the 58-60 kDa PFTAIRE substrates was increased (Chapter 4, Figure 4.9), which suggested that when the *in vitro* translated protein preparation was provided with a substrate it functioned as a protein kinase. However, this observation may also imply that the *in vitro* translated PFTAIRE required association with other proteins in the brain extract to become active. The GST-PFTAIRE fusion protein was shown to be phosphorylated by a kinase activity in brain extract (Figure 4.11) that associated with the PFTAIRE region of this molecule. This observation raises the question of whether other proteins, particularly other protein kinases, interact with PFTAIRE to regulate its activity. Other members of the cdc2 kinase family require reversible phosphorylation of specific amino acids in order to function as protein kinases (Chapter 1, Sections 1.2b and 1.3e). Ser99, Tyr100 and Ser243 of PFTAIRE, which are the amino acids corresponding to the regulatory phosphorylation sites of p34^{cdc2} (Thr14, Tyr15 and Thr161), are obvious sites to mutate to determine whether there are similar requirements for phosphorylation of these sites to regulate PFTAIRE kinase activity. In addition, consensus sequences for phosphorylation by cAMP-dependent protein kinase and protein kinase C that have been identified in the PFTAIRE sequence (Table 3.1) may also have implications for the regulation of PFTAIRE kinase activity.

b. Functional implications of the characterization of PFTAIRE kinase activity and future directions

Several questions remain to be answered about the kinase activity of PFTAIRE, with

regard to what its function is and how its function may be regulated by other proteins. To further explore the ideas that PFTAIRE may be important for maturation or reorganization of synapses, or for changes in the regenerative capacity of neurons, any observed changes in the expression of PFTAIRE mRNA in wild type mice in response to neuronal damage would necessitate determining whether there are corresponding alterations in localization of PFTAIRE protein and quantitative and qualitative changes in PFTAIRE kinase activity. However, this approach to understanding the function of PFTAIRE would still be indirect. Furthermore, if no obvious phenotypic changes occur in the nervous systems of PFTAIRE null mice or transgenic mice overexpressing PFTAIRE in neurons, the most direct way to begin to assess the function of PFTAIRE in the nervous system, may be to identify the PFTAIRE substrates found in PFTAIRE immune complexes. This question can only be undertaken by purification of these proteins or possibly by interactive cloning.

The possibility that the 58-60 kDa proteins, which are the major endogenous substrates that remain associated with PFTAIRE immune complexes, may be tau is provocative. As discussed previously in Chapter 4, proline-directed kinases, including ERK2, GSK-3 β and CDK5 are associated with tau during purification of microtubule-associated proteins from normal adult brain tissue and paired helical filaments. However, it is not clear whether these protein kinases affect the phosphorylation state of tau during normal development. The correlation between PFTAIRE expression and tau phosphorylation, and the possibility that PFTAIRE and tau are associated in PFTAIRE immune complexes suggest a function and a substrate protein for PFTAIRE in the nervous system. Synthetic peptides derived from tau containing various sites known to be phosphorylated *in vivo* and preparations of tau that do not contain phosphatase activities may be used with PFTAIRE immunoprecipitated from mouse brain protein extracts to determine whether PFTAIRE phosphorylates tau *in vitro*. Using transgenic mice that overexpress PFTAIRE it may be possible to obtain indirect evidence indicating whether PFTAIRE is involved in tau phosphorylation during development. In mice that overexpress PFTAIRE under the control of the NF-L promoter one might examine whether fetal tau is highly phosphorylated earlier in embryonic development than in wild type mice. Because the expression of the PFTAIRE transgene is driven by the NF-L promoter, it should be expressed at higher levels much earlier in development (E12) than the endogenous PFTAIRE. One might also examine whether fetal tau remains phosphorylated for a longer period during postnatal development of these mice. The PFTAIRE null mice may also be used for similar experiments to determine if tau is less

phosphorylated or dephosphorylated in the absence of functional PFTAIRE.

c. Conclusions

To conclude, the PFTAIRE protein is present in several populations of neurons and possibly in myelin-producing glial cells in the adult mouse CNS and PNS. The protein is localized predominantly in the nuclear and cytoplasmic compartments of neurons, but has also been detected in some axons, such as sciatic nerve axons. This distribution suggests the possibility that this kinase may perform multiple functions in the nervous system. PFTAIRE has been shown to phosphorylate protein substrates from brain protein extracts and 293 cell lysates that co-immunoprecipitate with this kinase activity, but the identities of these proteins remain to be determined. I have also shown that PFTAIRE is phosphorylated by other protein kinases present in adult mouse brain, which indicates that like other members of the cdc2 kinase family PFTAIRE may be regulated by the activities of other protein kinases and phosphatases, such as Wee1-, CAK- or CDC25-like proteins.

6.4. Consequences of ectopic expression of PFTAIRE in cell lines

Based on the spatial and temporal expression patterns of PFTAIRE mRNA and protein in the nervous system, the function of PFTAIRE is not predicted to be related to control of cell cycle like several CDKs nor does it seem likely that PFTAIRE would be involved in the neurogenesis or the early differentiation of immature neurons. On the basis of results obtained from kinase assays using PFTAIRE immunoprecipitated from adult mouse brain protein extracts, PFTAIRE also does not appear to be involved in phosphorylation of KSP repeats of NF-M and NF-H. By attempting to ectopically overexpress PFTAIRE in a variety of proliferating fibroblastic cell lines that express little or no endogenous PFTAIRE, I have found that it was not possible to obtain cell lines that constitutively overexpressed the kinase and the transient expression of PFTAIRE compromised the viability of the majority of these cell lines. These results, presented in Chapter 5, demonstrated that cells transfected with the pHermes-L/PF plasmid (PFTAIRE and lacZ) appeared more rounded and shrunken compared to pHermes-L (lacZ)-transfected and nontransfected cells (Figures 5.2 and 5.3). Indirect immunofluorescence analysis of PFTAIRE-overexpressing cells showed that PFTAIRE was highly expressed in nuclei and the overexpression of PFTAIRE correlated with morphological changes in nuclei, that included irregularities in shape, size and distribution of lamin proteins, compared to nontransfected cells in the same culture (Figures 5.5

and 5.6). The alterations in the distribution of lamin proteins in the PFTAIRE-transfected cells, the presence of fragmented or multiple nucleoli and the frequent occurrence of transfected cells as pairs suggested that these cells could be daughter cells in the G₁ phase of the cell cycle.

Nontransfected cells in the same culture rarely showed the nuclear morphology observed in PFTAIRE-transfected cells, suggesting that these changes were the result of PFTAIRE overexpression. Based on these observations it appears that PFTAIRE overexpression in proliferating cells may cause cell cycle arrest in the G₁ phase or a slowing of the progression through the cell cycle in PFTAIRE-transfected cells. The nuclear morphology that I observed in PFTAIRE-transfected cells, particularly the punctate lamin distribution, is similar to the lamin distribution observed in apoptotic cells in culture, where lamins were shown to localize at the nuclear periphery around condensed chromatin (Oberhammer et al., 1994). Thus it is possible that the PFTAIRE-transfected cells were daughter cells in G₁ undergoing apoptotic cell death.

Without directly labelling the DNA of PFTAIRE-transfected cells to determine if the chromatin was indeed condensed or nicked, it is not possible to conclude definitively that overexpression of PFTAIRE resulted in apoptotic cell death. Nevertheless, these observations are provocative and the changes in cellular morphology that occurred following transient expression of PFTAIRE may be related to the difficulties in obtaining permanent cell lines that constitutively overexpressed this kinase.

Other CDKs, cdc2-related kinases and cyclins have been implicated in mediating changes in cell cycle progression or apoptotic cell death when overexpressed or expressed at an inappropriate time. When Thr14 and Tyr15 of p34^{cdc2} were mutated to non-phosphorylatable residues, cells expressing the p34^{cdc2} mutants entered mitosis prematurely and suffered “mitotic catastrophes”, resembling apoptosis (Gould and Nurse, 1989; Norbury et al., 1991; Krek and Nigg, 1991b). Overexpression of p34^{cdc2} also leads to “mitotic catastrophes” (Heald et al., 1993) and premature activation of p34^{cdc2} in lymphoma cells was shown to be required for apoptosis induced by a lymphocyte granule protease (Shi et al., 1994). Permanent cell lines constitutively overexpressing the cdc2-related kinase PITALRE (p58^{GTA}), showed longer doubling times than wild type CHO cells due to a prolonged G₁ phase (Bunnell et al., 1990). Induction of apoptosis in HeLa cells is associated with activation of cyclin A-dependent kinase activity. Overexpression of

p34^{cdc2}, CDK2, CDK3 and cyclin A has been shown to promote apoptosis in a HeLa cell line stably overexpressing the anti-apoptotic activity of Bcl-2 when these cells were treated with apoptosis-inducing agents, such as tumor necrosis factor α (TNF- α) or staurosporine (Meikrantz et al., 1994). Expression of p34^{cdc2}, CDK2 and CDK3 dominant negative mutants in wild type HeLa cells suppressed apoptosis induced by TNF- α or staurosporine (Meikrantz and Schlegel, 1995). The overexpression of cyclin D1 in fibroblasts during the G₁ phase of the cell cycle has been shown to result in a reduction in cell size, acceleration of the G₁ phase of the cell cycle and cause cell death (Jiang et al., 1993; Quelle et al., 1993; Kranenberg et al., 1996).

Although it is not entirely clear how each of these proteins may mediate cell cycle arrest or apoptotic cell death, there is evidence indicating that cells undergoing apoptosis frequently seem to do so from late G₁ or early in S phase (Ucker et al., 1991; Meikrantz and Schlegel, 1995). Thus cell cycle arrest in late G₁ or early in S phase can potentiate apoptosis (Howard et al., 1993; Meikrantz et al., 1994), whereas arrest in G₀ or early G₁ seems to suppress apoptosis in response to various agents (Belizario and Dinarello, 1991; Shi et al., 1992; Howard et al., 1993). It is possible that perturbation of pRB function may be one mechanism by which cells in G₁ undergo apoptosis. The phosphorylation state of pRB is a key factor determining whether cells remain in G₁ or progress through this phase of the cell cycle to initiate DNA replication. pRB may exert its growth suppressive effects by binding to several different proteins, including several transcription factors, such as E2F (Taya, 1997). As discussed in Chapters 1 and 5, phosphorylation pRB by CDK4-, CDK6-cyclin D1 and inactivation of its growth suppressing function allows progression through the G₁ phase of the cell cycle. In addition, CDK2-cyclin E complexes which are active later in G₁ may also phosphorylate pRB (reviewed in Weinberg, 1995). Phosphorylation of pRB continues until M phase (Weinberg, 1995), presumably so that it is maintained in a hyperphosphorylated, inactive state until the next round of the cell cycle. Thus deregulation or interference with the activities of proteins that regulate pRB activity or proteins that are regulated by pRB binding may be key factors contributing to inappropriate cell cycle arrest or apoptosis. The observation that PFTAIRE-transfected cells may have been daughter cells in G₁ undergoing apoptosis is consistent with idea that ectopic overexpression of PFTAIRE might interfere with the normal activities of any of the proteins that are required for progression through G₁.

b. Functional implications of ectopic expression of PFTAIRE and future directions

The inappropriate expression of PFTAIRE is not well tolerated in proliferating cells that normally express little or no endogenous PFTAIRE protein. Additional experiments are required in order to determine whether PFTAIRE overexpression does contribute to apoptotic cell death in proliferating cells. Standard procedures for labelling the DNA of PFTAIRE-transfected cells, such as propidium iodide staining, Hoechst staining or *in situ* terminal transferase end labelling of DNA (TUNEL), can be employed to assess the condition of the chromatin these cells and determine whether the cells are undergoing apoptosis. The PFTAIRE mutant D228N, which is can function as a kinase inactive dominant negative when transfected Chapter 4, section 4.4b), can be used to determine whether the morphological changes observed following transfection of PFTAIRE plasmids were due specifically to the expression of this kinase activity. Although the morphology of cells transfected with the D228N plasmid has not been examined by immunofluorescence with PFTAIRE and lamin antibodies, the number of 293 cells surviving after transfection of this plasmid and antibiotic selection was twice the number of PFTAIRE-transfected cells (Chapter 5, Section 5.5b). This suggests that transfection of the D228N plasmid did not have the same adverse effect on cells as overexpression of PFTAIRE. The co-transfection of the PFTAIRE and D228N plasmids should not produce the same morphological changes associated with overexpression of PFTAIRE and would provide further evidence that PFTAIRE overexpression contributes to these changes. In addition, the use of an inducible PFTAIRE plasmid for transfections would provide a way of monitoring what happens to PFTAIRE-overexpressing cells before they arrest or die.

Although the observation that PFTAIRE overexpression may result in apoptotic cell death has not directly contributed to the elucidation of PFTAIRE function in the nervous system, it has raised additional questions to consider about PFTAIRE kinase activity and function. If PFTAIRE overexpression prevents proliferating cells from progressing through the cell cycle, is this the reason why PFTAIRE is expressed predominantly in neuronal cells that have permanently exited from the cell cycle? Is PFTAIRE interacting with the same proteins in proliferating cells and neurons? Does PFTAIRE perform different functions in different types of cells? Do the consequences of ectopic PFTAIRE expression have any bearing on its function in neurons? Would overexpression of PFTAIRE in neurons result in cell death? Based on the results obtained

following PFTAIRE overexpression in proliferating cells it may also be relevant to ask whether PFTAIRE expression is upregulated in neurons in neuropathological conditions.

6.5 Conclusion

I have identified a cDNA encoding a novel mammalian protein kinase, PFTAIRE, that is related to the family of cyclin-dependent kinases and cdc2-related kinases. The characterization of PFTAIRE has shown that unlike most proteins in this kinase family, the mRNA expression of PFTAIRE in adult mice is restricted predominantly to the nervous system, where it is expressed in several populations of neurons. During development PFTAIRE is expressed at very low levels in embryos. PFTAIRE mRNA expression increases dramatically on the day of birth and continues to increase throughout the first two weeks of postnatal development, suggesting a possible role in maturation of neurons. The characterization of PFTAIRE kinase activity has demonstrated that it phosphorylates proteins in brain protein extracts, which are co-immunoprecipitated with it.

Preliminary analysis of PFTAIRE null mice in Dr. Julien's laboratory has indicated that there is a loss of tau immunoreactivity in nervous tissue extracts from adult mice, immunoblotted with the AT8 antibody. The AT8 antibody reacts specifically with the tau epitope that contains phosphoserine at the position corresponding to Ser202 of human adult tau. Tau was detected on immunoblots of the nervous tissue protein extracts from PFTAIRE null mice with the Tau-1 antibody, which reacts specifically with tau when the AT8 epitope is dephosphorylated. This indicates that loss of PFTAIRE function did not affect the expression level of tau, but may result in alterations in the phosphorylation state of tau. This preliminary data, taken together with the data from two dimensional gel analysis of PFTAIRE immunoprecipitation-kinase reactions indicating that tau may be a substrate for PFTAIRE in PFTAIRE immune complexes, suggest that PFTAIRE may be involved in the phosphorylation of tau *in vivo*. Further studies will be required to determine if the 58-60 kDa PFTAIRE substrates from brain protein extracts are tau proteins. The transgenic mice that have been generated to overexpress PFTAIRE in neurons and the PFTAIRE null mice will provide additional approaches for studying the function of PFTAIRE in the mammalian nervous system.

Figure 6.1 Sequence alignment of murine PFTAIR and Drosophila PFTAIR.

Alignment of the amino acid sequences predicted from the murine PFTAIR (*Mus.* PFTAIR) cDNA and the Drosophila PFTAIR (*Dm.* PFTAIR) cDNA to show regions of homology. Identical amino acids are indicated by black boxes. The Drosophila PFTAIR sequence is from Sauer et al. (1996).

1 M S T R N C Q G T D S V I K H L D - - - T I P E D K - - - Mus. PFTAIRE
 1 M A T T T T T T T T T G A T N A K D G V T M R E K K G G A L Dm. PFTAIRE

24 - - - - - - - - - - K V R V Q R T Q S T F D P F E K P A Mus. PFTAIRE
 31 Q K L K K R L S H S F G R L T I S R E D G D E S T H H H H Dm. PFTAIRE

42 N Q V K R - Mus. PFTAIRE
 61 H H H H R G G H G G H H N H G N K V P Y N G Y N S E E Y L D Dm. PFTAIRE

47 - - - - - - V H S E N N A C I - - N F K S S S A G K E S P Mus. PFTAIRE
 91 R L E P N G N I P A D K T N C R Y G D W R S T D S T D H H D Dm. PFTAIRE

68 K V R R H S S - Mus. PFTAIRE
 121 R V Q R Q L S V S S D S K L L D E D I R E E M K Y H S H V V Dm. PFTAIRE

75 - - P S S P T S P K - Mus. PFTAIRE
 151 M R P K K P P R P K S E V F L N K Q E T H P R R K R F S A F Dm. PFTAIRE

83 - - - - - F G K A D S Y E K I F K I G E G S Y A T V Y K G K Mus. PFTAIRE
 181 G G D S P F G K Q E A Y V K I E P L G E G S Y A T V Y K G F Dm. PFTAIRE

108 S K V N G K L V A L K V I R L O E E E G T P F T A I R E A S Mus. PFTAIRE
 211 S K L T Y Q R V A L K E I R L O E E E G A P F T A I R E A S Dm. PFTAIRE

138 L L K G L K H A N I V L L H D T I H T K E T I T L V F F E Y V Mus. PFTAIRE
 241 L L K E L K H S N I V T L H D T I V H T R E T I T L F V F E Y C Dm. PFTAIRE

168 H T D L C O Y M D K H P G G L H P D N V K I F L F Q L L R G Mus. PFTAIRE
 271 D T D L S O Y M E K H P G G L D H R N V R L F L F Q L L R G Dm. PFTAIRE

198 L S Y I H Q R Y I I H R D L K P O N L L T S D T G F L K L A Mus. PFTAIRE
 301 L S Y C H K R R V L H R D V K P O N L L T S D C G F L K L A Dm. PFTAIRE

228 D F G I A R A K S V P S H T Y S N F V V T L N Y R P P D V L Mus. PFTAIRE
 331 D F G L A R A K S V P S H T Y S H E V V T L N Y R P P D V L Dm. PFTAIRE

258 I G S T F Y S T C L D M W G V G C I F V F M I Q G V A A F P Mus. PFTAIRE
 361 L G S T E Y S T S L D M W G V G C I F V F M V T G V A A F P Dm. PFTAIRE

288 G M K D I Q D O L E R I F L V L G T P N E D T W P G V H S L Mus. PFTAIRE
 391 G I R D T Y D O L D K I F K L L G T P T E D T W P G V T H F Dm. PFTAIRE

318 P H F K P E R F T V Y N S K S I R Q A W N K I S Y V N H A F Mus. PFTAIRE
 421 P G Y K P H K L G F Y R P R K L G H N F P R L Y D I I E G F Dm. PFTAIRE

348 D L A S K L I O C S P K N R I S A Q A A L S H E Y F S D L P Mus. PFTAIRE
 451 T I A N G F L O L N P E Q R I G A D D A L Q H P Y F A Q L P Dm. PFTAIRE

378 P R I W E I T D M S S I F T V P N V R L Q P F A G E S M R A Mus. PFTAIRE
 481 K K L Y E L P D E T S I F T V E G V Q L Y T E P - - - - - Dm. PFTAIRE

488 F G K N N S Y G K S L S N S K H . Mus. PFTAIRE
 505 - - - - - - - - - - - H R Q N K Dm. PFTAIRE

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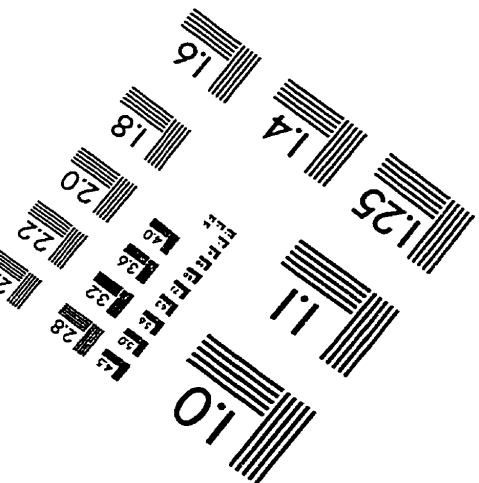
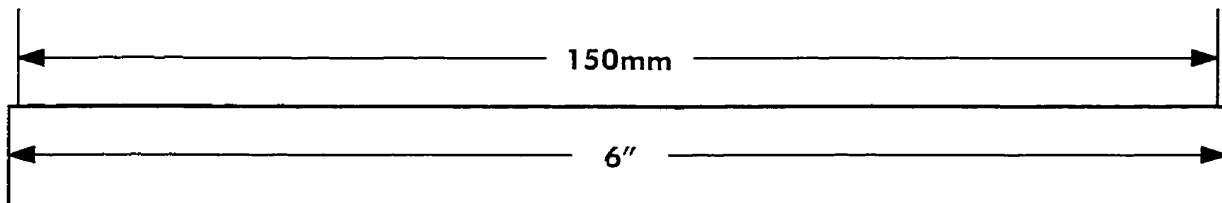
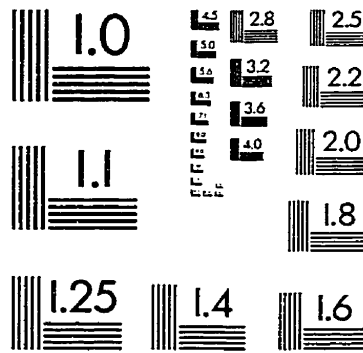
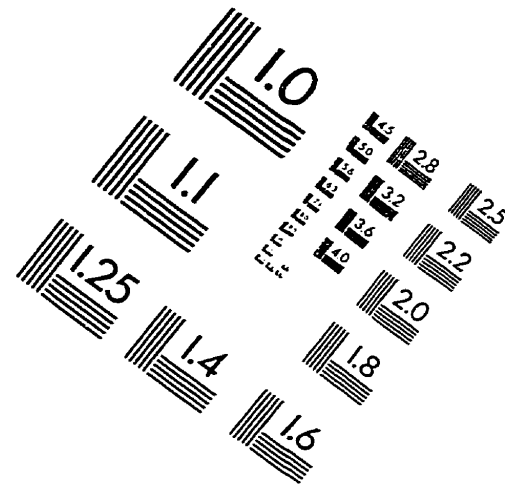
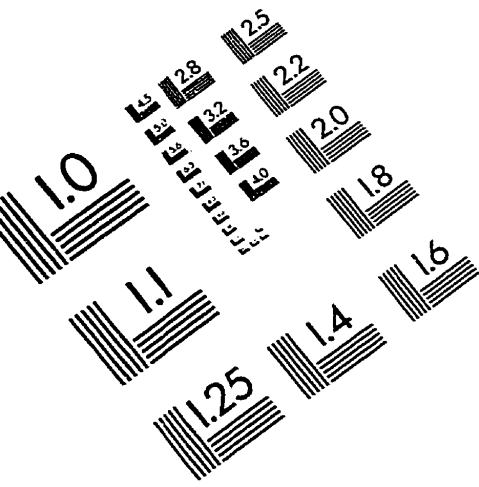
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IMAGE EVALUATION TEST TARGET (QA-3)



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