THE REGULATION OF IRF -4 ACTIVITY IN LYMPHOID CELLS AND INVOLVEMENT IN HTLV-I-INDUCED T CELL LEUKEMOGENESIS

By

Yael MAMANE

A thesis submitted to the Faculty of Graduate Studies and Research

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Microbiology and Immunology

McGill University, Montreal

July 2001

To my parents, Daniel and Annie for their encouragement, love and support.

À mes parents Daniel et Annie pour leurs encouragements, amour et support.

To my sisters, Eve and Myriam for their support, sense of humor and of course of style.

À mes soeurs Eve et Myriam pour leur support, leur sens de l'humour et bien sûr de style.

To Anna for all her support and cheering up.

A Anna pour son support et ses encouragements.

To Shmuel for all his love, thoughtfulness and energy.

You are my joy and inspiration.

הקדשה מיוחדת

אני מבקשת להקדיש את עבודת התיזה של לאהובי שמואל.
מעולם לא שיערתי שבמהלך נסיעתי הראשונה לישראל אפגוש את הנפש התאומה שלי. פגשתי אותו הוא הקריב למעננו קורבן גדול. הוא החליט לעזוב את משפחתו האוהבת, חבריו וארצו היפה , כדי להיות איתי בקנדה.
לעולם אהיה אסירת תודה לו על מעשהו.
עבודה זו היתה אפשרית לא רק בזכות עבודה קשה והתמדה, אלא גם בזכות הקרבתו למעני. תודה , שמואל.
אני רוצה להודות לרותי, דוד , גיל ומור על היותם משפחה-מחותנת הנחמדה ביותר שאי פעם יכולתי לקוות לה. אני מצטערת על העצב שגרמתי לכם עקב עזיבתו של שמואל. אני מבטיחה להחזיר לכם אותו בחזרה.
אני מקדישה גם לכם את עבודתי.

באהבה לכולם

יעל

ABSTRACT

The Human T cell Leukemia Virus (HTLV) is the etiologic agent of adult T cell leukemia (ATL), an aggressive and fatal leukemia of $CD4^+$ T lymphocytes, and is also associated with a neurological demyelinating disease called Tropical Spastic Paraparesis (TSP) or HTLV-I Associated Myelopathies (HAM). The oncogenic potential of HTLV-I resides in the viral Tax oncoprotein, a positive regulator of viral and cellular gene transcription. Interestingly, all HTLV-1 infected cells and Jurkat cells transiently transfected with the HTLV-I Tax gene, express constitutively the Interferon Regulatory Factor-4 (IRF-4), a lymphoid-specific member of the IRF family of transcription factors, indicating that Tax may function as an indirect transactivator the IRF-4 gene. The overexpression of IRF-4 in HTLV-1 infected cells may play a role in viral-mediated cellular transformation and thus in adult T cell leukemia. IRF-4 expression studies revealed its presence in B cells, activated T cell, macrophages and T cells infected with HTLV-I or HTLV-II. In attempt to understand the regulation of IRF-4 expression, promoter analyses were undertaken using genomic footprinting and EMSA. These promoter analyses revealed the involvement of the NF-κB and NF-AT family members in IRF-4 regulation. Using IRF-4 as bait in a yeast two hybrid screen, a novel interaction between IRF-4 and the FK506 binding protein 52 (FKBP52), a 59kDa member of the immunophilin family with peptidyl-prolyl isomerase activity (PPIase) as well as chaperone-like functions, was characterized. Inhibition of IRF-4 DNA binding activity as well as transcriptional potential was shown to require functional PPIase of this immunophilin. FKBP52 seems to induce a conformational change in IRF-4 by cis-trans prolyl isomerization which interferes with IRF-4 DNA binding and transactivation. These studies suggest the direct involvement of the immunophilin FKBP52 in the regulation of IRF-4 function by a novel post-translational modification. Several other potential IRF-4 interacting partners were found during a second two-hybrid screen: eIF4γ1, c-src, Gi3, RhoANEF, RhoGDI and

PP2A. These interactions could be involved in HTLV-I induced leukemogenesis. Novel IRF-4 regulated genes were also analyzed using an IRF-4 stably expressing Jurkat cell line and cDNA array technology. Several genes potentially regulated by IRF-4 such as RhoA, HSC70, RP-A, cyclin B1, PCNA, EB1, NIP3, MAPKK3 were identified. The deregulation of these genes by IRF-4 could lead to an increase in cellular proliferation and activation, a decrease in apoptosis and in DNA repair; Events that are hallmarks of HTLV-I, induced T cell leukemogenesis.

RÉSUMÉ

Le rétrovirus HTLV (Human T Lymphotropic Virus) constitue l'agent étiologique de la leucémie à cellules T de l'adulte (ATL), une leucémie agressive et fatale des lymphocytes T $CD4^+$ qui est également associée à une myélopathie chronique appelée Paraparésie spastique tropicale (Tropical Spastic Paraparesis ou TSP) ou atteinte neurologique connue sous le nom de HTLV-I Associated Myelopathy (HAM). Le potentiel oncogénique de HTLV-I est associé à l'oncoprotéine virale Tax, qui est un activateur de la transcription de gènes viraux et cellulaires. De façon intéressante, les cellules infectées par HTLV-I et les cellules T Jurkat transfectées avec le gène codant la protéine Tax, expriment de façon constitutive le facteur régulateur d'Interféron-4 (IRF-4), membre de la famille IRF exprimé de façon spécifique dans les cellules lymphoïdes, indiquant que Tax pourrait constituer un activateur indirect du gène IRF-4. Une expression plus forte d'IRF-4 dans les cellules infectées par HTLV-I pourrait ainsi jouer un rôle dans la transformation cellulaire et par conséquent dans la leucémie à cellules T de l'adulte. Les études sur l'expression de la protéine IRF-4 ont révélé sa présence dans les cellules B, les cellules T activées, les macrophages et les cellules T infectées par le virus HTLV-I ou HTLV-II. Afin d'étudier la régulation de l'expression du gène IRF-4, l'analyse du promoteur de gène a été réalisée par détection d'empreinte génomique *in vivo* et par des expériences de retard de migration sur gel et a montré l'implication des membres des familles NF-κB et NF-AT dans la régulation de ce promoteur. L'utilisation d'IRF-4 comme «appât» dans la technique de criblage double hybride a mis en évidence une nouvelle interaction entre IRF-4 et la protéine FKBP52 (FK506 Binding Protein 52), une protéine de 59 kDa, membre de la famille des immunophilines et qui possède une activité

de peptidyl-prolyl isomérase (PPIase) ainsi que des fonctions similaires à celles des protéines chaperones. Il a ainsi été montré que l'inhibition de l'activité de fixation à l'ADN et du potentiel transactivateur d'IRF-4 par cette immunophiline, nécessite une activité enzymatique PPIase fonctionnelle. FKBP52 induirait un changement conformationnel d'IRF-4 caractérisé par une isomérisation cis-trans des résidus proline, qui interfère avec la capacité d'IRF-4 de lier l'ADN et d'activer la transcription. Ces études suggèrent un rôle directe de l'immunophiline FKBP52 dans la régulation de la fonction d'IRF-4 par une nouvelle modification post-traductionnelle. Plusieurs autres partenaires potentiels d'interaction avec IRF-4 ont été identifiés lors d'un second criblage double hybride, parmi lesquels eIF4γ1, c-src, Gi3, RhoANEF, RhoGDI et PP2A. L'interaction de ces protéines avec IRF-4 pourrait également être impliquée dans le développement des leucémies induites par HTLV-I. De nouveaux gènes cibles du facteur IRF-4, parmi lesquels RhoA, HSC70, RP-A, cyclin B1, PCNA, EB1, NIP3, MAPKK3, ont également été identifiés dans les cellules Jurkat exprimant IRF-4 de façon stable par criblage de macro-arrangements d'ADNc. La dérégulation de ces gènes par IRF-4 pourrait conduire à une augmentation de la prolifération et de l'activité cellulaire et une diminution de l'apoptose et de l'activité de réparation de l'ADN, qui sont des événements caractéristiques du développement des leucémies à cellules T induites par HTLV-I.

ACKNOWLEDGEMENTS

I would like to especially thank my supervisors: Dr. John Hiscott and Dr. Rongtuan Lin. Dr. Hiscott, thank you for your support, guidance and encouragement/pressure. I also want to acknowledge your attempts to make me call you by your first name. Maybe now that I have a Ph.D. I might just do so (probably not!). Thank you for making me shorten my already huge thesis! I wish you of course continued success and happiness. May the years to come bring you many conferences in warm and exotic places.

Thank you Dr. Lin for all your technical help and good advice. I can only hope one day to be as efficient as you. I will always remember waiting in line at the Louvre in Paris and how it only took us two hours to see "everything" in this museum. I also wish you all the best.

林博士，

言传身教

教益匪浅

师恩深重

铭记于心

Tell me, I will forget.
Show me, I may remember.
Involve me, and I will understand.

Thank you both for your teachings.
I understand.

During my Ph.D. studies I had the opportunity to meet warm and wonderful lab-mates. First I need to acknowledge Dr. Pierre Genin and Dr. Michèle Algarté for all their help with answering my numerous technical questions. Thank you Pierre for never giving blood because of your so-called hypertension for our monocyte extractions. I also need to thank Christophe "Coco" Heylbroeck (soon to be MD) for the heated discussions on religion and politics. I will miss all of you in the "French Connection". A special thanks to Sonia Sharma, Jill Bartlett and Dr. Nathalie Grandvaux: "The IRF-4 girls" or "The HTLV ladies". Sonia, it's been lots of fun working with you in the land of 531. I wish lots of success. Thank you Dr. Marc Servant for being a constant source of knowledge and encouragement. I wish you the best in your new career.

I am also thankful to all the other past and present members of my lab for useful discussions and making this lab an interesting place to work in: Dr. Louisa Petropoulos, Dr. Hannah Nguyen, Dr. Carmela DeLuca, Dr. Hakju Kwon, Dr. Cecile LePage, Tudor Baetu, Jing Lian, Benjamin Ten Oever, Eduardo Hernandez, Sonia Cisternas, Nadine Pelletier, Will Spencer, Delphine Duguay, Meztli Arguello, Maria Ricci, Dr. Marco Sgarbanti and Guillaume Martel. A special thanks to Benjamin Ten Oever and Maria Ricci for their help in proofreading this thesis. I would especially like to thank Mrs. Mary Wiebe for all her help and organization, without her we would be covered in a mountain of paperwork.

I am also very grateful to the McGill scholarship funds, the "Fonds pour les Chercheurs et à l'Aide à la Recherche" (FCAR/ FRSQ) as well as the Medical Research Council of Canada (MRC) for supporting me financially during my Ph.D. career.

Finally, I am also thankful to Drs. Antonis Koromilas, Stéphane Richard and Steve Michnick for their support and advice. I wish to thank Drs. Harinder Singh, Matthew Fenton and Tak Mak for various reagents they provided for my research. I am also indebted and extremely grateful to Dr. Bernard Turcotte, my Honor's project supervisor, who initiated me to scientific research.

PREFACE

In accordance to the "Guidelines for Thesis Preparation", the candidate has chosen to present the results of her research in classical form. A general introduction is presented in chapter I and appears in the following review articles:

1. **Nguyen, H., Hiscott, J. and Pitha, P.** 1997. The Growing Family of IRF Transcription Factors. *Cytokine & Growth Factor Review* **8**:293-312.

2. **Hiscott, J., Pitha, P., Genin, P., Nguyen, H., Heylbroeck, C., Mamane, Y., Algarte, M., Lin, R.** 1999. Triggering the Interferon Response: The Role of IRF-3 Transcription Factor. *Journal of Interferon and Cytokine Research* **19**:1-13.

3. **Mamane, Y., Heylbroeck, C., Genin, P., Algarte, M., Servant, M., LePage, C., DeLuca, C., Kwon, H., Lin, R., Hiscott, J.** 1999. Review: Interferon Regulatory Factors: The Next Generation. *Genes* **237**:1-14.

4. **Mamane, Y. and Hiscott, J.** 2000. Human T cell Leukemia Virus-I: Molecular Biology and Pathogenesis. Fundamentals of Molecular Virology, Editor N. Acheson, John Wiley and Son Publishers. NY, USA. (in press)

5. **Genin, P. and Hiscott, J.** 2000. Antiviral actions of Interferons. Fundamentals of Molecular Virology, Editor N. Acheson, John Wiley and Son Publishers. NY, USA. (in press)

The materials and methods used in this thesis are presented in chapter II. The results are described in chapters III to VI and appear in part in the following journal articles:

6. **Mamane, Y., Sharma, S., Petropoulos, L., Lin, R., Hiscott, J.** 2000. Posttranslational Regulation of IRF-4 Activity by the Immunophilin FKBP52. *Immunity* **12**:129-140.

7. **Sharma, S., Mamane, Y., Grandvaux, N., Bartlett, J., Petropoulos, L., Lin, R., and Hiscott, J.** 2000. Activation and Regulation of the IRF-4 Transcription Factor in Human T cell Leukemia Virus-I Infected Cells. *AIDS Research and Human Retroviruses* **16**: 1613-1622.

8. **Sharma, S., Grandvaux, N., Mamane, Y., Lin, R., and Hiscott, J.** 2001. Regulation of the IRF-4 promoter by NF-κB, NF-AT and Sp1 in HTLV-I-infected ATL-derived cells. Submitted to *Molecular and Cellular Biology*.

Chapter III presents the introductory studies leading to the basis for the work presented in Chapters IV, V and VI. Specific contributions to the work described in Chapters III and VI are as follows:

> Sonia Sharma was responsible for the IRF-4 expression studies using Western blot analysis, RT-PCR and Luciferase reporter assays. Sonia Sharma also participated in immunoprecipitation data in Chapter IV.
>
> Nathalie Grandvaux was responsible for genomic footprinting analysis of the IRF-4 promoter in Chapter III. She was also involved in the IRF-4 regulated gene studies presented in Chapter V.

I would like to acknowledge the participation of both Sonia Sharma and Nathalie Grandvaux for their dedication and effort in the work presented in Chapters III and VI.

The candidate was also involved in collaboration with other researchers in the laboratory, which resulted in the following publications:

9. **Lin, R., Mamane, Y. and Hiscott, J.** 1998. Structure-function analysis of IRF-3: Localization of the C-terminal transactivation and autoinhibition domains. *Molecular and Cellular Biology* **19**: 2465-2474.

10. **Genin, P., Mamane, Y., Kwon, H., Wainberg, MA. and Hiscott, J.** 1999. Differential regulation of CC chemokine gene expression in Human Immunodeficiency Virus-I- infected myeloid cells. *Virology* **261**:205-215.

11. **Lin, R., Genin, P., Mamane, Y., and Hiscott, J.** 2000. Selective DNA Binding and Association with CBP Co-activator Contributes to Differential Activation of Alpha/Beta Interferon Genes by Interferon Regulatory Factors 3 and 7. *Molecular and Cellular Biology* **20**: 6342-6353.

12. **Lin, R., Mamane, Y., and Hiscott, J.** 2000. Multiple Regulatory Domains Control IRF-7 Activity in Response to Virus Infection. *Journal of Biological Chemistry* **275**:34320-34327.

13. **Lin, R., Genin, P., Mamane, Y., Sgarbanti, M., Battistini, A., Harrington Jr., W., Barber, G., and Hiscott, J.** 2001. HHV-8 encoded vIRF-1 represses the interferon antiviral response by blocking IRF-3 recruitment of CBP/p300 Coactivators. *Oncogene* **20**: 800-811.

TABLE OF CONTENTS

CHAPTER III THE REGULATION OF IRF-4 EXPRESSION IN LYMPHOID CELLS AND IN HTLV-I INFECTED T CELLS

CHAPTER IV POSTTRANSLATIONAL REGULATION OF IRF-4 BY THE IMMUNOPHILIN FKBP52

LIST OF FIGURES AND TABLES

CHAPTER I

GENERAL INTRODUCTION

CHAPTER III

CHAPTER IV

CHAPTER V

CHAPTER VI

CHAPTER VII

LIST OF ABBREVIATIONS

-/-	knockout
aa	amino acids
AD	activation domain
ATF	activating transcription factor
ATL	adult T cell leukemia
bHLH	basic helix-loop-helix
CAT	chloramphenicol acetyl transferase
CBP	CREB binding protein
CDK	cyclin dependent kinase
CKI	CDK inhibitory protein
CKII	casein kinase II
CNS	central nervous system
CML	chronic myeloid leukemia
ConA	concavalinA
CRE	c-AMP response element
CREB	c-AMP-responsive element binding factor
CREM	c-AMP-responsive element modulator factor
CsA	cyclosporin A
CTL	cytotoxic T lymphocyte
Cyp	cyclophilin
DBD	DNA binding domain
DMSO	dimethyl sulfoxide
dsRNA	double stranded RNA
EBV	Epstein -Barr virus
EIF4	Elongation initiation factor 4
ELISA	Enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
ER	endoplasmic reticulum
FL	full-length
FKBP	FK506 binding protein
GAF	IFNgamma activated factor
GAS	IFNgamma activated site
GFP	green fluorescent protein
GC	germinal center
G-CSF	granulocyte-colony stimulating factor
GRB2	growth factor receptor-bound protein 2
HAM	HTLV-I associated myelopathies
HHV-8/ KSHSV	Human herpes virus-8/ Kaposi's Sarcoma
	Herpes simplex virus
HIV-1	Human immunodeficiency virus-1
HLA	human leucocyte antigen
HTLV-I	Human T cell leukemia/ lymphoma virus-1
HMG	high mobility group

HSC70	heat shock cognate 70-kDa protein
HSP	heat shock protein
IAD	IRF association domain
IB	immunoblotting
ICAM	intercellular adhesion molecule
ICS	interferon consensus site
ICSAT	interferon consensus binding protein in activated T-cells or in adult T-cell leukemia
ICSBP	interferon consensus binding protein/ IRF-8
IFN	interferon
IFNAR	interferon alpha/beta receptor (type I receptor)
IFNGR	interferon gamma receptor (type II receptor)
Ig	immunoglobulin
IgLλ	immunoglobulin λ light chain rearranged gene
IκB	inhibitory factor kappa B
IKK	IκB kinase
IL	interleukin
IL-2R	interleukin-2 receptor
iNOS	inducible nitric oxide synthetase
Iono	ionomycin
IP	immunoprecipitation
IRF	interferon regulatory factor
IRF-E	interferon regulatory factor-element
ISG-15	interferon stimulated gene-15
ISGF	interferon stimulated gene factor
ISRE	interferon stimulated response element
IV	intravenous
IVDU	intravenous drug user
JAK-STAT	Janus kinase-signal transducer and activator of transcription
LFA-1	lymphocyte function-associated 1
LPS	lipopolysaccharide
LSIRF	lymphoid specific IRF
LTR	long terminal repeat
MAD	mitotic arrest defective
MAPK	mitogen activated protein kinase
MAPKK3	mitogen activated protein kinase kinase 3
M-CSF	macrophage-colony stimulating factor
MHC	major histocompatibility complex
MSCV	murine stem cell virus
MUM1	multiple myeloma 1
NDV	Newcastle's disease virus
NES	nuclear export signal
Neo	neomycin, G418
NF-AT	nuclear factor of activated T cells
NF-κB	Nuclear factor-kappa B

NK	natural killer cells
NLS	nuclear localization sequence
NRE	negative regulatory domain
nt	nucleotide
PA	particle agglutination
PAGE	polyacrylamide gel electrophoresis
PBL	peripheral blood lymphocyte
PCNA	proliferating cyclic nuclear antigen
PEL	primary effusion lymphomas
Phe	phenylalanine
Pip	PU.1 interaction partner
PKA	protein kinaseA
PKC	protein kinase C
PKR	protein kinase R (dsRNA activated kinase)
PMA	phorbol 12-myristate 13-acetate
PML	promyelocytic leukemia
PP2A	protein phosphatase 2A
PPIase	peptidyl-prolyl isomerase
PRD	positive regulatory domain
Pro	proline
PTHrP	parathyroid-related protein
Puro	puromycin
RAR	retinoic acid receptor
Rb	retinoblastoma
RBC	red blood cells
RE	restriction enzyme
RhoA	ras homolog gene family member A
RhoANEF	RhoA guanine nucleotide exchange factor
RhoGDI	Rho GDP dissociation inhibitor
RNA Pol III	RNA polymerase III
RP-A	replication protein-A
RT-PCR	reverse transcription-polymerase chain reaction
RxRe	Rex responsive element
SDS	sodium dodecyl sulfate detergent
Ser	serine
SRE	serum responsive element
SRF	serum responsive factor
STLV	Simian T cell Leukemia Virus
TBP	TATA binding protein
TCR	T cell receptor
TGFβ	transforming growth factor β
Thr	threonine
TK	thymidine kinase
TNF	tumor necrosis factor
TPR	tetratricopeptide repeats
TSP	tropical spastic paraparesis

VCAM	vascular cell adhesion molecule
vIRF	viral IRF
WCE	whole cell extract

CHAPTER I

GENERAL INTRODUCTION

1. Human T cell Leukemia Virus-I

HTLV-I was the first human oncoretrovirus discovered in 1980 by Dr. Robert Gallo. It was isolated from a patient previously diagnosed with cutaneous T cell lymphoma (162, 171)(Figure 1). This condition was later characterized as Adult T cell Leukemia (ATL). In 1985, a group of West Indian patients with a neurological disease known as Tropical Spastic Paraparesis (TSP) were found to be seropositive for HTLV-I, and the disease was therefore referred to as HTLV-I associated myelopathies (HAM). Other diseases have also been associated with HTLV-I infection such as uveitis, arthropathy and dermatitis (221). HTLV-II was later identified also in a T cell line established from a patient with hairy-cell leukemia. To date, the role of HTLV-II in malignancy is still unclear.

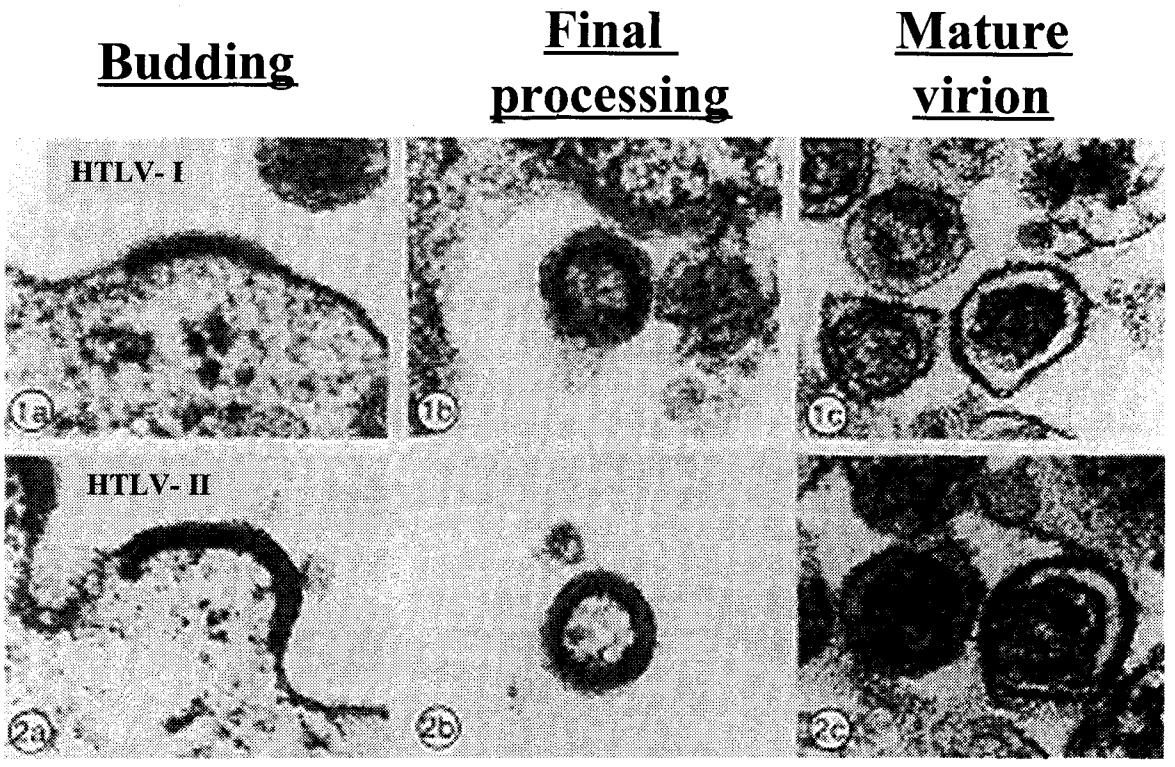
1.1 HTLV-I Gene Expression

1.1.1 Genome and structural proteins

The mature HTLV-I virion is spherical and enveloped with a diameter of 110-140 nm. It contains two identical single-stranded RNAs associated with nucleocapsid protein (p15 or NC). The capsid (p24 or CA) protein surrounds the RNAs and nucleocapsid complex as well as the integrase, reverse transcriptase, protease which cleaves viral structural proteins, and tRNApro which is required as a primer for reverse transcription. The matrix (p19 or MA) protein further surrounds the capsid. The whole structure is finally covered by the envelop which is constituted of lipid bilayer of cellular origins and of viral glycoproteins spikes (gp46 and gp21 cleaved from the gp62 precursor) (Figure 2) (55, 242).

Figure 1: HTLV-I and HTLV-II viruses seen by electron microscopy. (A) HTLV-I and HTLV-II are budding and maturing from an infected T lymphocyte (Cann Fields Virology, 1996) **(B)** HTLV-I is attacking a CD4+T cell (The Big Picture Book of Viruses by the Garry Lab, www.virology.net/big_virology/BVRetro.html).

A


Budding
Final processing
Mature virion
HTLV- I
1a
1b
1c
HTLV- II
2a
2b
2c

B

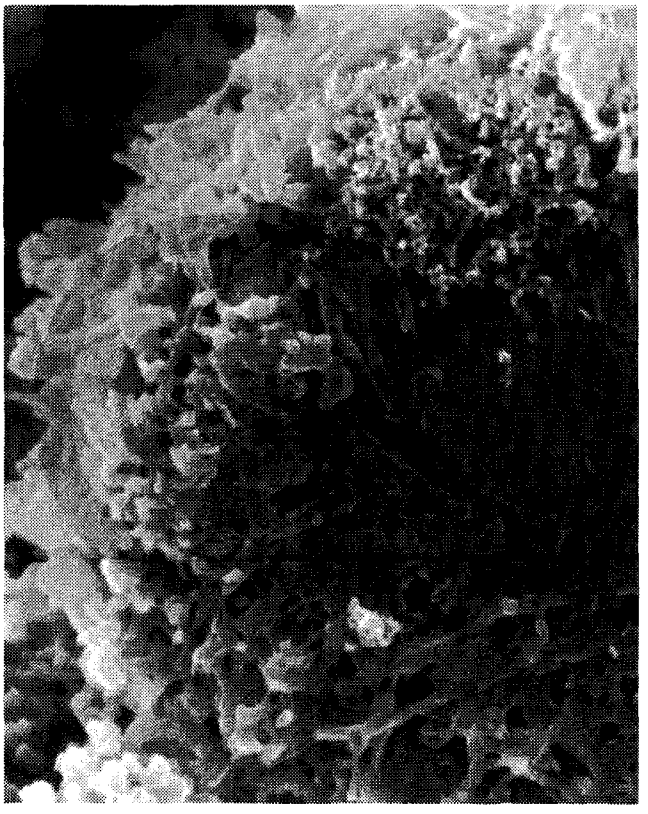
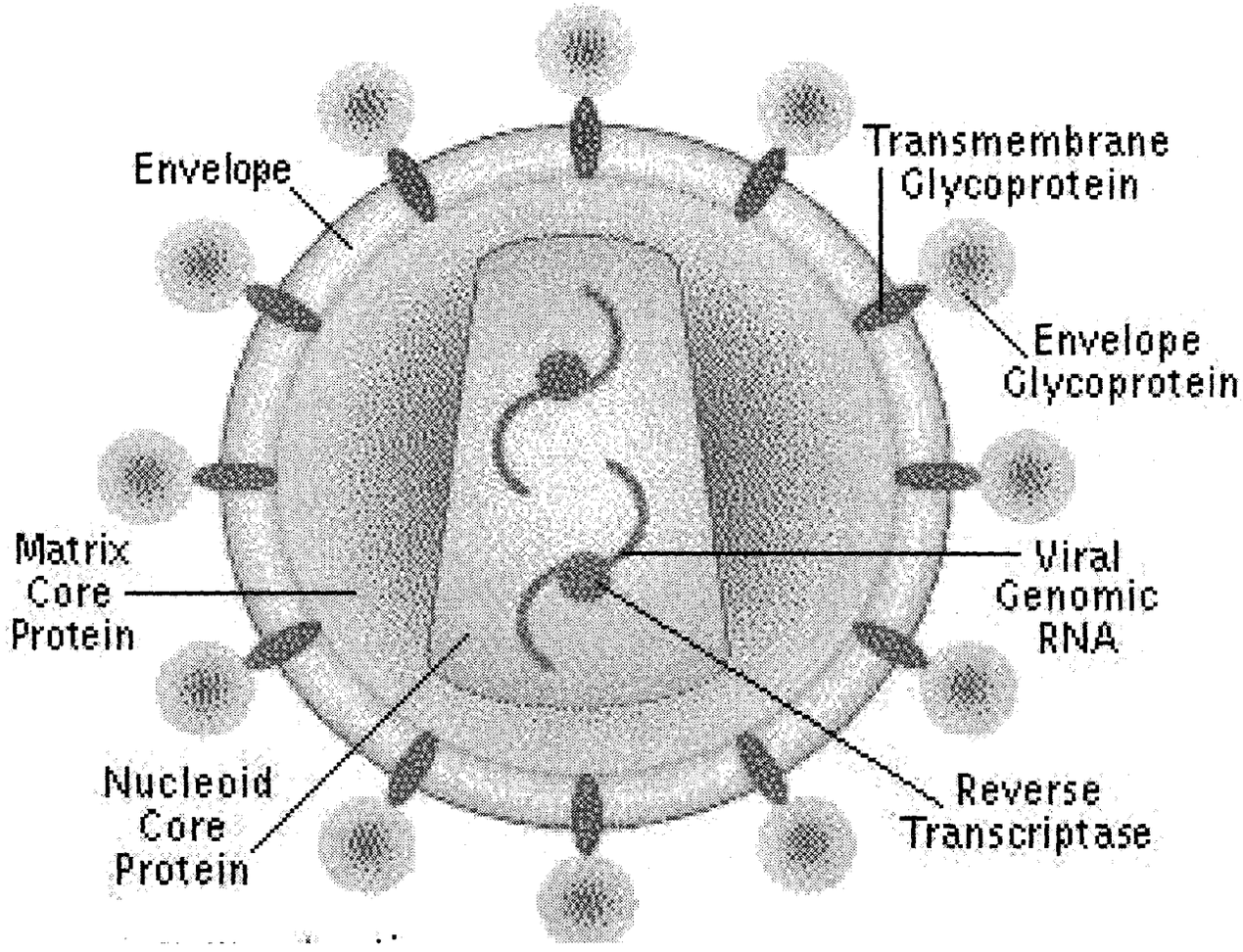


Figure 2: **HTLV-I structure schematic**. HTLV-I virion has a diameter of 110-140 nm and contains two identical single-stranded RNAs associated with nucleocapsid protein (p15 or NC). The capsid (p24 or CA) protein surrounds the RNAs and nucleocapsid complex as well as the integrase, reverse transcriptase, tRNAproline and the protease. The matrix (p19 or MA) protein further surrounds the capsid. The whole structure is finally covered by the envelope which is constituted of lipid bilayer of cellular origins and of viral glycoproteins spikes (gp46 and gp21 cleaved from the gp62 precursor). This structure is very similar to that of HIV-I. (Adapted from "The Big Picture Book of Viruses by the Garry Lab", www.virology.net/big_virology/BVRetro.html).


Envelope
Transmembrane Glycoprotein
Envelope Glycoprotein
Matrix Core Protein
Viral Genomic RNA
Nucleoid Core Protein
Reverse Transcriptase

The virus life cycle can be divided into two phases: receptor binding, fusion with cell membrane, uncoating of viral core, nuclear transport or viral core, reverse transcription and integration into cellular DNA. All these processes are accomplished in the absence of *de novo* viral gene expression by the viral structural proteins and the enzymatic proteins packaged in the virion. The second stage uses the host cellular machinery for transcription and translation of viral proteins, assembly and budding of immature virions from the cellular membrane and finally maturation (Figure 3) (55, 242).

The overall genetic structure of HTLV-I is similar to other retroviruses; it contains *gag*, *pol* and *env* genes that code for the viral matrix (MA), capsid (CA) and nucleocapsid (NC), enzymes such as reverse transcriptase, integrase, protease (PRO), and envelop protein consisting of surface glycoprotein (SU or gp46) and transmembrane protein (TM or gp21). In HTLV, a reading frame that spans the 3' part of the gag region and the 5' part of the pol region encodes the protease. Synthesis of the protease as part of the gag polyprotein precursor is accomplished by ribosomal frameshifting, a process in which the ribosome slips to the -1 position and changes the translational reading frame. A second ribosomal frameshift event is necessary to express the *pol* gene. In addition to viral structural genes, HTLV-I encodes in its 3' region (pX with four open reading frames (ORFs)) several unique regulatory genes: Tax, Rex, $p12^{I}$, p13 and p30 (55). To produce all viral proteins, three different species of mRNA are generated: full-sized genomic RNA encoding the Gag and Gag/ Pol fusion proteins, single-spliced mRNA encoding the Env proteins and double-spliced mRNA encoding the HTLV-I regulatory proteins (9, 20) (Figure 4).

Figure 3: **HTLV-I life cycle**. The life cycle of HTLV-I is very similar to other retroviruses such as HIV-I. The virus life cycle is described by the following events: receptor binding, fusion with cell membrane, uncoating of viral core, nuclear transport of viral core, reverse transcription of viral RNA into DNA and integration of proviral DNA into cellular DNA, transcription and translation of viral proteins, assembly and budding of immature virions from the cellular membrane and finally maturation.

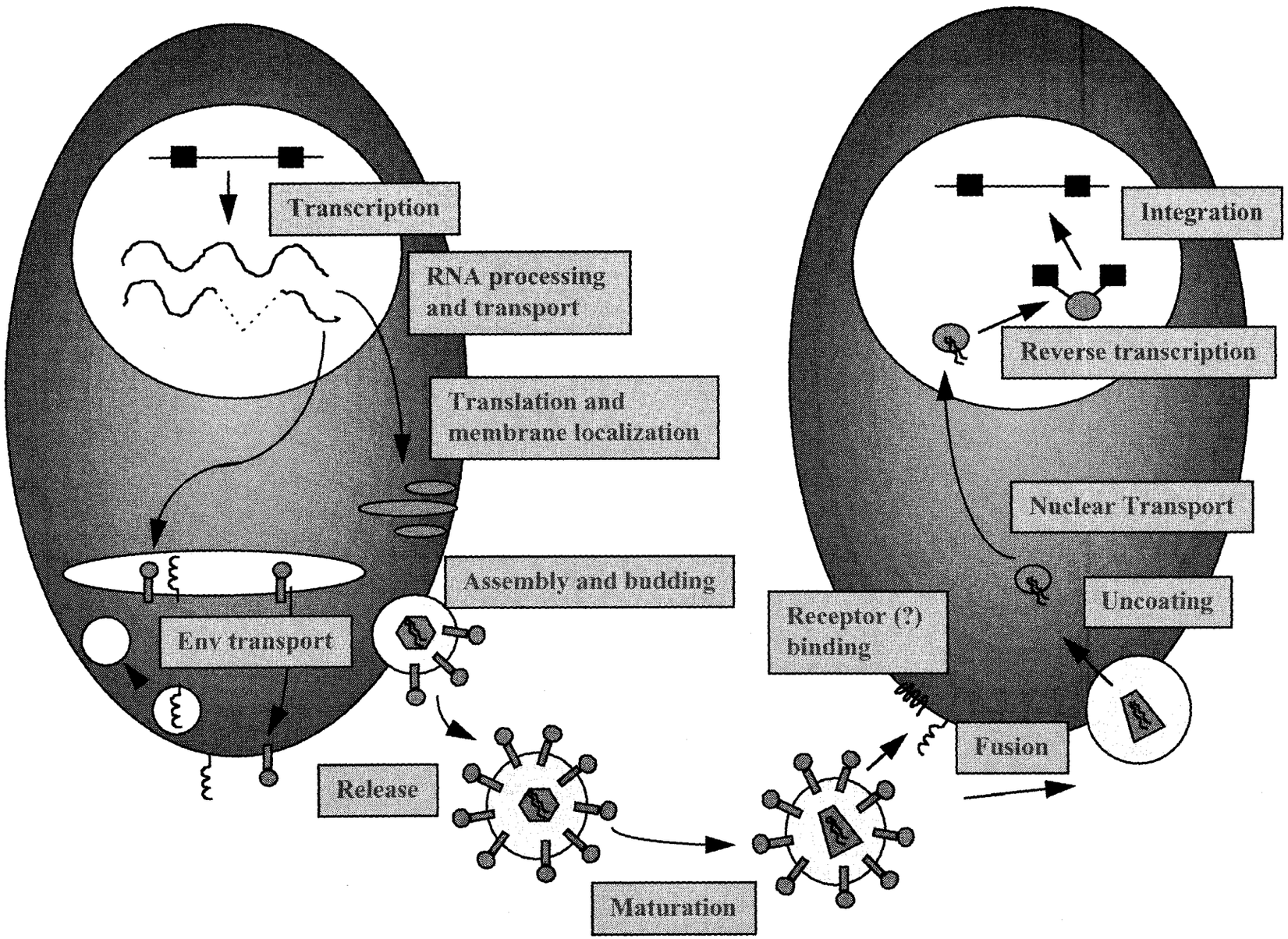
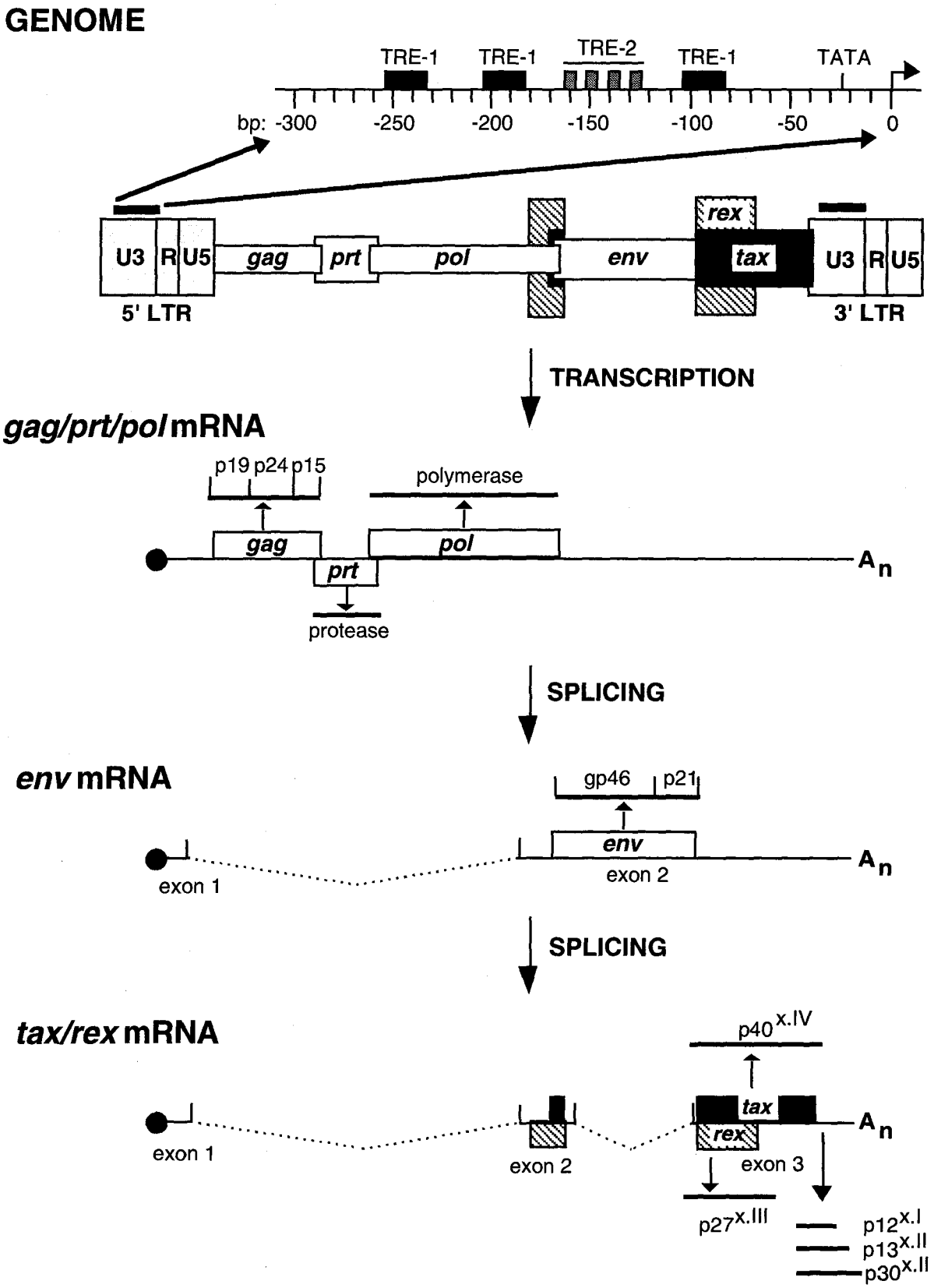

Transcription
RNA processing and transport
Translation and membrane localization
Assembly and budding
Env transport
Release
Maturation
Receptor (?) binding
Fusion
Uncoating
Nuclear Transport
Reverse transcription
Integration

Figure 4: HTLV-I genome and mRNAs coding for viral proteins. The integrated proviral form of HTLV-I is illustrated as well as the three size classes of mRNA required for complete synthesis of viral structural and regulatory proteins: 1) unspliced mRNA coding for the capsid, nucleocapsid, matrix, reverse transcriptase, integrase and protease; 2) single-spliced mRNA coding for the envelope proteins; and 3) double-spliced mRNA coding for the viral regulatory proteins Tax, Rex, p12, p13 and p30. In the U3 region of the HTLV-I LTR, which is expanded above the structure of the proviral DNA, three 21-bp repeats (TRE: Tax responsive element) are depicted which are required for Tax-mediated transactivation of the viral LTR. The TREs contain several consensus binding sites for the host cellular transcription factors of the CREB/ATF family.


GENOME
TRE-1
TRE-1
TRE-2
TRE-1
TATA
bp: -300
-250
-200
-150
-100
-50
0
U3
R
U5
gag
prt
pol
env
rex
tax
U3
R
U5
5' LTR
3' LTR
TRANSCRIPTION
gag/prt/pol mRNA
p19 p24 p15
polymerase
gag
pol
prt
protease
An
SPLICING
env mRNA
gp46
p21
env
exon 1
exon 2
An
SPLICING
tax/rex mRNA
p40x.IV
tax
rex
An
exon 1
exon 2
exon 3
p27x.III
p12x.I
p13x.II
p30x.II

1.1.2 Rex and RNA processing and transport

Rex is a 27 kDa nucleolar phosphoprotein produced from doubly spliced pX-ORF-III mRNA (Figure 4) and regulates viral RNA processing/transport, as well as the production of the three mRNA species. Rex acts posttranscriptionally to induce the cytoplasmic expression of singly spliced or unspliced mRNAs encoding viral structural proteins (103). Although the exact mechanism of action has not been elucidated, it is generally accepted that Rex shuttles between the nucleus and the cytoplasm in a process that involves the binding of Rex to specific regions of viral RNAs and their subsequent escort into the cytoplasm. Rex acts through *cis*-acting elements, referred to as Rex responsive elements (RxRe), within the 3' region of the HTLV-I Long Terminal Repeat (LTR). The RxRe is a highly stable 255-nucleotide stem-loop RNA structure that is a integral sequence element of all viral mRNAs. In addition to its role in mediating Rex responsiveness, the RxRe also plays a role in the 3' processing of viral transcripts. The viral polyadenylation signal is separated from the 3' cleavage site by the RxRe sequence, a distance that does not allow processing of the 3' ends of viral transcripts. Formation of the correct RxRe secondary structure, however, brings the two elements into close proximity with each other, thereby permitting correct polyadenylation (70). A cellular protein called Sam68 is believed to enhance Rex activities (176).

Rex possesses two important structural domains. A region in the amino terminus of the protein (aa 1 to 19) which is rich in basic amino acids functions as a nuclear and nucleolar targeting signal (NLS). A second essential region in the Rex protein, located between aa79 and aa99 contains a leucine-rich activation domain which functions as a nuclear export signal (NES). This region was shown to mediate binding to RxRe, enhancing the export of RxRe-containing mRNAs from the nucleus by binding to cellular cofactors. Rex binding to the RxRe also prevents splicing. In the absence of Rex, the unspliced and incompletely spliced viral transcripts are in the nucleus and either spliced to completion or subjected to degradation. When Rex is present, the transcripts are exported from the nucleus to the cytoplasm, where they are either translated or packaged as genomes into progeny virions (20, 221, 242).

HIV-I has an analogous regulatory protein, Rev, which binds to and mediates its actions through the Rev-responsive element (RRE) located within the *env* gene. The HTLV-I Rex protein is capable of binding to the HIV-I RRE and rescuing Rev-deficient HIV-I mutants. This indicates some functional conservation between HTLV Rex and HIV Rev although there is no sequence homology between the two proteins (20, 70).

Human CRM1 (hCRM1, exportin 1) was found to be a receptor for various NES sequences, including the activation domain of Rev. hCRM1 belongs to the importin β family, the members of which act as carriers to transport the proteins between the cytoplasm and the nucleus (234). hCRM1 also promotes the export of the Rex protein from the nucleus (70). Protein nuclear import is generally mediated by basic nuclear localization signals (NLS) that serve for the importin alpha (Imp alpha) NLS receptor. Imp alpha in turn binds to importin beta (Imp beta), which targets the resultant protein complex to the nucleus. Both HIV-I Rev and HTLV-I Rex fail to interact with Imp alpha but instead bind directly to Imp beta for import to the nucleus. The import of Rev and Rex are both dependent on the integrity of the Ran GTPase cycle.

1.1.3 Tax and transcription

The oncogenic potential of HTLV-I resides in the 353aa, 40kDa viral Tax oncoprotein encoded by the doubly spliced pX-ORF-IV mRNA (242) (Figure 4). Tax is a positive regulator of viral gene transcription that acts via 21bp repeats cAMP responsive element (CRE) within the HTLV LTR. Tax also stimulates the expression of cellular genes involved in early leukemogenic events, including cell cycle regulatory proteins, cytokines/growth factors (IL-2, IL-2R-α, GM-CSF, IL-6) and transcription factors (CREB, fos/jun, NF-κB/Rel, Egr/Krox, c-*myc*) (79). Since Tax is not a DNA binding protein *per se*, Tax transactivation occurs indirectly via physical interaction between Tax and host proteins that are targets for transactivation including: CREB, $p67^{SRF}$, Ets-1, ATF, and NF-κB/Rel proteins (figure 5) (79, 242, 246).

CREB/CREM/ATF. Tax is potent transactivator of gene expression from the HTLV-I LTR. Three imperfect 21bp repeats located in the U3 region of the HTLV-I LTR are cis-acting enhancer elements required for Tax activation. Each 21-bp repeat contains a central domain sharing homology with cAMP response element (CRE) (Figure 6). The cyclic AMP response element binding protein (CREB), cyclic AMP response element modulator (CREM) and the activating transcription factor (ATF) family of cellular transcription factors were shown to bind to the 21-bp repeats and to Tax. The Tax-ATF/CREB/CREM interaction is mediated through the basic leucine zipper domain found in these cellular transcription factors. The formation of the Tax-CREB-21-bp complex leads to the recruitment of the transcriptional coactivator CREB binding protein (CBP) (9, 64, 104). The G/C-rich sequences immediately flanking the CRE motif in the 21-bp repeat are crucial for stable Tax/CREB/ATF2/DNA ternary complexes. Surprisingly, Tax has been shown to contact directly to these G/C-rich sequences via interaction with the minor groove (110, 111).

Figure 5: **Schematic of Tax**. The oncogenic potential of HTLV-I resides in the 353aa, 40kDa viral Tax oncoprotein. Tax possesses at the N-terminus, a nuclear localization signal (NLS: black) although it is found in both the cytoplasm and the nucleus, and the CREB binding domain between aa2-59. A zinc finger domain (Zn: pink) is adjacent to the NLS and is believed to be involved in protein-protein interaction. Tax possesses to powerful activation domains (AD-I and AD-II). Within AD-I (blue), aa81-95 are involved in CBP binding and aa123-204 in dimerization and NF-κB activation. Within AD-II (silver), a region between aa284-325 HTLV-I transactivation. Tax does not have a DNA binding domain and therefor affects transcription by modulating other DNA binding-transcription factors. The function of a cluster of cysteine (C) and the alanines (AAA) residues found within the molecule remains unknown. Interspersed within the molecule are residues which are important for CREB mediated activity (3aa, 315aa, 317aa, 319aa and 325aa) or NF-κB mediated activity (113aa, 130aa, 160aa, 258aa).

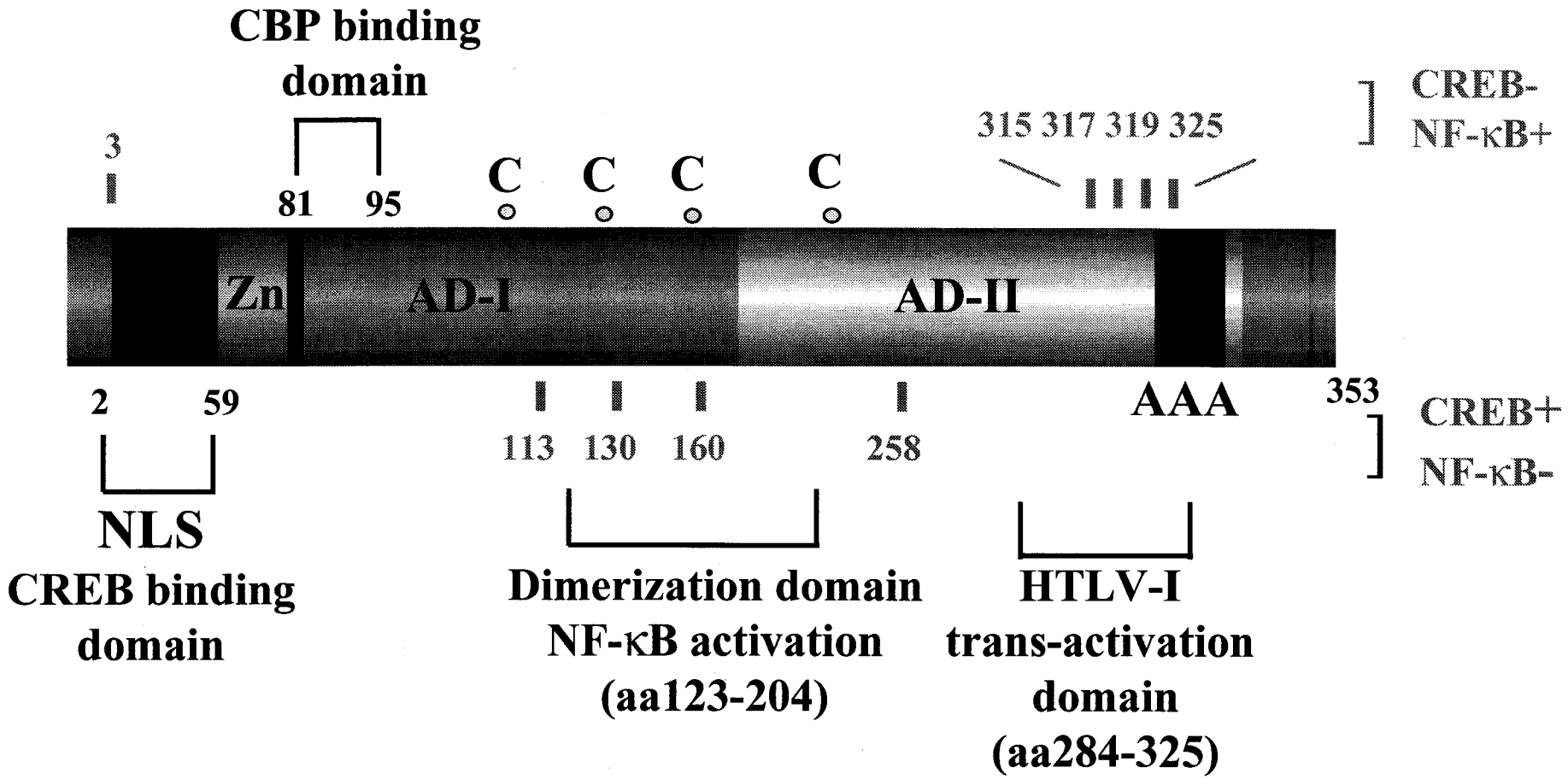
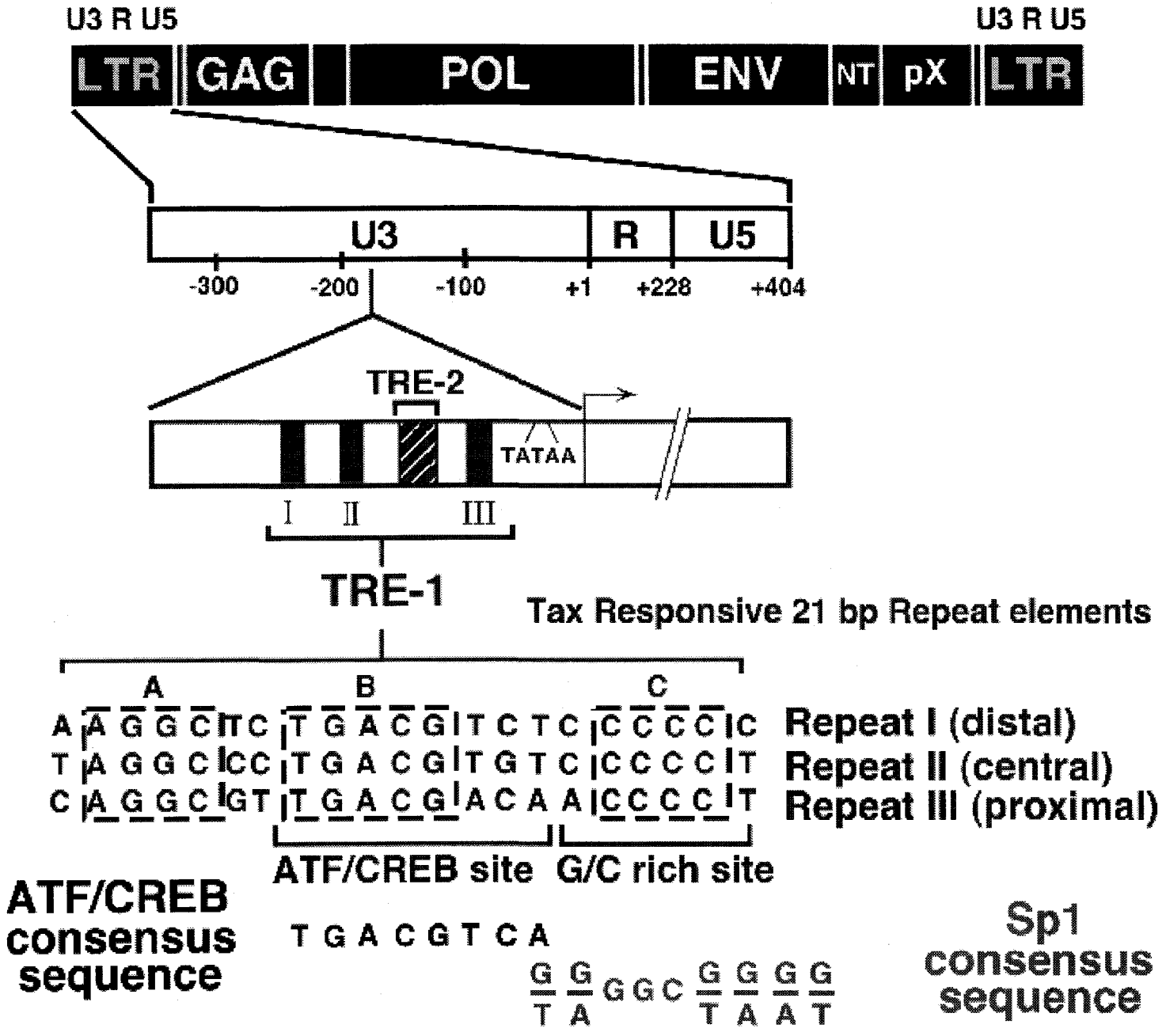

CBP binding domain
3
81
95
C
C
C
C
315 317 319 325
CREB-
NF-κB+
Zn
AD-I
AD-II
2
59
113
130
160
258
AAA
353
CREB+
NF-κB-
NLS
CREB binding domain
Dimerization domain
NF-κB activation
(aa123-204)
HTLV-I
trans-activation
domain
(aa284-325)

Figure 6: **Human T cell Leukemia Virus-I Long Terminal Repeats**. In the U3 region of the LTR which is expanded above the structure of the proviral DNA, three 21-bp repeats (TRE: Tax responsive element) are depicted which are required for Tax-mediated transactivation of the viral LTR. They are located at positions -251 to -231 (TRE-1.I; distal), -203 to -183 (TRE-1.II; central) and -103 to -83 (TRE-1.III; proximal). The TRE-1s contain several consensus binding sites for the host cellular transcription factors of the CREB/ATF family. Each 21-bp repeat of TRE-1 contains three completely conserved domains designated A, B and C from promoter distal end to promoter proximal end. These three domains comprise 13 nucleotides of the 21-bp repeat. Domain B contains the first five of eight bp of the cAMP element response (CRE: TGACGTCA) and is sufficient for the Tax-mediated transactivation in combination with either domain A or domain C. ATF-1, ATF-2, CREB and CREM bind to the three TRE-1. In addition, Fos and Jun can also bind TRE-1.2 (central) and Sp1 and Sp3 to TRE-1.III (proximal). A second TRE, TRE-2, is located between the promoter proximal repeat and the promoter central repeat an binds Myb, Ets, Sp1 and TIF-1 (Yao, 2000).

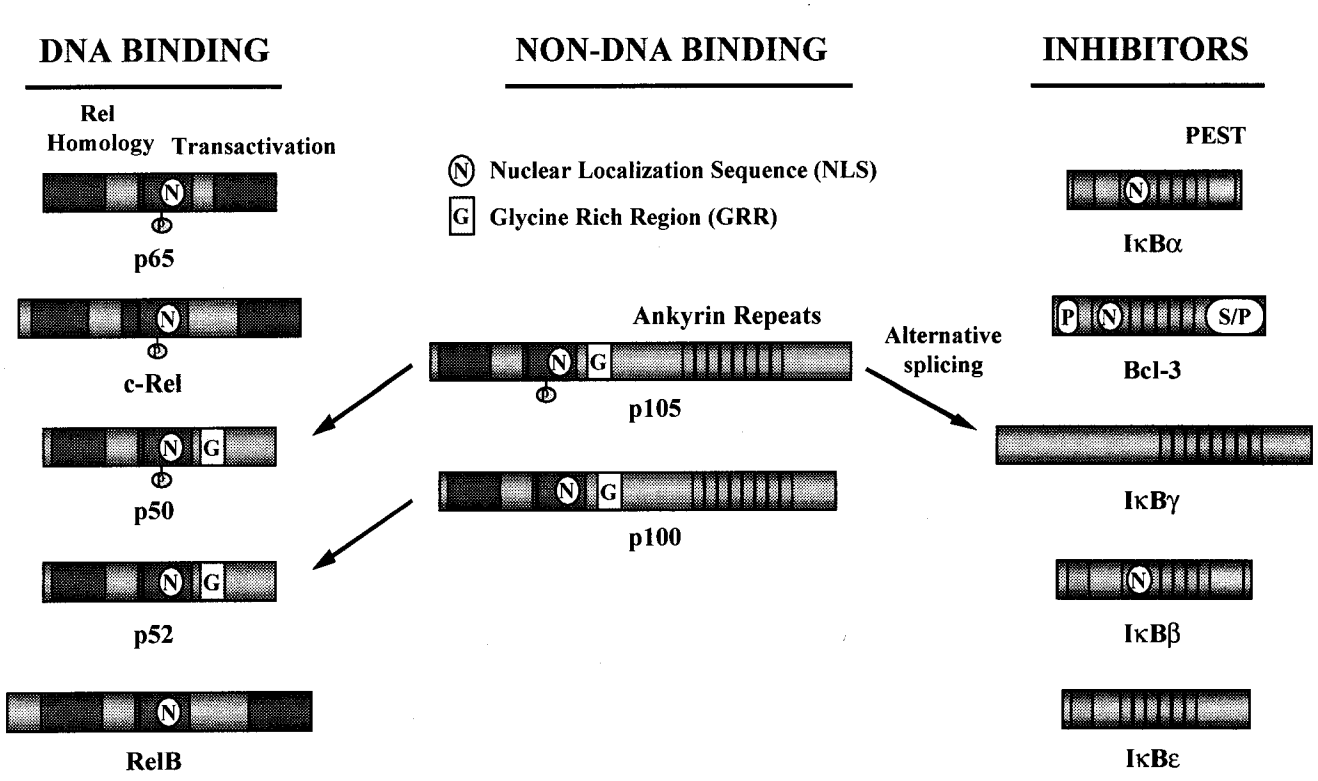

U3 R U5
U3 R U5
LTR
GAG
POL
ENV
NT
pX
LTR
U3
R
U5
-300
-200
-100
+1
+228
+404
TRE-2
TATAA
I
II
III
TRE-1
Tax Responsive 21 bp Repeat elements
A
B
C
A A G G C T C T G A C G T C T C C C C C
T A G G C C C T G A C G T G T C C C C T
C A G G C G T T G A C G A C A A C C C C T
Repeat I (distal)
Repeat II (central)
Repeat III (proximal)
ATF/CREB site
G/C rich site
ATF/CREB consensus sequence
T G A C G T C A
G/T G/A G G C G/T G/A G/A G/T
Sp1 consensus sequence

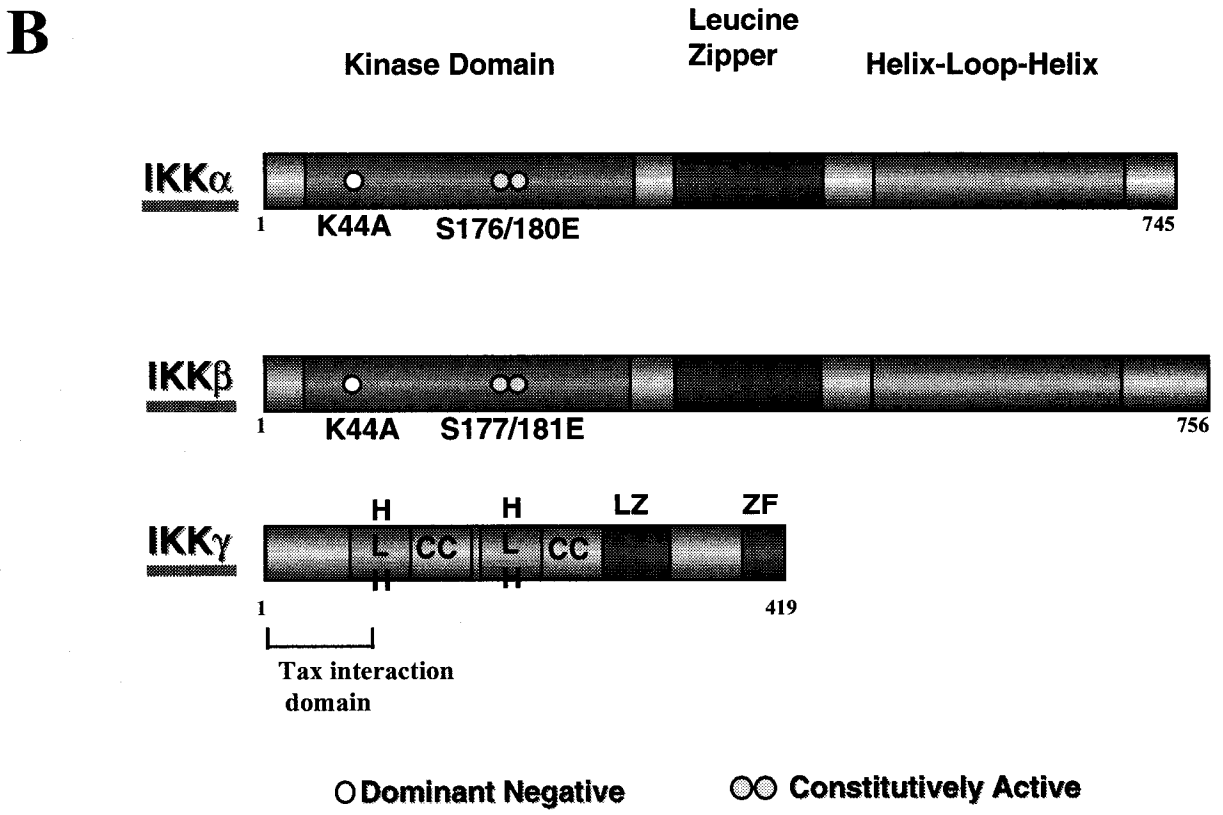
CBP. CBP is a 256kDa protein which is part of the RNA polymerase II holoenzyme complex. The recruitment of CBP promotes a strong transcriptional activation by integrating the transcriptional enhancer complex (Tax-CREB-CBP) with the general transcriptional machinery. This effect is mediated through chromatin remodeling induced by the histone acetylase activity of CBP. Therefore, Tax acts by increasing the binding of CREB to the viral CRE and recruiting the co-activator CBP to the viral promoter, leading to an increase in viral replication (9, 104). Tax directly contacts the KIX domain of CBP in order to anchor the coactivator to the CREB complexes at the promoter. It is also believed that Tax competes with other transcription factors for binding to CBP potentially leading to aberrant gene expression and transformation (241).

PCAF is another cellular coactivator with histone acetyltransferase activity. As with CBP/p300, PCAF is implicated in transcriptional control. Jiang et al. have demonstrated that PCAF is recruited by Tax *in vivo* to the HTLV-I LTR to activate transcription. Strikingly, the ability of PCAF to stimulate Tax transactivation is not solely dependent on the PCAF histone acetyltransferase activity in contrast to CBP/p300 (89). PCAF may help recruit other transcription factors to the viral LTR.

NF-κB. One of the important targets of interaction for Tax protein is the NF-κB/IκB transcription complex. The NF-κB/Rel transcription factors are a family of dimer forming proteins that bind to the consensus DNA sequence 5'-GGGANNYYCC-3', found in the promoter regions of cellular genes implicated in immunoregulation, cell growth control, lymphoid development and apoptosis (160). NF-κB is composed of homodimers and heterodimers of the Rel family of transcription factors which include p65 or RelA, c-Rel, RelB, as well as p50 and p52 which are processed amino-terminal fragments of the NF-κB precursors p105 and p100, respectively. NF-κB/Rel proteins are coupled to the inhibitory IκB molecules (IκBα or MAD3, IκBβ and IκBε) that are responsible for cytoplasmic retention of the latent NF-κB complex (Figure 7A).

Figure 7: NF-κB/ IκB family members. (A) NF-κB exists as homo and heterodimers of related proteins, which share a conserved DNA-binding and dimerization domain, called the Rel homology (RH: blue) domain. The major NF-κB complexes are composed of either p65 (RelA) and p50 or c-Rel and p50 subunits. In unstimulated cells, the NF-κB dimers are maintained in an inactive form in the cytoplasm by the inhibitory proteins IκB which include IκBα, IκBβ and IκBε. All NF-κB proteins share the Rel homology domain (RHD: blue). Within the RHD is the DNA binding domain (DBD: green) and a region essential for NF-κB dimer formation. The NF-κB1/p105 and NF-κB2/p100 contain a long C-terminal domain with seven ankyrin repeats (pink) and a PEST region (yellow). Cleavage of the C-terminal region of p105 and p100 occurs at a site near a flexible glycine-rich hinge (G: light yellow) to generate the transcription factors NF-κB1/p50 and NF-κB2/p52, respectively. All NF-κB/Rel members contain a nuclear localization signal (N) flanking the 3' region of the RHD. c-Rel, p65, and RelB contain transcriptional transactivation ability, mediated through transactivation domain (red). Some of the inhibitors such as IκBα, IκBβ and BCL-3 also contain a nuclear localization signal (N). BCL-3 also possesses proline-rich (P) as well as serine-and proline-rich domains (S/P). **(B)** Signal-mediated phosphorylation of IκBα is mediated by a multi-protein complex called the IκB Kinase complex composed of three identified subunits. Two of these polypeptides, IKKα and IKKβ (also known as IKK1 and IKK2) are the catalytic subunits of the kinase and phosphorylate IκBα and IκBβ. The third component of the IKK complex is IKKγ. IKKα and IKKβ possess a N-terminal kinase domain (blue), followed by a leucine zipper motif (red) and a C-terminal helix-loop-helix domain (pink). Certain mutations within their kinase domain can lead to dominant negative effect (white circle) or constitutively active kinase activity (yellow circle). IKKγ is a smaller protein (419aa) with no kinase activity. It possesses two helix-loop-helix domains (HLH; pink), two coiled-coiled domains (CC; yellow), a leucine zipper (LZ, red) and zinc finger motif (ZF, purple). Recent structure-function analysis of IKKγ revealed that the N-terminal domain is responsible for interactions with IKKα and IKKβ, the central domain (aa 201-319) constitutes the IKKγ self-association domain and mediates the RIP interaction whereas the amino-terminus is involved in Tax interaction (Ye et al., 2000).


A
DNA BINDING
NON-DNA BINDING
INHIBITORS
Rel
Homology
Transactivation
Nuclear Localization Sequence (NLS)
Glycine Rich Region (GRR)
PEST
p65
IκBα
Ankyrin Repeats
Alternative
splicing
c-Rel
Bcl-3
p105
p50
IκBγ
p100
p52
IκBβ
RelB
IκBε

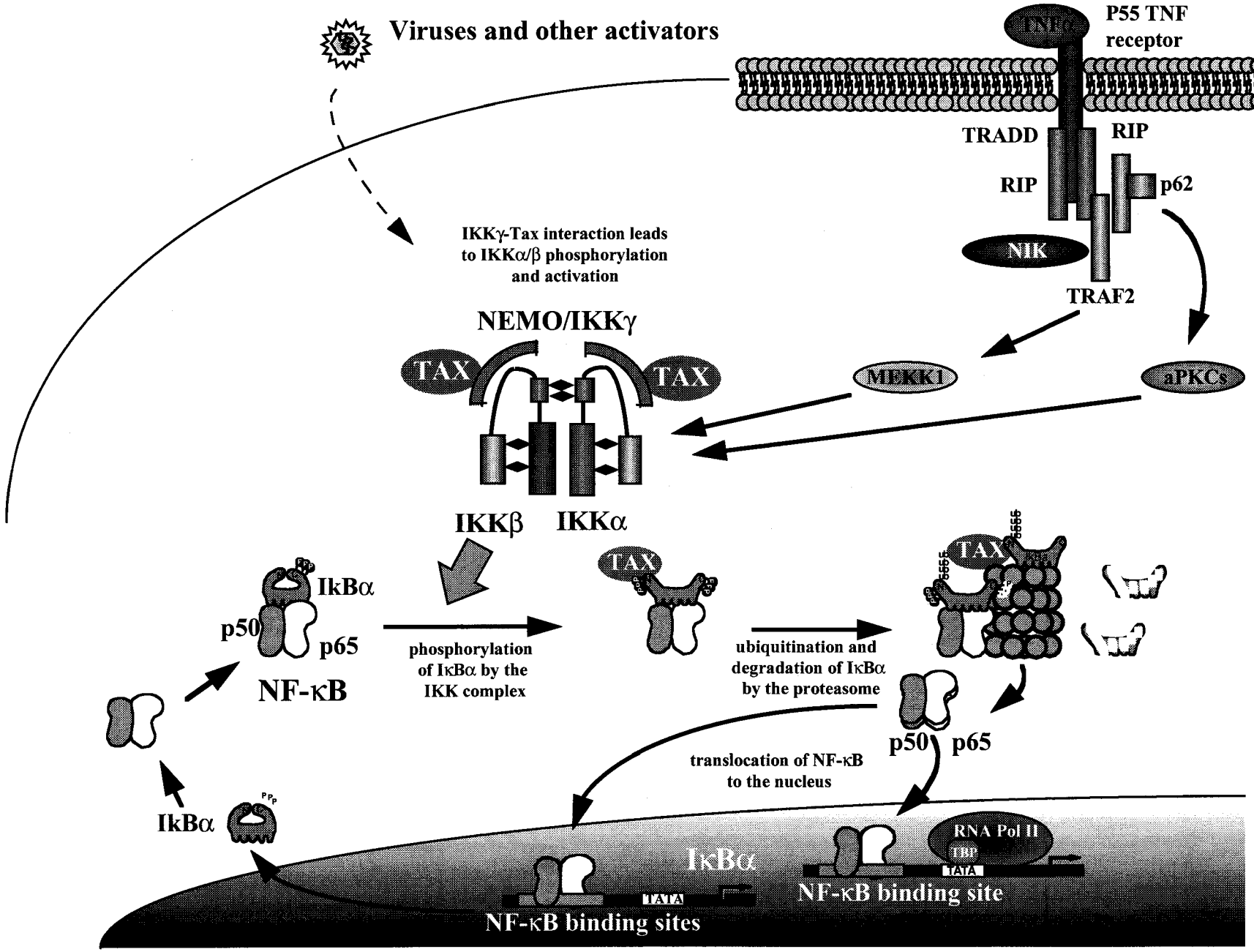

B
Kinase Domain
Leucine
Zipper
Helix-Loop-Helix
IKKα
1
K44A
S176/180E
745
IKKβ
1
K44A
S177/181E
756
IKKγ
H
H
LZ
ZF
L
CC
L
CC
1
419
Tax interaction
domain
Dominant Negative
Constitutively Active

Multiple inducers (cytokines, virus infection, and mitogens) lead to the phosphorylation at serine residues 32 and 36; phosphorylation at these sites represents a signal for subsequent ubiquitination and degradation of IκBα. Loss of IκB permits NF-κB/Rel dimer translocation to the nucleus and target gene activation (160, 205).

IKK complex. Recently, a novel kinase complex - the IκB kinase (IKK) complex - involved in IκBα phosphorylation at Ser-32/Ser-36 was identified (94, 95). This kinase complex of 700-900 kDa possesses two catalytic subunits (IKKα and IKKβ) as well as a regulatory subunit protein (NEMO/IKKγ) (Figure 7B) (26, 94, 237). This kinase complex is itself activated by phosphorylation through multiple upstream activators such as MEKK1 and atypical PKCs (222, 244) (Figure 8). The third component of the IKK complex, IKKγ does not exhibit kinase activity, but rather constitutes a regulatory subunit (136, 181, 240). Interestingly, a physical interaction of IKKγ with HTLV-1 Tax oncoprotein was reported and is involved in constitutively activating IKK catalytic subunits, thus bypassing the requirement for engagement of upstream activators (26, 74, 75, 91).

Three recent observations indicated that the cytokine-responsive IKKs are the primary cellular targets of Tax in the NF-κB pathway: First, kinase-inactive forms of IKKα and IKKβ interfered with Tax-mediated NF-κB activation. Second, mutants of Tax that were unable to interact with IKKs also prevented Tax-mediated activation of endogenous IKK and NF-κB. Third, Tax associated with constitutively active IKKs to form higher order complexes that were resistant to dissociation *in vitro* (26). Thus, Tax interferes with the NF-κB pathway via direct Tax/IKK interaction, which leads sequentially to chronic IKK activation, continuous IκB turnover, and persistent NF-κB activation.

Figure 8: **NF-κB signaling pathway and HTLV-I Tax**. After stimulation by multiple activators (LPS, TNFα, PMA, viruses, mitogens), the IKK complex composed of IKKα, IKKβ and NEMO/IKKγ is activated and phosphorylates IκBα at Ser-32 and Ser-36. The phosphorylated IκBα is subsequently ubiquitinated on lysine residues 21 and 22 by the E3 ubiquitin ligase and targeted for degradation by the proteasome. NF-κB dimers (p50/RelA, p50/p50) are then free to translocate to the nucleus, bind DNA and activate target gene transcription. In the case of TNFα stimulation through the TNF receptor, MEKK1 and atypical PKCs are involved in the activation of the IKK complex in the context of Tax. Cumulative molecular studies indicate that Tax expression in HTLV-I infected cells targets the NF-κB/IκB transcription complex, as well as other host proteins, at multiple regulatory levels via protein-protein interactions: Tax 1) stimulates IκBα phosphorylation via interaction with and constitutive activation of the IKKs. Tax physically associates with IKKγ in order to recruit another kinase to phosphorylate IKKα and IKKβ; 2) physically associates with NF-κB2 (p100), IκB and other family members and directs these proteins to the proteasome for degradation; and 3) indirectly mediates the transcriptional activation of c-Rel, NF-κB2 and IκBα genes, leading to constitutive NF-κB activity and aberrant gene expression.


Viruses and other activators
TNFα
P55 TNF receptor
TRADD
RIP
RIP
p62
NIK
TRAF2
IKKγ-Tax interaction leads to IKKα/β phosphorylation and activation
NEMO/IKKγ
TAX
TAX
MEKK1
aPKCs
IKKβ
IKKα
IkBα
p50
p65
NF-κB
phosphorylation of IκBα by the IKK complex
TAX
TAX
ubiquitination and degradation of IκBα by the proteasome
p50
p65
translocation of NF-κB to the nucleus
IkBα
IκBα
RNA Pol II
TBP
TATA
NF-κB binding site
TATA
NF-κB binding sites

This association of IKK with Tax was shown to be mediated specifically through the IKKγ component of the IKK complex (26, 75, 91). Genetic complementation analyses with IKKγ-deficient rat fibroblast cells that failed to activate NF-κB in the presence of Tax (240) suggested that IKKγ was required for the assembly of the Tax-responsive IKK complex. In human T cells, interference with IKKγ expression by antisense expression inhibits Tax-mediated activation of NF-κB but not of CREB/ATF (26). Harhaj et al. isolated mutant T cell lines deficient in IKKγ expression that were blocked in NF-κB activation when stimulated by both T-cell co-stimulatory signals and HTLV-I Tax. This signaling defect was rescued by expression of exogenous IKKγ (74). Although significant IκB kinase activity can be reconstituted *in vivo* by overexpressing Tax, IKKγ, and either IKKα or IKKβ, interaction of Tax with IKKα and IKKβ does not induce kinase activity in the absence of IKKγ expression, demonstrating that IKKγ functions as a molecular adaptor and provides a site for Tax binding in the assembly of Tax/IKK complexes (91). Interactions with Tax require sequences at the C-terminal region of IKKγ; by contrast, the N-terminal region of this protein (amino acids 1-120) is required for the formation of a stable and active IKK complex (26, 75).

Cumulative molecular studies indicate that Tax expression in HTLV-I infected cells targets the NF-κB/IκB transcription complex, as well as other host proteins, at multiple regulatory levels via protein-protein interactions: 1) Tax stimulates IκBα phosphorylation via interaction with and constitutive activation of the IKKs. Tax physically associates with IKKγ in order to recruit another kinase to phosphorylate IKKα and IKKβ; 2) Tax physically associates with NF-κB2 (p100), IκB and other family members and directs these proteins to the proteasome for degradation; and 3) Tax indirectly mediates the transcriptional activation of c-Rel, NF-κB2 and IκBα genes, leading to constitutive NF-κB activity and aberrant gene expression (Table 1) (52, 79, 205, 238).

Table 1: Tax regulated genes. Genes transactivated or transrepressed by Tax and the cellular transcription factors involved. NF-κB: Nuclear Factor-κB, ATF/CREB: Activation Transcription Factor/cAMP Responsive Element Binding, SRF: Serum Response Factor, bHLH: basic Helix-Loop-Helix, EGR-1: Early Growth Response-1.

Genes induced by Tax

Transcription factors

1) Cytokines and receptors

IL-1, IL-2, IL-6, IL-8, IL-15, TNFα, TNFβ, IFNβ, GM-CSF	NF-κB
IL-2Rα , Immunoglobulin κ light chain, T cell receptor, βT cell receptor α chain, MHC class I	NF-κB

2) Transcription factors and protooncogenes

c-fos, erg-1,	SRF
fra-1	NF-κB
c-myc, c-rel	EGR-1
c-sis	

3) Cell adhesion molecules

gp34	NF-κB
Endothelial leukocyte adhesion molecule-I (ELAM-I)	NF-κB
Intercellular cell adhesion molecule-I (ICAM-I)	NF-κB
Vascular cell adhesion molecule I (VCAM-I)	NF-κB
Vimentin	?
OX-40	

4) Inhibitors of apoptosis: A20

NF-κB

5) Inhibitors of nucleotide excision repair: PCNA

?

6) Viruses

CMV	NF-κB
HIV-I	NF-κB
HTLV-I	CREB/ATF

7) Acute phase proteins

angiotensinogen	NF-κB
Serum amyloid A precursor	NF-κB
complement factors	NF-κB

Genes repressed by Tax

1) DNA repair enzymes: β–polymerase

bHLH

2) Pro-apoptotic factors: *bax, p53*

bHLH

3) Protein tyrosine kinases

lck	bHLH
zap-70	?

SRF. Serum response factors (SRF) are also involved in Tax-mediated transactivation. SRF bind to serum responsive element (SRE) within promoters of the *c-fos*, *egr-1* and *fra-1* genes. Tax binds specifically to $p67^{SRF}$ and enhances the transcription of these protooncogenes which are involved in cell growth and proliferation (20, 205).

NF-AT. NF-AT represents a family of enhancer binding proteins that participate in the regulation of a large number of cytokine genes. This family consists of four classical members NFAT1 (p, c2), NFAT2 (c, c1), NFAT3 (c4) and NFAT4 (x, c3). A fifth member has been recently added, NFAT5/TonEBP (98). In normal T cells, the NF-AT proteins are sequestered in the cytoplasm as hyperphosphorylated inactive precursors. Mitogenic T cell stimuli results in rapid dephosphorylation of NF-ATs by the phosphatase calcineurin. Dephosphorylation of NF-ATs leads to nuclear translocation and activation of their DNA binding activity.

HTLV-I Tax transactivates the IL-2 gene through an enhancer termed CD28 responsive element (CD28RE). This activation by Tax is partially mediated by NFAT1. HTLV-I infected T cells were also shown to possess constitutively dephosphorylated and activated NFAT1 (63). This factor is also involved in Tax-mediated as Fas ligand promoter transactivation in HTLV-I infected T cells which may serve a mechanism of viral pathogenesis (178).

SMAD. HTLV-I infected cells have been found to be resistant to growth inhibition by transforming growth factor β (TGFβ). Tax can perturb Smad-dependent TGFβ signaling even though Tax does not interact with any Smads. It seems that Tax interferes with the recruitment of CBP/p300 into the transcription initiation complexes on TGFβ-responsive elements (143). This novel function of Tax as a repressor of TGFβ signaling may contribute to HTLV-I-induced leukemogenesis.

AP-1. Tax can also activate AP-1 family members: cJun, JunB, Fra-1 and cFos DNA binding and transactivation activities were increased in the presence of Tax (86). AP-

1 signaling is crucial in T cell activation, proliferation and transformation. Thus, AP-1 is also a candidate involved in dysregulated phenotypes seen in HTLV-I-infected T cells.

Nuclear receptors. The nuclear receptor superfamily includes the steroids, thyroids, retinoids, and peroxisome proliferator-activated receptors. Steroids and retinoids are widely reported to exert antiproliferative and anti-inflammatory effects in T lymphocytes. Doucas et al. demonstrated that Tax is a potent repressor of steroids and retinoids receptor transcription. PML expression can restore nuclear receptor signaling by blocking the Tax effect. Inhibition of nuclear receptor signaling is therefore potentially involved in uncontrolled proliferation of HTLV-I-infected T cells (35).

1.1.4 Other viral regulatory proteins

$p12^I$ or Rof. It is small hydrophobic protein encoded by the 3' end of the HTLV-I genome. It is encoded by the singly or doubly spliced pX-ORF-I mRNA (242). $p12^I$ binds specifically to both the β and γc chains of the IL-2R, an interaction that stabilizes the immature form of the IL-2R β and γc chains and decreases its cell surface expression. $p12^I$ may therefore interfere with the normal IL-2 dependent proliferation by inducing heterodimerization of the IL-2R β and γc chains which is sufficient for adequate IL-2 signaling. The $p12^I$ /IL-2R β and γc chains interaction may have important implications in the immunosuppressive effect of HTLV-I *in vivo* as well as in the IL-2-independent HTLV-I-mediated T cell proliferation. $p12^I$ also interacts with the 16-kDa component of the H+ vacuolar ATPase and cooperates with the bovine papillomavirus E5 protein in cell transformation (46, 147). $p12^I$ can also associate with MHC class-I molecules and cause their intracellular degradation by the proteasome. Chronic production of this protein could protect infected cells from being destroyed by cytotoxic T cells and ensure virus propagation *in vivo* (168).

$p13^{II}$ or Tof. It is encoded only by the singly spliced pX-ORF-II mRNA (242). p13 is found in the mitochondria and its expression leads to modification in mitochondria morphology and architecture. Current studies are trying to determine the effect of p13 on apoptosis, senescence, oxidative stress and ATP production. A yeast two-hybrid with p13 revealed two interacting clones: a member of the nucleoside monophosphate kinase superfamily and the actin-binding protein 280 (82). Functional roles for such interactions have yet to be elucidated.

$p30^{II}$. It is encoded only by the doubly spliced pX-ORF-II mRNA (242). p30 is found in the nucleolus of transfected cells and is believed to act as a transcription factor (55). Abrogation of p12, p13 and p30 expression in the HTLV-I genome had no effect on viral replication or immortalization of primary lymphocytes (179). The function of these viral proteins is still controversial but they are believed to play a role in infection *in vivo*.

1.2 HTLV-I induced transformation

HTLV can infect different cell types - T cells, B cells, macrophages, immature bone marrow cells and neural cells from different species but HTLV can only transform human T cells *in vivo*. Several observations show the importance of Tax in transformation: 1) the Tax coding region is always maintained in leukemic cells although other viral genes are frequently found deleted; 2) Tax immortalizes normal human T cells; 3) Tax transforms a rodent fibroblastic cell line and 4) Tax transgenic mice develop tumors (188, 247).

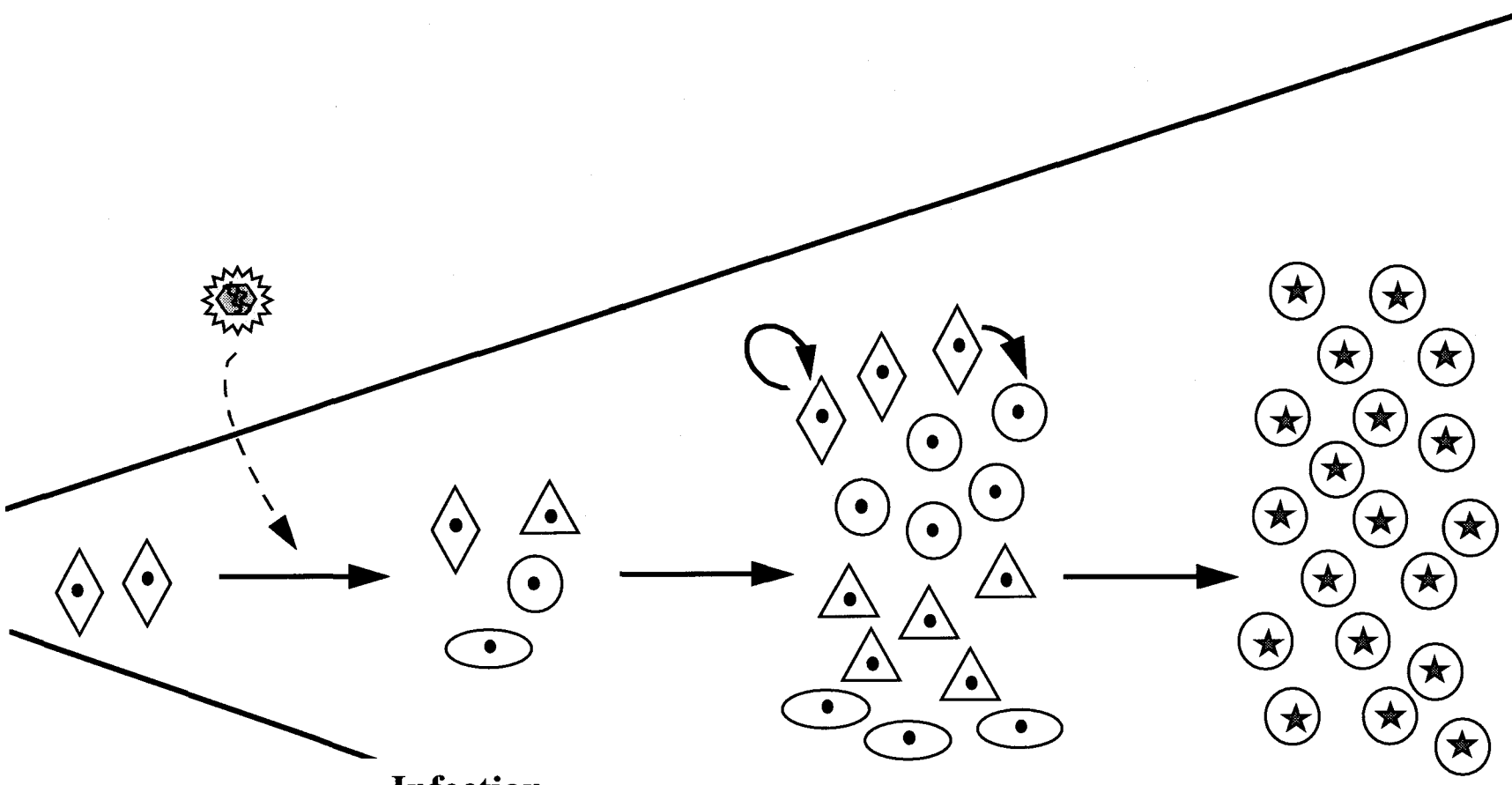
The precise mechanisms by which HTLV-I transforms human T cells remain unclear but the viral transactivator Tax appears to play a critical role in leukemogenesis: 1) Tax leads to T cell activation and proliferation by generating an IL-2 -IL-2 receptor autocrine loop; 2) Tax induces the G1 to S phase transition in the cell cycle; 3) Tax physically associates with cellular transcription factors to activate gene transcription and 4) Tax upregulates RNA polymerase III (247).

1.2.1 IL-2 autocrine loop

The human T cell lymphotropic virus (HTLV-I) is the etiologic agent of adult T cell leukemia (ATL), an aggressive and fatal leukemia of CD4+ T lymphocytes (20) and is also associated with a neurological demyelinating disease, tropical spastic paraparesis (TSP). ATL and TSP are geographically localized to regions of the world where HTLV infection is endemic. Based on the phenotype of ATL cells *in vivo* and HTLV-I transformed T cell lines, infection of CD4+ T cells initiates a multi-step oncogenic process that is characterized by an early proliferation of HTLV-I infected T cells, resulting in polyclonal expansion of infected cells. Increased proliferation of HTLV-I infected T cells in the early stages of infection has been shown to be dependent on the production of interleukin 2 (IL-2) or T cell growth factor. High levels of interleukin-2 production and upregulation of IL-2 receptor alpha expression result in an autocrine stimulatory loop that drives the early stages of T cell proliferation. Over a period of decades *in vivo*, a growth factor independent, monoclonal population of leukemic T cells emerges, likely as a consequence of the rapid growth of a single leukemic clone bearing multiple mutations in oncogenes and/or tumour suppressor genes (Figure 9) (221).

Growth factors are necessary to traverse T cells from G1 to S phase of the cell cycle. In HTLV-I infection, Tax appears to act as a mitogen by inducing the expression of early mitogenic genes and the IL-2Rα chain, a component of the high-affinity IL-2 receptor (IL-2R). In addition Tax transactivates IL-2 gene expression and triggers an autocrine/paracrine mechanism, which leads to continuous T cell proliferation. This growth phase is characterized as IL-2-dependent. Over time, T cell proliferation becomes IL-2 independent by constitutively activating the downstream signaling pathway - the Jak-Stat cascade more specifically Jak1, Jak3, Stat3 and Stat5 (20, 208, 221). Targeting the Jak-Stat pathway could prove to be an attractive anti-leukemic therapy (119).

Figure 9: **Leukemic transformation of T cells by HTLV-I.** HTLV-I induces T cell proliferation and leukemogenesis. HTLV-I infects CD4+ T cells and causes at first a polyclonal/ IL-2-dependent growth phase. After decades of proliferation, a monoclonal T cell population of leukemic T cells emerges and leads to Adult T cell Leukemia.



-Infection

-Polyclonal T cell proliferation
-IL-2 autocrine/ paracrine loop
-IL-2 dep. growth phase

-Emergence of monoclonal leukemic T cell
-Accumulation of mutations
-IL-2 indep. growth phase

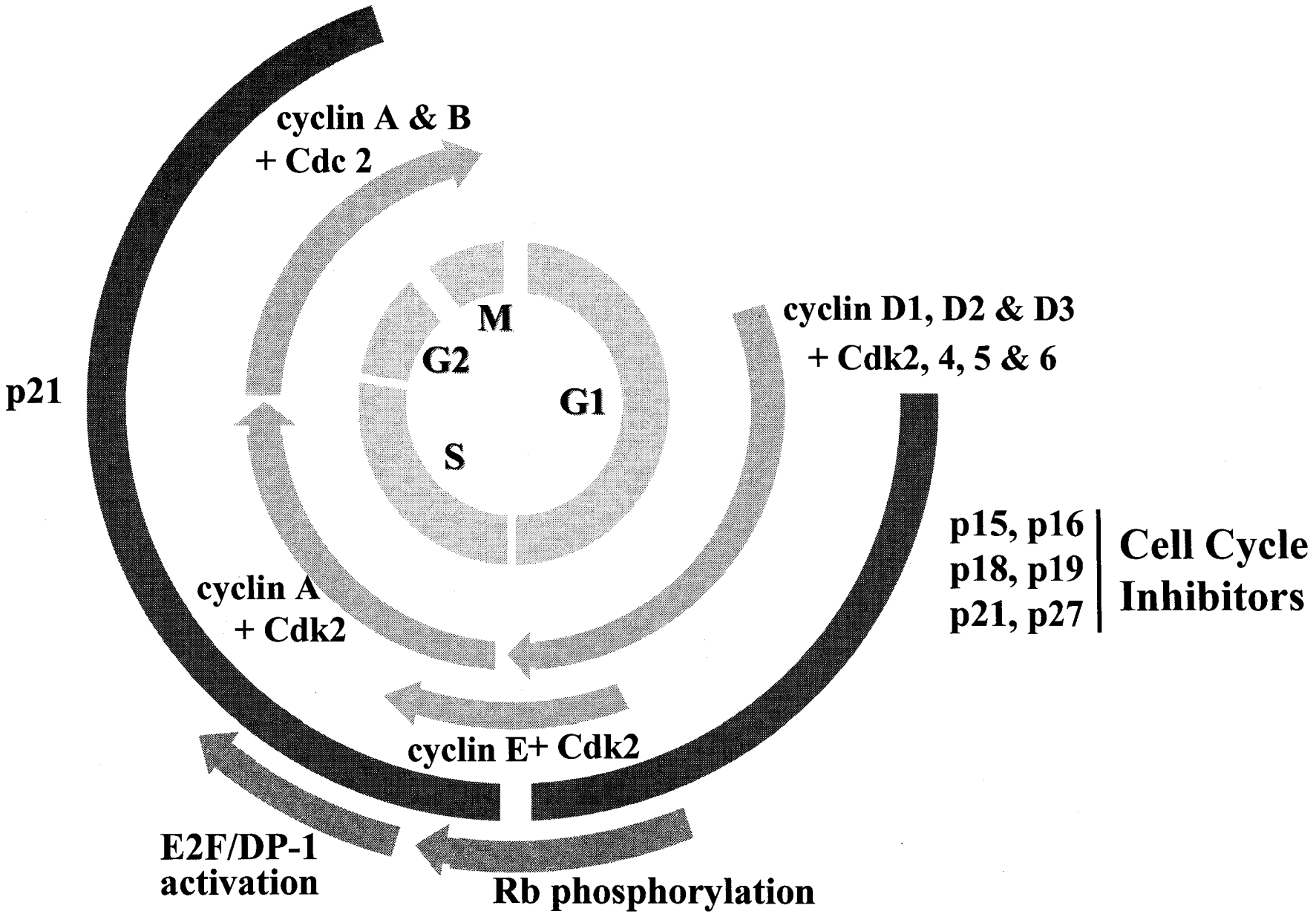
$p12^I$ is small hydrophobic protein encoded by the 3' end of the HTLV-I genome, $p12^I$ also binds specifically to both the β and γc chains of the IL-2R, an interaction that stabilizes the immature form of the IL-2R β and γc chains and decreases cell surface expression. $p12^I$ may therefore interfere with the normal IL-2 dependent proliferation by inducing heterodimerization of the IL-2R β and γc chains which is sufficient for adequate IL-2 signaling. The $p12^I$ /IL-2R β and γc chains interaction may have important implications in the immunosuppressive effect of HTLV-I *in vivo* as well as in the IL-2-independent HTLV-I-mediated T cell proliferation (147).

1.2.2 Cell cycle dysregulation

Normal transition from one phase of the cell cycle to the next is regulated at "checkpoints" which are in part governed by cyclin-dependent kinases (CDKs) assembled with their partner cyclins. Phosphorylation and dephosphorylation further regulate active CDK-cyclin complexes. CDKs can also be inactivated through physical interaction with CDK inhibitory proteins (CKI). D- and E-type cyclins are important in cell cycle transition from G1 to S (Figure 10). The cyclin D-CDK complex specifically phosphorylates retinoblastoma (Rb) protein which releases the transcription factor E2F. E2F action facilitates the transition from G1 to S and induces cellular proliferation. Tax plays a role in cell cycle progression by direct interference with the cell cycle inhibitor, $p16^{INK4A}$ (122, 206). Interestingly, $p16^{INK4A}$ is a tumor suppressor which is often deleted in human cancer and structurally possesses ankyrin motifs similar to IκBα.

$p16^{INK4A}$ binds to the cyclin-dependent kinases, CDK4 and CDK6 and inhibits kinase activity which results in the suppression of G1 phase progression. The binding of Tax to $p16^{INK4A}$ induces a reduction in the $p16^{INK4A}$-CDK4 complex, with subsequent activation of CDK4 kinase (206). The activated CDK4 kinase phosphorylates the tumor suppressor Rb protein which in turn releases E2F and induces transcription of genes involved in cellular progression from G1 to S. By inhibiting $p16^{INK4A}$, Tax therefore causes unregulated cell cycle progression and cellular proliferation.

Figure 10: **Cell cycle regulation**. The cell cycle is divided into 5 phases: G1 (gap1) where DNA repair takes place before S (synthesis) phase which is the duplication of cellular DNA, followed by G2 (gap2) where DNA repair takes place before M (mitosis) which leads to the generation of two identical cells. The fifth phase G0 which is an extension of G1 where cells are quiescent and non-replicating. Normal transition from one phase of the cell cycle to the next is regulated at "checkpoints" which are in part governed by cyclin-dependent kinases (CDKs) assembled with their cyclin partners. Phosphorylation and dephosphorylation further regulate active CDK-cyclin complexes. CDKs can also be inactivated through physical interaction with CDK inhibitory proteins (CKI). D- and E-type cyclins are important in cell cycle transition from G1 to S. The cyclin D-CDK complex specifically phosphorylates retinoblastoma (Rb) protein which releases the transcription factor E2F. E2F action facilitates the transition from G1 to S and induces cellular proliferation.


cyclin A & B
+ Cdc 2
M
G2
G1
S
cyclin D1, D2 & D3
+ Cdk2, 4, 5 & 6
p21
p15, p16
p18, p19
p21, p27
Cell Cycle
Inhibitors
cyclin A
+ Cdk2
cyclin E+ Cdk2
E2F/DP-1
activation
Rb phosphorylation

Tax was also shown to mediate an increase in cyclin D3-dependent CDK4 and CDK6 activity in cells with no $p16^{INK4A}$ expression. Rb phosphorylation and E2F activation accompanied this increase in kinase activity. A Tax and cyclin D3 physical association was also detected in $p16^{INK4A}$ null cells and correlated with increased phosphorylation of CDKs. Tax seems to have two effects: inactivation of a CKI ($p16^{INK4A}$) and activation of cyclin D-CDK through a CKI-independent route (154).

Tax can also bind and sequester $p15^{INK4B}$ in a similar fashion to $p16^{INK4A}$. $p15^{INK4B}$ is also a cell cycle inhibitor of CDK4 and has been shown to be important in the regulation of cell growth and transformation especially in T lymphocytes. Tax binding to $p15^{INK4B}$ inactivated its function and made cells resistant to growth arrest induced by TGFβ (207). Inactivation of $p15^{INK4B}$ gene has been found in acute lymphoblastic leukemia and murine primary T-cell lymphoma independently of $p16^{INK4A}$ alteration. $p15^{INK4B}$, like $p16^{INK4A}$ is a tumor suppressor. In contrast to $p15^{INK4B}$ and $p16^{INK4A}$, Tax was also shown suppress the expression of $p18^{INK4C}$ in HTLV-I-infected cells through the E-box element of the p18 promoter (207). Tax has been previously implicated in repression of gene expression through the modulation of the basic helix-loop-helix family of transcription factors which bind to E-box motifs (223). Genetic alterations in $p18^{INK4C}$ have only been found in a few cases of human tumors. However, $p18^{INK4C}$ deficient mice provided suggestive data for its role in T cells. The T and B cells of these mice exhibited a higher proliferative rate upon mitogenic stimulation, suggesting an important role of $p18^{INK4C}$ in the regulation of lymphocytes (207).

Another cell cycle inhibitor, $p19^{INK4D}$ expression was also decreased in HTLV-I-infected T cells when compared to uninfected T cells (207). Tax was not involved in this downregulation and the mechanism of $p19^{INK4D}$ repression still remains unclear.

1.2.3 RNA polymerase III upregulation

RNA polymerase III is responsible for 5-10% of all nuclear transcription. It synthesizes a variety of essential cellular products, including tRNA, 5S rRNA, the 7SL RNA component of the signal recognition particle, and the U6 small nuclear RNA that is required for splicing of mRNA. Common features of these transcripts are that they are short (usually less than 200bp) and are not translated. The synthesis of Pol III products is clearly an essential component of cellular metabolism and can be regarded as housekeeping function. Although Pol III transcription is found in all cells with nuclei, it is strongly regulated in response to a variety of external stimuli. It is tightly linked to growth conditions, increasing in response to mitogenic signals and falling when serum factors or nutrients are limiting. It is also subject to cell cycle control, being activated at the G1/S transition and repressed at mitosis. Many different viruses have potent effects upon the rate of Pol III transcription. Furthermore, the majority of transformed and tumor cell types display abnormally elevated Pol III products. Therefore the level of Pol III transcription is tightly linked to the cellular growth rate (17).

Recent data suggests that RB and its relatives p107 and p130 are responsible for Pol III regulation. During G0 and early G1, RB and p130 bind and repress the Pol III-specific factor TFIIIB, shortly before S phase they dissociate from TFIIIB, allowing it to associate with Pol III, TBP and other factor to increase transcription. When cells enter mitosis, Pol III transcription is again suppressed by phosphorylation of TFIIIB. During the cell cycle, TFIIIB is also bound and repressed by tumor suppressor p53 (17).

As mentioned, a variety of viruses have been found to stimulate Pol III transcription. SV40, Hepatitis B virus and HTLV all target RNA Pol III in order to increase cellular growth and proliferation. HTLV-I Tax seems to activate TFIIIB in infected T cells. The mechanism is still unclear but the tumor suppressor p53 is believed to be involved. p53 in ATL cells is transcriptionally inactivated by Tax. Since p53 can no

longer repress TFIIIB in ATL cells, an increase in Pol III activity would lead to an increase in cellular proliferation (17).

1.2.4 p53 tumor suppression inactivation

The long latency period in the development of ATL led to speculation that increased HTLV-I mediated T cell proliferation would permit the accumulation of genetic changes that result in leukemogenesis. The tumor suppressor p53 is a target for alterations in HTLV-I induced transformation. p53 levels were normal or even slightly higher in HTLV-I transformed and ATL cells but p53 transactivation activity was reduced. This tumor suppressor induces the expression of many genes: 1) MDM2 which is involved in DNA repair; 2) Gadd45 which serves as a marker for DNA damage; 3) p21 which arrests cells in G1/S to correct DNA damage; and 4) Bax which acts as a pro-apoptotic protein. p53 also downregulates Bcl-2, an anti-apoptotic protein. By inhibiting p53 activity, Tax inhibits DNA repair and apoptosis. Tax can also inhibit p53 mediated transactivation by binding to CBP and preventing p53-CBP complex formation (248). A recent study in Tax transgenic mice seems to suggests that p53 is not critical for initial tumor formation, but contributes to later-stage tumor progression (172).

1.2.5 DNA mutations and genomic alterations.

The failure to maintain proper cell cycle control is recognized as contributing factor to genetic instability, a hallmark of human and experimental cancer. The mitotic spindle checkpoint prevents the onset of anaphase and subsequent commitment to cellular division until chromosomes are aligned properly on a bipolar spindle. Experiments in yeast have shown that the disruption of genes required for spindle checkpoint during mitosis (M), dramatically increased the rate of chromosome loss. Several proteins in yeast and in humans have been identified as factors required for preanaphase arrest or delay in response to microtubule depolymerization. These factors are called mitotic arrest-defective (MAD) proteins. Recent studies have shown that Tax interacts with human MAD1 (HsMAD1) protein and compromises the functions of HsMAD1 by preventing homodimerization and heterodimerization with

HsMAD2, thus leading to the loss of the M checkpoint. ATL cells are karyotypically abnormal and are frequently present as pleiomorphic multinucleated giant cells. The expression of Tax correlates with progressive accumulation of damaged DNA in cells (138, 167). This Tax-HsMAD1 interaction provides a molecular explanation for HTLV-I-induced karyotypic abnormalities and potentially transformation seen in ATL cells (19, 90).

Tax was also shown to repress DNA polymerase β expression, which is involved in DNA repair. A decrease in DNA repair would lead to an increase in the accumulation of DNA mutations and ultimately contribute to cellular transformation (169) (Table 1). Tax can also bind and repress DNA topoisomerase I. This result suggests that Tax has a novel potential to affect cellular processes, such as transcription and maintenance of genomic stability, in which DNA topoisomerase I is involved (249). Genomic instability could be involved in cellular transformation induced by HTLV.

1.3 Epidemiological aspects

HTLV-I is not a ubiquitous virus. 15 to 25 million people are estimated to be infected worldwide. Endemic areas are recognized in southern Japan, tropical Africa, the Caribbean, Central and South America, and a few regions in the Middle East and Melanesia (Figure 11). In these endemic zones, 0.5-50% of the population, depending on age and gender, have specific antibodies against HTLV-I (54, 200, 218). It is estimated that the cumulative lifetime risk of developing a life-threatening or debilitating disease due to HTLV-I infection is approximately 5%, increasing to 8-10% when the patient has other illnesses (37).

The transmission of HTLV-I is fairly difficult in human populations. It requires the passage of infected lymphocytes by 1) pregnancy 2) breast feeding for more than 6 months 3) sexual contact favoring the male to female transmission through infected semen cells and 4) blood exposure containing infected cells (blood transfusions and intravenous drug users). Breast feeding is probably the most common mode of transmission. Sexual transmission is relatively inefficient when compared to HIV. There is gender bias in sexual transmission; The risk of HTLV-I transmission from husband to wife is about 60% while the risk from wife to husband is 0.4% over a 10-year period. Blood transfusion is also fairly inefficient. Because HTLV-I is not present in plasma, this retrovirus is not found in the clotting-factor concentrates prepared from plasma for hemophiliacs. Transmission among IV drug users is becoming quite common and is believed to occur from the sharing of needles and syringes containing contaminated white blood cells. HTLV infection is not transmitted by casual contacts. Therefore the risk of transmission for health care workers for example is less than hepatitis B and equal or lower than that of HIV (37, 54, 200, 218).

HTLV-I is very genetically stable unlike HIV. This is probably due to clonal expansion of infected cells through mitosis, conjugated to the minimal use of the viral reverse transcriptase. This genetic stably has been used by epidemiologists as a

molecular marker to study viral transmission *in vivo* and follow the migrations of infected human populations (54).

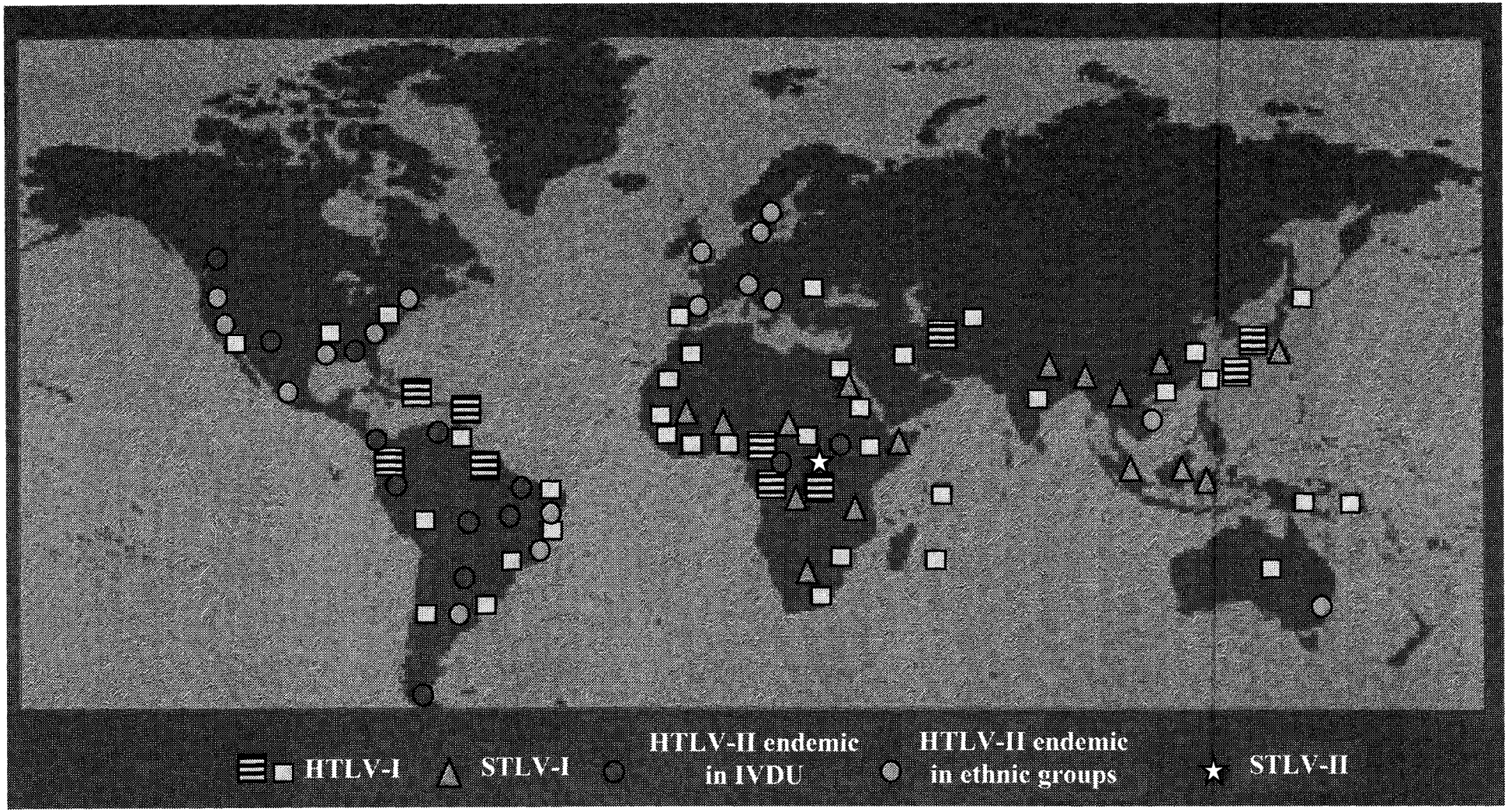
Today, 6 major sub-types of HTLV-I have been documented. Sub-type A or the cosmopolite sub-type is the most geographically disperse; It is found in Japan, the Americas, Caribbean, North and West Africa, the Middle East, India and Europe. Recently a group of scientist identified the presence of HTLV-I cDNA (sub-type A) in a Chilean mummy over 1,500 years old. The HTLV-I DNA was virtually identical to the ones found in today's Chilean and Japanese HTLV-I-infected people. This lead to the belief that the Japanese are the ancestors of Chileans by having migrated to South America thousands of years ago through the Bering Straight (115).

Sub-type B is mainly found in Central Africa (Zaire) and diverges by 3% in the *env* gene compared to sub-type A. Sub-type C is found in Melanesia, Papua New-Guinea and the Solomon's Isles. It diverges by 6-8% in gp21 (env protein) compared to sub-type A. Sub-type D is mainly found in Central Africa (Cameroon, Gabon, and Pygmy populations). It has an intermediate position between sub-types B and C. Finally, two new sub-types, E and F, were identified in the Congo and Gabon, respectively (54, 225).

In 1982, a simian retrovirus (Simian T cell Leukemia Virus-I: STLV-I) was isolated in Japan and found to be genetically similar to HTLV-I (90% homology to sub-type A). ATL-like manifestations have been detected in gorillas, macaques and in African green monkeys infected with STLV-I but no HAM/TSP has ever been reported. It is believed that HTLV-I was transmitted to humans from monkeys by a deep bite or accidental cut around 30,000-40,000 years ago. This hypothesis is strengthened by the fact that the sequence of gp21 of STLV-I in chimpanzees is 98% identical to that of HTLV-I sub-type B (54, 225). The current distribution of HTLV-I and its simian homologue, is believed to be the result of at least 4 events: 1) transmission of STLV-I between different monkey species 2) transmission of STLV-I to humans 3) persistence of HTLV-I in isolated human populations and 4) global and more recent

distribution of HTLV-I (mainly Sub-type A) subsequent to migration of virus-infected populations (54).

Figure 11: HTLV-I, HTLV-II and STLV worldwide distribution. HTLV-I endemic regions: yellow stripped square, presence of HTLV-I in non-endemic regions: smaller yellow square, Simian T cell Leukemia Virus-I (STLV-I): pink triangle, HTLV-II endemic in ethnic groups: green circle, HTLV-II endemic in intravenous drug users (IVDU): orange circle; and Simian T cell Leukemia Virus-II (STLV-II) distribution: white star. Adapted from Slattery et al., 1999.


HTLV-I
STLV-I
HTLV-II endemic in IVDU
HTLV-II endemic in ethnic groups
STLV-II

1.4 Viral Pathogenesis

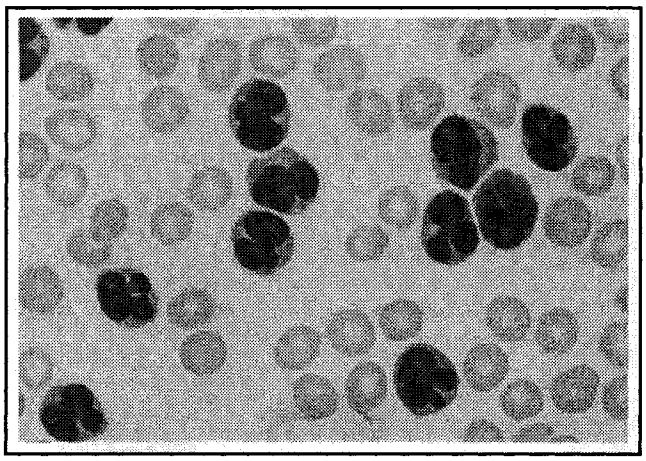
1.4.1 Adult T cell Leukemia

ATL develops in a small proportion of HTLV-I infected people (0.5-1%) after a long latency period with a mean disease onset of 55 years. ATL is classified into four clinical subtypes: acute, chronic, smoldering and lymphoma types, based on the number of abnormal CD4+ T cells, tumor lesions and the clinical course (218). The acute phase of ATL is the most severe; patients with acute ATL have elevated abnormal T cell count, hypercalcemia, prominent skin lesions, hepatospenomegaly and suffer from serious bacterial, viral, fungal and protozoan infections. Although less frequent, digestive tract, lung, central nervous system, bones and/or other organs may be involved. ATL cells have a highly indented or lobulated nuclei with markedly condensed chromatin and were therefore named "flower cells" (Figure 12). (218).

HTLV does not carry viral oncogenes like other retroviruses, which usually contribute to cellular transformation. ATL occurs in a multistep process leading to the acute phase of ATL. The phases are divided according to the clinical manifestations seen in patients: pre-ATL, smoldering, chronic, lymphoma and acute. The prognosis of ATL is poor with a median survival time for acute disease of 6.2 months (Table 2) (20, 221). The viral oncoprotein Tax is believed to be crucial in early stages of leukemogenesis. Later in transformation, the inactivation of cellular tumor-suppressors such as p53, p15 and p16 are likely involved since most viral proteins are no longer expressed in ATL cells (218).

Figure 12: Clinical feature of Adult T cell Leukemia. (A) ATL cells showing the typical convoluted nuclei - "Flower cells" and **(B)** Prominent skin lesions on patients with ATL (Cann Fields Virology, 1996).

A



B

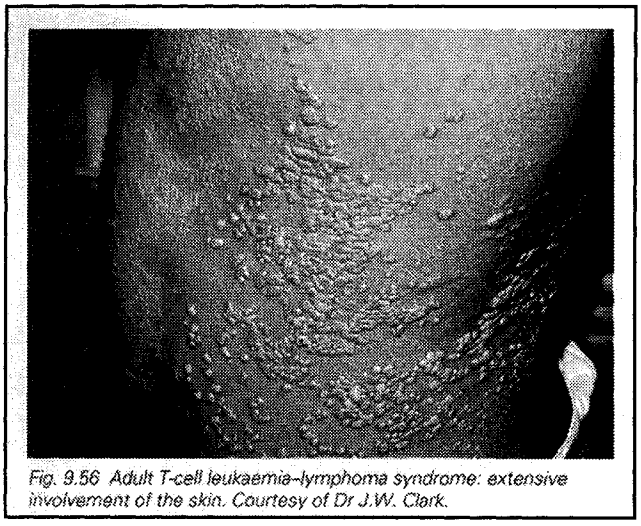


Fig. 9.56 Adult T-cell leukaemia–lymphoma syndrome: extensive involvement of the skin. Courtesy of Dr J.W. Clark.

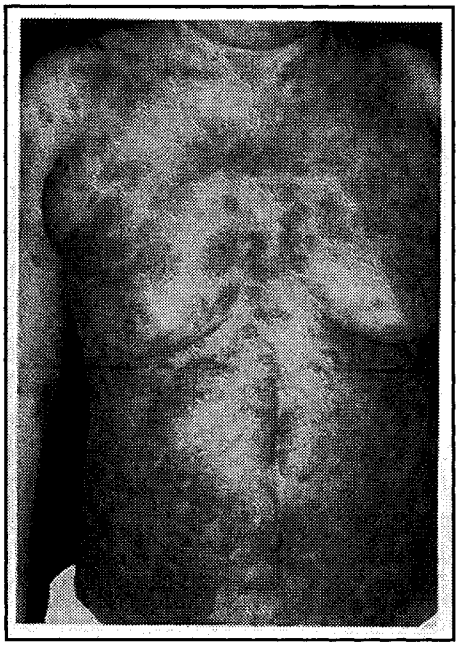
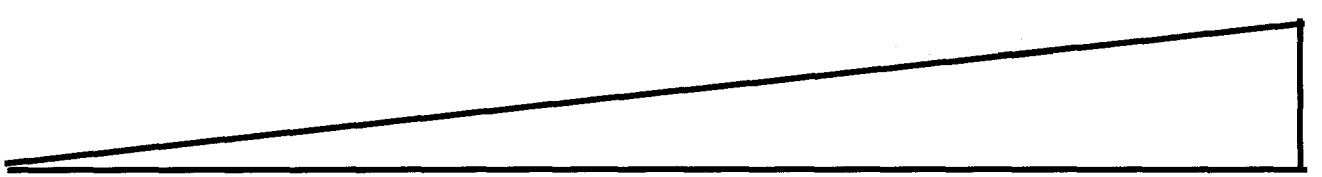


Table 2: Characteristics of the clinical phases of Adult T cell Leukemia. ATL is classified into four clinical subtypes: acute, chronic, smoldering and lymphoma types, based on the number of abnormal CD4+ T cells, tumor lesions and the clinical course. Patients who develop symptoms will progress to the acute phase of the disease which is the most aggressive and always fatal.

	Pre-ATL	Smoldering	Chronic	Lymphoma	Acute
Clinical picture	-no clinical signs for decades	- less aggressive -normal white cell count -skin lesions -5% abnormal T cells	- less aggressive -normal white cell count -prominent skin lesions -5% abnormal T cells -modest bone marrow and visceral involvement	-skin lesions -1% abnormal T cells -visceropathy	- very aggressive -elevated white cell count -prominent skin lesions - >5% abnormal T cells -prominent skin lesions -visceropathy
Survival time	-	> 10 months	> 10 months	10 months	6 months

Progression

Proportion of patients



Almost all ATL patients suffer from the complications of hypercalcemia. A parathyroid-related protein (PTHrP) acts as a humoral factor of hypercalcemia in some solid tumors. ATL patients, HAM/TSP and HTLV-I infected-asymptomatic patients all express elevated levels of PTHrP. The basis of increased PTHrP expression was shown to be Tax induced transactivation of the PTHrP gene promoter. These observations explain the elevated expression of the PTHrP in ATL patients and the high incidence of hypercalcemia in ATL (229).

1.4.2 HTLV-I Associated Myelopathies

HTLV-I infection is also associated with a neurological disorder termed HAM/TSP. The prevalence of HAM amongst HTLV-I seropositive patients is estimated at 1 in 1500 individuals. The latency period is shorter (1-3 years) and disease onset occurs earlier in life at a mean of 35 years. Onset has occurred in patients as young as 6 years of age. Women are more frequently affected than men with a 3:1 ratio (37). Clinical manifestations include progressive demyelination of motor neurons in the spinal cord, spasticity, bladder disturbances and lower extremity muscle weakness. PCR analyses have demonstrated a correlation between the amount of HTLV-I proviral DNA in spinal cord lesions and the percentage of infiltrating CD4+ T lymphocytes in patients with HAM/TSP. This observation suggests that a viral reservoir may be maintained in the infiltrating CD4+ T lymphocytes in these lesions.

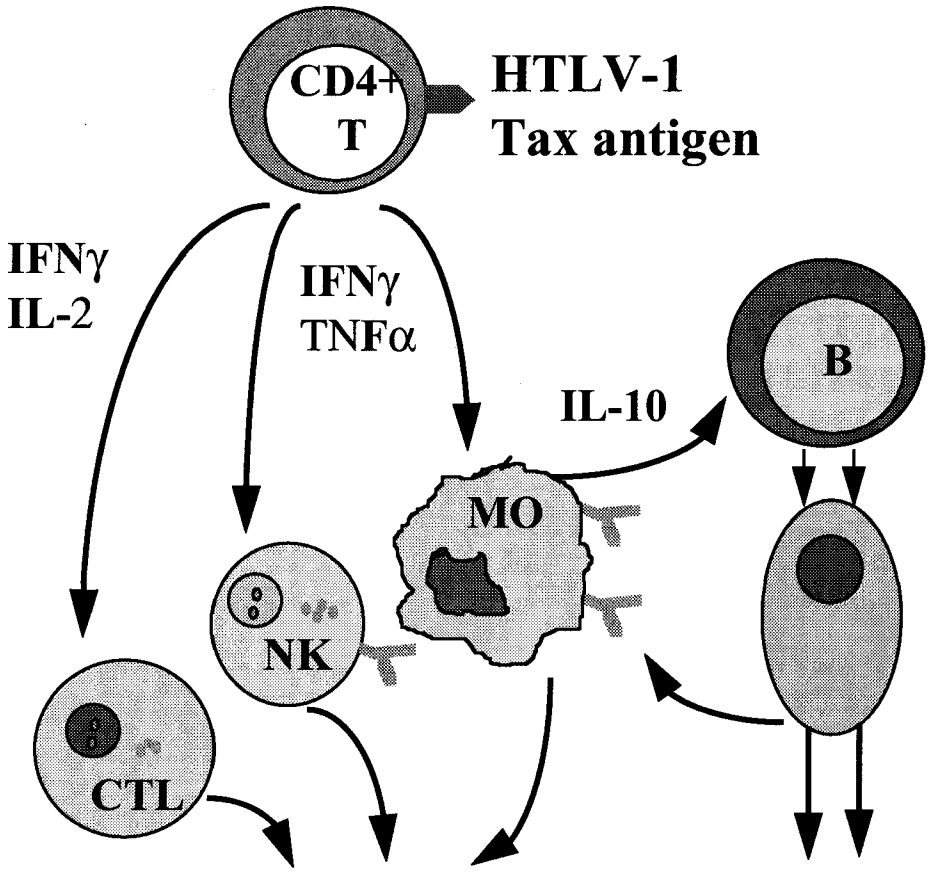
Three possible mechanisms have been suggested for the pathogenesis of HAM/TSP. 1) The first model - the autoimmune model - suggests that HTLV-I activates autoreactive T lymphocytes, which then migrate to the central nervous system (CNS), where they recognize target antigens presented on the surface of CNS constituent cells. 2) The second direct infection model proposes that tissue destruction is caused by persistent HTLV-I activation in the infected CNS constituent cells or by a cellular immune attack against the infected CNS cells. 3) The third "bystander damage" model proposes that the supply of infected CD4+ T lymphocytes from blood flowing to the CNS is crucial for the development of CNS lesions. The consequent lymphocyte proliferation involves an activated replication of HTLV-I in infected T

cells and proliferation of immunocompetent T lymphocytes responding to viral antigens on infected cells. Continuous inflammation in a restricted area such as the spinal cord or CNS may reflect sustained antigen stimulation, defective immunoregulation, or both (Figure 13) (20, 221).

Many studies have also indicated that HTLV-I proviral load in peripheral blood lymphocytes (PBL) of HAM/TSP patients was very high (16 fold higher) compared to ATL or HTLV-I-seropositive asymptomatic carriers. It is still unknown why HTLV-I proviral load is so high in HAM/TSP patients but it is believed to be a critical point in the pathogenesis of the disease (23, 151). As well, the specific cytotoxic T lymphocyte (CTL) activity to the HTLV-I pX (Tax, Rex, p12, p13 and p30), which is the immunodominant target antigen, is detected at a very high frequency in PBL and in the cerebrospinal fluid of HAM/TSP patients. These facts suggested that CD8+ HTLV-I-specific CTL activity is involved in immune-mediated damage in the spinal cord of these patients (23, 151).

Several groups have demonstrated a relationship between immunogenetic background and the pathogenesis of HAM/TSP. The frequency of the DR1 and DR4 alleles of the HLA class II was higher in patients with HAM/TSP, and that the frequency of HLA-A26 of the HLA class I was higher in ATL patients (88, 151). Further investigation in this field could prove highly useful in determining disease susceptibility and even disease prevention.

Figure 13: HTLV-I Associated Myelopathies pathogenesis model. In the central nervous system, CD4+ T cells infected with HTLV-I present at their surfaces Tax antigen which can be recognized in the context of MHC. These infected T cells also secrete cytokines such as IFNγ and TNFα which can activate cytotoxic T cells (CTL), natural killer cells (NK), macrophages (MO). These activated immune cells then secrete CC-chemokines and adhesion molecule which could damage infected as well as healthy brain and spinal cord tissue (bystander model). B cells are also activated and differentiate into immunoglobulin secreting plasma cells. Anti-Tax antibodies and complement activation could lead to inflammation and demyelination. Adapted from Casseb, 2000.



CC-chemokines
Adhesion molecules

Anti-Tax antibodies
and complement activation

Inflammatory lesion in the spinal cord
Demyelination

HTLV-I is responsible for ATL and HAM/TSP, yet patients tend to develop either ATL or HAM/TSP and never both. It has long puzzled scientists what factors determine the pathogenesis of HTLV-I. The genetic variation of HTLV-I and the genetic makeup up of the patients are believed to be important (88). The type of T cell proliferative response also determines the pathogenesis of HTLV-I: high T cell proliferative response occurs in HAM/TSP patients while low T cell proliferative response is seen in ATL and asymptomatic carriers (23, 221) (Table 3).

1.4.3 Other HTLV-I associated diseases

Several other diseases have been associated with HTLV-I infection such as infective dermatitis which is characterized by severe exudative dermatitis with crusting of the scalp, neck, ears, hands and groin area (105). HTLV-I-induced uveitis is a sight threatening intraocular inflammatory disorder characterized by blurred vision, retinal exudates and hemorrhages (187). HTLV-I-induced arthropathy is a chronic arthritis of the joints. Sjogren's Syndrome induced by HTLV-I infection is a chronic inflammatory disorder characterized by lacrimal and salivary insufficiency resulting in xerostomia (dryness of mouth). Occasionally, these other HTLV-I-associated diseases occur in conjuction with ATL or HAM/TSP (151). As well, many opportunistic infections occur in HTLV-I infected individuals due to their state of immune suppression in the later stages of disease progression; *Pneumocystis carinii* pneumonia, cytomegalovirus pneumonia, cryptococcal meningitis, disseminated fungal infections, bacterial lung abscesses and bacterial sepsis have been found in HTLV-I infected patients (221).

Table 3: **Adult T cell Leukemia versus HTLV-I Associated Myelopathies**. The different characteristics of ATL and HAM caused by Human T cell Leukemia Virus-I infection. Patients infected with HTLV-I develop either ATL or HAM. It is believed that viral genetic variation, the different genetic backgrounds of patients, viral load and T cell proliferative response are involved in the development of either ATL or HAM. Adapted from Casseb, 2000.

Characteristics	HAM/TSP	ATL
Underlying lesion	Inflammatory process, demyelination	Neoplastic
Characteristic in host	High viremia PBMC/CNS	Low viral load
Genetic background	HLA A2, A26, A33, B7...	DRB1*1011-DRB1*0301
Frequency of disease	14/ 1464	1/ 10 000
Activation	IL-2, IL-2R, IL-6, TNFα, CC-chemokines, TGFβ	IL-2, Il-2R soluble
Apoptosis	Not altered	Decreased
Treatment	Anti-inflammatory	Antiretroviral
Prognosis	Fair	Poor

Rheumatoid arthritis very much resembles HTLV-I-associated arthropathy. The precise mechanism of proliferation of synoviocytes (cells in the synovial liquid found in joints), synovial cell hyperplasia, infiltration of various inflammatory cells and joint destruction in rheumatoid arthritis and HTLV-I-associated arthropathy remain misunderstood. Recently, Nakazawa et al. demonstrated that HTLV-I Tax- CBP complex through the activation of CREB/PKA signaling pathway, lead to synoviocyte activation. Tax could therefore be involved in the pathogenesis of HTLV-I-associated arthropathy (152).

Other conditions have also been reported to be associated with HTLV-I infection such as polymyositis, alveolitis and thyroiditis (218).

1.4.4 HTLV-II

HTLV-II was identified in a T cell line established from a patient with an atypical hairy-cell leukemia in 1982 (20). Although most hairy-cell leukemias are of B cell origin, HTLV-II infected patients have T cell leukemia which is characterized by infection of CD8+T cells and atypical circulating lymphocytes. HTLV-II infection is quite rare but is found clustered in specific groups of Native American tribes in South, Central and North America and in intravenous drug users (Figure 11) (20). An alarming trend has been documented in HTLV-II infected intravenous drug users; HTLV-II is mutating 100 times faster in these patients than in other HTLV-II infected individuals (226). Considering the possible effect of evolutionary rate of retroviruses on virulence and pathogenesis, the HTLV-II infected-intravenous drug user population should be considered as a potential source of new and more aggressive viral variants. The discovery of HTLV-II in two groups of Pygmies and non-Pygmies in Central Africa is quite interesting in that it suggests a possible African origin for the virus (226).

Complications occur with HTLV-II infection such as spontaneous lymphocyte proliferation, mycosis fungoides, neurological complications similar to HAM/TSP and opportunistic infections (20). HTLV-II has also been found in HIV-I infected individuals. The effect of HTLV-II infection in these patients remains controversial.

HTLV-I and HTLV-II share significant homology at the nucleotide level (65% homology) but differ drastically in their clinical manifestations and distribution (20). It is believed that the Tax protein is in part responsible for these differences. Alignment between Tax1 (from HTLV-I) and Tax2 (from HTLV-II) reveals extensive conservation (greater than 77% amino acid identity). Semmes et al. demonstrated that Tax1 was a potent inducer of micronuclei formation in cells while Tax2 lacked this ability. It is known that damaged cellular chromosomes present as light-microscope visible micronuclei. Tax1 expression has been previously shown to be associated with increased cellular DNA damage which is reflected by an increase in cellular micronuclei (124, 138). DNA damage/micronuclei induction is believed to lead to aberrant cellular growth and potentially cellular transformation. The fact that Tax1 and not Tax2 can induce DNA damage through micronuclei formation could in part explain the differences in the pathogenesis of HTLV-I and HTLV-II (195).

1.4.5 HTLV cellular receptor

To this date, the cellular receptor for HTLV entry remains a mystery. HTLV-I can infect many cell types such as B and T cells, monocytes, fibroblasts, neuronal cells from different species such as mouse, cat, dog, pig, bovine as well as primate. Interestingly, HTLV-I can only transform human CD4+ T cells *in vivo* (20, 157, 216). Several candidates have been proposed as the HTLV-I receptor such as IL-2 receptor alpha, 70KDa Heat shock cognate protein (HSC70), vascular cell adhesion molecule-1 (VCAM-1) and the adhesion molecule LFA-1 (216). Recently, HSC70, ICAM-1, ICAM-3 and VCAM-1 were shown to act as cell fusion-enhancing factors by promoting syncytia formation of HTLV-I infected cells but not as entry factors for the virus (28, 44, 224). The search of the entry factor for cell-free HTLV remains to be determined.

1.4.6 HTLV and HIV

Several epidemiological surveys have indicated an increasing number of individuals co-infected with HIV and HTLV. This trend is particular common amongst intravenous drug users and, although still controversial, evidence suggests that coinfection with both viruses may accelerate the progression to AIDS and worsen the consequences of HTLV infection (144). The transmission modes for HTLV-I are identical to those of HIV: through sexual contact, blood transfusion, pregnancy and breast milk. In the case of HTLV-I, the passage of HTLV-I infected cells is required for disease transmission, whereas with HIV, a low number of free virus particles are sufficient to transmit the disease (20). With co-infection of HIV and HTLV, there also arises the potential problem of recombinant viruses, which could broaden viral tropism and lead to accelerated disease dissemination and progression (108).

1.4.7 Detection and therapies

Serological, virological or molecular examinations can detect HTLV-I infection. As with other human retroviruses, once acquired, HTLV-I infection is life-long after proviral integration in host cellular DNA. Specific antibodies against HTLV-I can be detected by enzyme-linked immunosorbent assay (ELISA), particle agglutination (PA), immunofluorescence, Western blot or radioimmuno-precipitation. ELISA and PA are frequently used as screening assays. To discriminate between HTLV-I and HTLV-II, which is less pathogenic than HTLV-I, Western blot is usually necessary as a confirmation assay (218). Clonal integration of the HTLV-I proviral DNA in infected T cells can be demonstrated by Southern blot analysis, PCR and/or ligation PCR. Single proviral integration of the full-sized HTLV-I genome is usually detected in all types of ATL cells. However, patterns of multiple or defective HTLV-I infection can also occur (218).

Standard chemotherapeutic regimens that have proven to be highly successful in the treatment of malignant leukemia and lymphomas have failed miserably in the treatment of ATL. These conventional chemotherapy regimens (even in combination) have produced a 4-year survival rate of less than 5%. A combination of AZT (zidovudine) and interferon alpha (IFNα) was shown to produce a synergistic response and induce a rapid remission within 1 month, with a maximum response reached after several months. Despite initial successes, IFNα/AZT combination therapy does not appear to be a cure since relapse occurred when patients in remission were taken off treatment (7, 56). Therapy with monoclonal antibodies against the alpha chain of the IL-2 receptor induced only a transient response. The use of other drugs such as the immunosuppressants cyclosporin A and FK506 had only minimal effects on the progression of HTLV-I induced Adult T cell Leukemia (20, 218, 221).

Recently, the use of arsenic and IFNα has been suggested for the treatment of ATL. *In vivo* studies have demonstrated that arsenic trioxide and IFNα synergistically induced cell cycle arrest and apoptosis in HTLV-I-transformed cells (41). The combined use of arsenic and IFNα induced a degradation of the Tax protein, associated with an upregulation of IκB which lead to a sharp decrease in RelA (NF-κB) DNA binding complexes. Inactivation of the NF-κB pathway probably accounts for cell death seen in HTLV-I-transformed cells treated with arsenic and IFNα. Such specific targeting of the viral oncoprotein Tax by arsenic and IFNα treatment is reminiscent of promyelocytic leukemia/retinoic acid receptor fusion degradation by arsenic in acute promyelocytic leukemia (92). These results provide a strong rational for combined arsenic and IFNα therapy in ATL patients (41).

Prevention of opportunistic infections is also essential in ATL patients because nearly half of these infected individuals suffering from late-stage ATL develop severe infection during chemotherapy. Prophylaxis is given against viral, fungal and bacterial infections. Hypercalcemia can also be corrected by hydration, elcatonin and/or biphosphonates (218).

HAM/TSP treatments are also only partially effective. IFNα has been used but shown to be only a short-term treatment of HAM. Administration of anti-IL-2 receptor antibodies to patients induced clinical improvement with selective down-regulation of activated T cells concomitantly with a reduction in proviral load in PBL. Taylor et al. also reported the efficacy of lamivudine, which is a reverse transcriptase inhibitor used to treat HIV-1 infection. They described a remarkable reduction in HTLV-I proviral load (151).

HAM/TSP patients also show a bias towards Th1 response with the detection of elevated IFNγ, TNFα, and granulocyte-macrophage colony-stimulating factor but not IL-4 in PBLs. Pentoxifylline may be therapeutic in HAM/TSP patients because it induces an immune deviation from Th1 to Th2 in these patients (151). Various immunosuppressants such as cyclosporin A, FK506 and anti-inflammatory steroids have also been used with moderate effects (3). It seems that a therapeutic strategy targeting HTLV-I-infected CD4+T cells would be useful in decreasing viral load, the Th1 bias and neurological manifestations due to infiltrating cells seen in HAM/TSP patients (151).

The best cure for HTLV is the development of a vaccine. There is evidence of the existence of natural immunity in humans. In Japan, scientists have shown that maternal antibodies from HTLV-I-infected mothers protect infants from milk-borne transmission. These antibodies are present during the first 6-12 months of life (37). It is also unlikely that antigenic variation will present as great as a problem in HTLV-I vaccine development as with HIV-I. These advantages favor a vaccine development

consisting for example of recombinant env proteins. A promising field involves DNA plasmid- based vaccination against HTLV (1).

2.Interferons

Interferons (IFNs) are a family of multi-functional cytokines that were first discovered as mediators of cellular resistance against viral infection and were later shown to play diverse roles in the immune response to pathogens, immunomodulation and hematopoietic development (156, 203). Type I IFNs (IFNα and IFNβ) are produced by virus-infected host cells and constitute the primary response against virus infection. Type II IFN (IFNγ) is a TH1 cytokine produced by activated T cells and natural killer cells, is crucial in eliciting the proper immune response and pathogen clearance (Figure 14 and Table 4). IFNs elicit their effects through the transcriptional activation of target genes that possess specific consensus DNA-binding recognition sites within their promoters. These genes are regulated through the JAK-STAT signaling pathway and through the interferon regulatory factors (IRFs), a growing family of transcription factors with a broad range of activities (156). Recent reviews have detailed the discovery and characterization of both the JAK-STAT pathway and the IRF transcription factors (14, 125, 156, 203, 212).

2.1 Interferon signaling

Type I and type II IFNs possess their own cellular receptors on cell surfaces: IFNAR and IFNGR (Figure 15). IFNAR stimulation results in the activation of Janus family protein tyrosine kinases, Tyk2 and Jak1, which are associated with the IFNAR1 and IFNAR2 chains, respectively. This activation is followed by site-specific tyrosine phosphorylation of Stat1 and Stat2 transcription factors. These two phosphorylated Stats in combination with IRF-9/ ISGF3γ/ p48 form the heterotrimer transcription factor complex, ISGF3, which translocates to the nucleus and binds to ISRE to activate IFN-inducible genes (Figure 16). In the case of IFNGR signaling, involves the IFNGR1 and IFNGR2 chains. IFNγ binding to IFNGR results in the activation of Janus kinases, Jak1 and Jak2. This activation is followed by site-specific tyrosine phosphorylation of Stat1 and homodimerization.

Figure 14: **Interferon system and actions**. Infection by pathogenic viruses leads to secretion of antiviral cytokines such as the IFNs, IL-6, TNF-α, IL-1 and other pro-inflammatory cytokines or chemokines. The signal triggered by IFN binding to specific membrane receptors leads to the activation of cytoplasmic factors that translocate to the nucleus and stimulate ISG expression. IFNs are also modulator of cell growth, block protein synthesis and induce apoptosis.

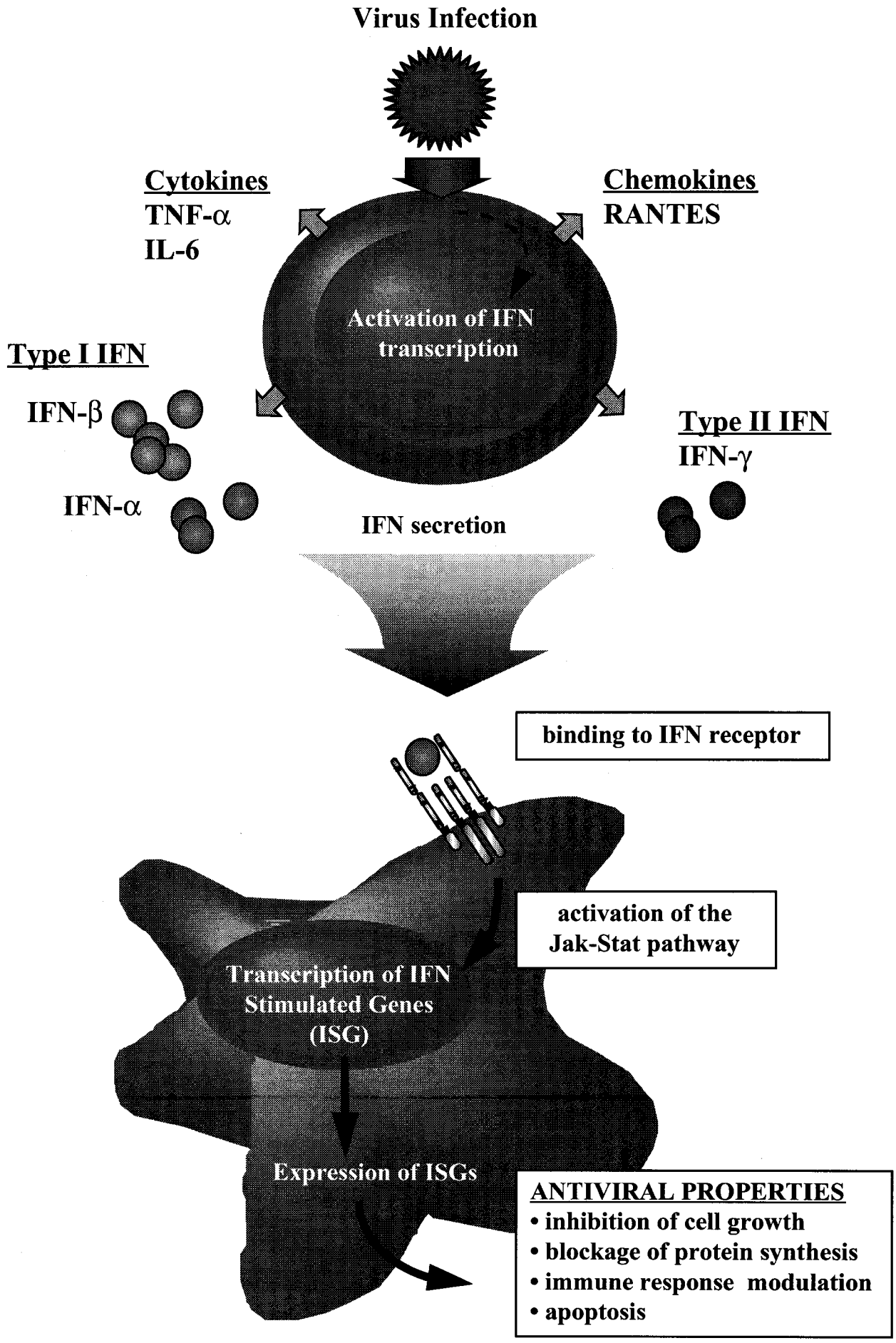
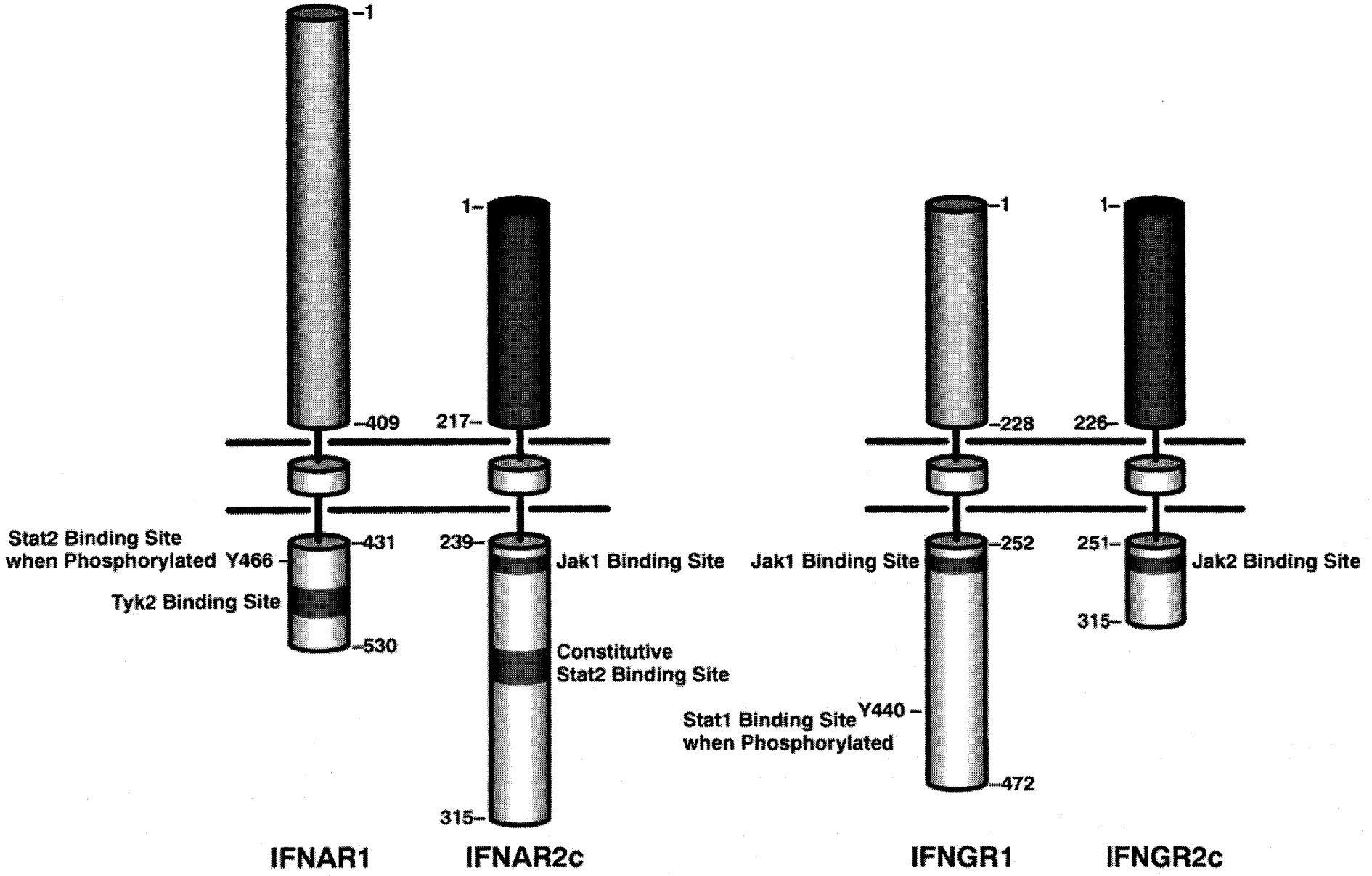

Virus Infection
Cytokines
TNF-α
IL-6
Chemokines
RANTES
Activation of IFN transcription
Type I IFN
IFN-β
IFN-α
Type II IFN
IFN-γ
IFN secretion
binding to IFN receptor
activation of the Jak-Stat pathway
Transcription of IFN Stimulated Genes (ISG)
Expression of ISGs
ANTIVIRAL PROPERTIES
• inhibition of cell growth
• blockage of protein synthesis
• immune response modulation
• apoptosis

Table 4: **Characteristics of Interferon proteins.** IFNs are divided into Type I (IFNα and IFNβ) and Type II (IFNγ). IFN-α, previously called leukocyte IFN, is produced by peripheral blood leukocytes in response to viral infection or double-stranded RNA. This heterogeneous group of proteins, of molecular weights 16 to 27 kDa, shares high homology in their amino acid sequence (165 aa residues). IFN-β, also called fibroblast IFN, is a glycosylated protein of 28 to 35 kDa, produced by leukocytes or epithelial cells. Twenty-one non-allelic IFN-A genes and pseudo-genes encoding the different IFN-α proteins have been identified and are clustered together with IFN-B gene on the short arm of human chromosome 9. Similarly, 10 different murine IFN-A genes grouped in a proximal region of chromosome 4 centromer have been identified. Human and murine IFN-A genes are intronless and maintain a high degree of homology (80 to 95%) at the nucleotide sequence level, suggesting that the gene cluster was derived from a common ancestor gene by successive duplications. The gene encoding IFN-β is unique, intronless and is derived from the same ancestor gene as the IFN-A genes. The IFN-γ is encoded by a unique gene (IFN-G) containing 3 introns and located in the long arm of human chromosome 12. This gene has a weak homology with type I IFN genes. The mature protein is a mixture of two polypeptides of 20 to 25 kDa molecular weight, differing by differential use of N-glycosylation sites. Secretion of IFN-γ by lymphocytes is modulated by mitogenic stimuli, antigens or soluble mediators such as IL-1, IL-2 or IFN-γ itself. Although IFN-γ possesses an antiviral activity, it is primarily an immune modulator than an antiviral agent.

INTERFERONS	TYPE I INTERFERONS		TYPE II INTERFERONS
	IFNα	IFNβ	IFNγ
Principal Producing cells	Leukocytes Fibroblasts Macrophages Epithelial cells		T lymphocytes Macrophages
Inducing Agents	Virus double-stranded RNA		Mitogens Antigens Interleukin-2
Chromosomal Locations	human chr 9 murine chr 4		human chr 12 murine chr 10
Gene Number	More than 15 human genes and 10 murine genes	1 unique gene	1 unique gene
Intron	0	0	3
Molecular Weight (kDa)	16to27	28to35	20to25
aa (mature protein)	165	165	146

Figure 15: **Interferon receptors**. Type I receptor binds to IFNα and IFNβ and Type II receptor bind to IFNγ. Signaling by IFNs is mediated by a pathway that includes the JAK kinases (Janus tyrosine kinases) and the STAT proteins (Signal Transducers and Activators of Transcription).


1
409
1
217
Stat2 Binding Site
when Phosphorylated Y466
431
Tyk2 Binding Site
530
239
Jak1 Binding Site
Constitutive
Stat2 Binding Site
315
IFNAR1
IFNAR2c
IFN α/β Receptor
1
228
1
226
Jak1 Binding Site
252
251
Jak2 Binding Site
315
Stat1 Binding Site
when Phosphorylated
Y440
472
IFNGR1
IFNGR2c
IFN γ Receptor

These two phosphorylated Stat1s form the transcription factor complex, GAF (IFN gamma-activated factor, which translocates to the nucleus and binds to GAS sites (IFN gamma-activated site; consensus sequence TTCNNNGAAA) to activate target genes (Figure 16) (Table 5) (212).

There is in fact a novel form of crosstalk which occurs between IFNα/β and IFNγ signaling, in which IFNγ is dependent on a weak IFNAR stimulation by spontaneously produced IFNα/β. Evidence has been provided for the physical association between IFNAR1 and IFNGR2 receptor chains. This docking site may be utilized by IFNγ–induced activation of the ISGF3 complex (212).

2.2. Interferon and viruses

Interferons (IFNs) are a large family of multifunctional secreted proteins involved in antiviral defense, cell growth regulation and immune activation. The biomodulatory activities pertinent to this group of cytokines have been extensively exploited at the clinical level, and are being used in therapy for many hematological malignancies and multiple sclerosis. Viral infection induces transcription of multiple IFN genes; Newly synthesized IFN interacts with neighboring cells through cell surface receptors, resulting in the prompt and efficient synthesis of a group of over 30 new cellular proteins through the activation of the JAK-STAT family of cellular transcription factors. These events represent the means by which IFNs induce the antiviral state that constitutes the primary host defense in innate immunity.

The ability of IFNs to confer an antiviral state to uninfected cells is their defining activity. IFNs provide an early line of defense against viral infections—hours to days before cellular and humoral immune responses. This vital role has been demonstrated by numerous animal studies in which animals that can not mount an IFN response succumb to infection by a non-lethal virus inoculation (203, 212).

Figure 16: Interferon signal transduction. The binding of IFNs to the IFN receptors induces the Jak-STAT pathway. The binding of a cytokine to its receptor rapidly induces the tyrosine phosphorylation of the receptor by JAK kinases; these phosphorylated tyrosines provide a docking site for the STAT proteins. The STATs are phosphorylated by Jak kinases, released from the receptor and dimerize with one another. Dimeric STATs then translocate into the nucleus where they modulate expression of target genes by direct DNA binding. A remarkable feature of this system is that newly induced STAT-DNA binding activity can be detected in the nucleus within minutes of cytokine binding. This timing accurately reflects the rapidity of their activation and ability to exert biological actions.

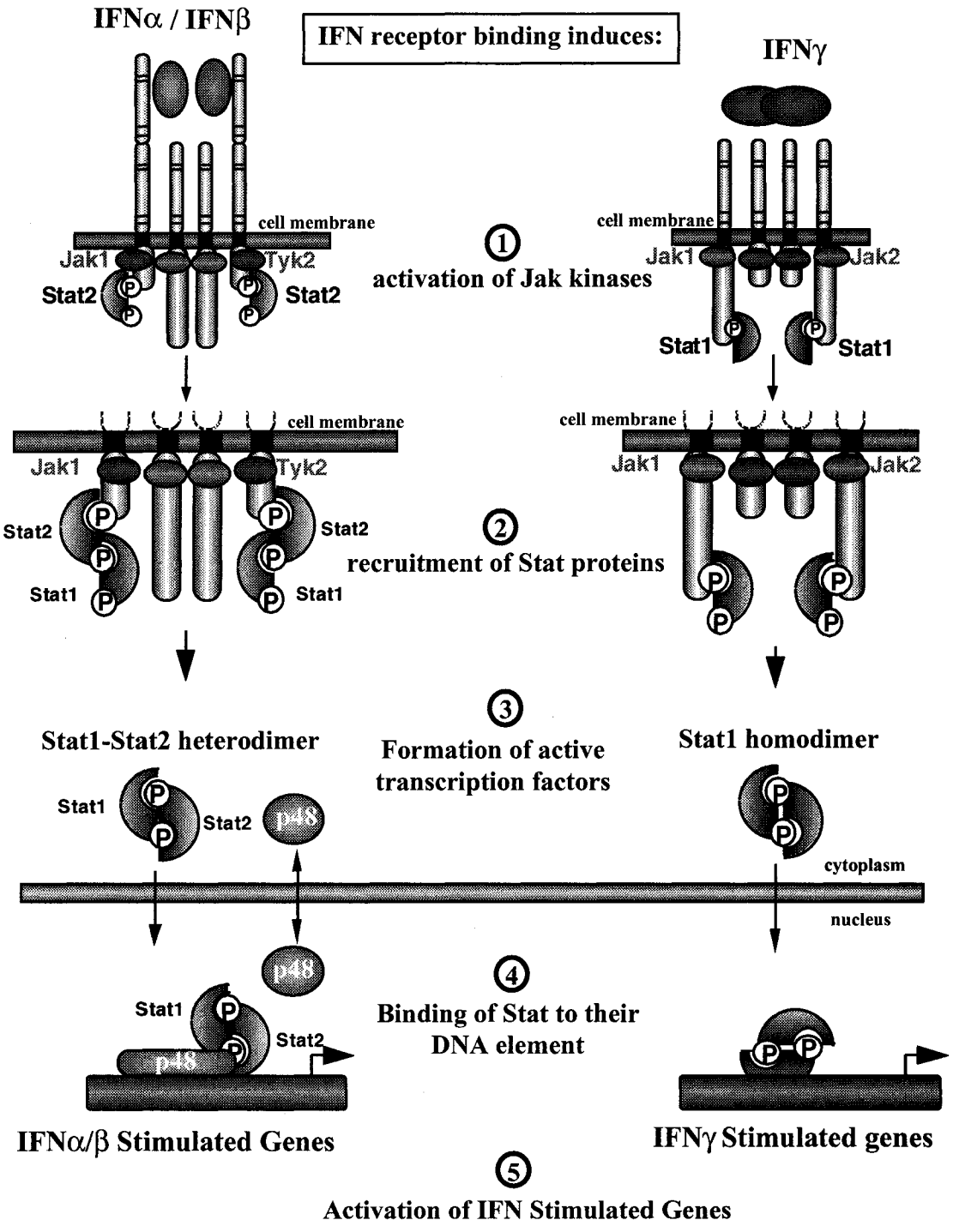

IFNα / IFNβ
IFN receptor binding induces:
IFNγ
cell membrane
Jak1
Tyk2
Stat2
Stat2
cell membrane
Jak1
Jak2
Stat1
Stat1
① activation of Jak kinases
cell membrane
Jak1
Tyk2
Stat2
Stat2
Stat1
Stat1
cell membrane
Jak1
Jak2
② recruitment of Stat proteins
Stat1-Stat2 heterodimer
③ Formation of active transcription factors
Stat1 homodimer
Stat1
Stat2
p48
cytoplasm
nucleus
p48
Stat1
Stat2
p48
④ Binding of Stat to their DNA element
IFNα/β Stimulated Genes
IFNγ Stimulated genes
⑤ Activation of IFN Stimulated Genes

Table 5: **Interferon stimulated genes**. A non-exhaustive list of ISGs, classified according to the type of IFN inducer is presented in the Table. The abbreviations correspond to: **AP**-Amino peptide; **CRG**-cytokine responsive gene; **FcγRI**-IgG-Fc receptor; **GBP**-guanylate-binding protein; **ICSBP**-interferon consensus sequence binding protein; **IDO**-indoleamine-2,3-dioxygenase; **IFP**-interferon induced Protein; **iNOS**-inducible nitric oxide synthetase; **IRF**-interferon regulatory factor; **ISG**-Interferon stimulated gene; **MHC**-major histocompatibility complex; **Mx**-myxovirus inhibiting protein; **PKR**-RNA-dependent protein kinase; **Rbp**-RNA-binding protein; **RING**-really interesting protein; **SOD**-superoxide dismutase.

IFNα/β		IFNα/β and IFNγ		IFNγ	
Gene	**Function**	**Gene**	**Function**	**Gene**	**Function**
ISG15	cytokine immuno-modulator	**CRG-2**	chemokine	**FcγRI**	IgG-Fcγ receptor
RNase L	mRNA degradation	**MHC class I**	Antigen presentation	**MHC class II**	Antigen presentation
2-5(A) synthetase	Oligoadenylate synthesis	**Rbp-27**	Inhibition of Rev-dependent HIV activation	**iNOS**	Macrophage-specific effector
MxA	inhibition of virus	**GBP**	GTP binding	**Trp-tRNA synthetase**	Protein biosynthesis
PKR	Protein synthesis inhibitor	**IDO**	Tryptophan degradation	**Leucine AP**	exopeptidase
Lysyl oxidase	Reversion of ras-transformed phenotype	**IRF-1**	Transcription factor	**MnSOD**	superoxide scavenger
IRF-2	Transcription factor	**IFP-35**	Leucine zipper protein	**Phagocyte gp91-phox**	cytochrome b subunit of NADPH
6-16	unknown			**RING4**	Peptide transporter
				RING12	Component of the proteasome
				ICSBP	Transcription factor

Any stage in virus replication appears to be fair game for inhibition by IFNs (Figure 17): entry and/or uncoating (simian virus 40 (SV40), retroviruses); transcription (influenza virus, vesicular stomatitis virus (VSV)); RNA stability (picornaviruses); initiation of translation (reoviruses, adenovirus, and vaccinia); maturation, assembly and release (retroviruses, VSV). The best-characterized IFN-induced antiviral pathways utilize the dsRNA-dependent protein kinase (PKR), the 2-5A system, and the Mx proteins (Figure 18) (203, 211, 213).

2'-5' oligoadenylate synthetases (2'5'-AS)

This system is a multienzyme pathway in which IFN, virus or dsRNA stimulates the unique enzymatic activity of 2-5A oligoadenylate synthetase. 2-5A oligoadenylate synthetase produces a series of short, 2',5'-oligoadenylates (2-5A) that activate a ribonuclease - the 2-5A–dependent RNase (Figure 18). 2-5A binds to inactive RNase L, inducing the formation of an active enzyme that produces extensive cleavage of viral and host RNA (203, 213).

Protein kinase PKR

PKR is a serine-threonine kinase with multiple functions in translation control and possibly transcription. PKR is normally present at low levels, but its expression is increased by IFN treatment; binding dsRNA subsequently activates the inactive PKR, leading to its autophosphorylation and phosphorylation of host substrates. No RNA sequence specificity is required for dsRNA to bind to PKR. The antiviral effect of PKR is due to its phosphorylation of the alpha subunit of initiation factor eIF2, which is a component of the translation initiation complex (Figure 18). Phosphorylation results in the formation of an inactive complex that involves eIF2-GDP and the recycling factor eIF2B, resulting in rapid inhibition of translation. Apoptosis may also play a role in the antiviral effect of PKR, since overexpression of PKR leads to the suppression of encephalomyocarditis virus replication (203, 213).

Figure 17: Biological functions of interferons in cells. The ability of IFNs to confer an antiviral state on cells is their defining activity. Any stage in virus replication appears to be fair game for inhibition by IFNs including entry and/or uncoating, transcription, RNA stability, initiation of translation, maturation, assembly and release of viral particles. The ability of a cell to resist to many viruses, after IFN treatment, is produced by the antiviral and immunomodulatory effects of the ISGs.

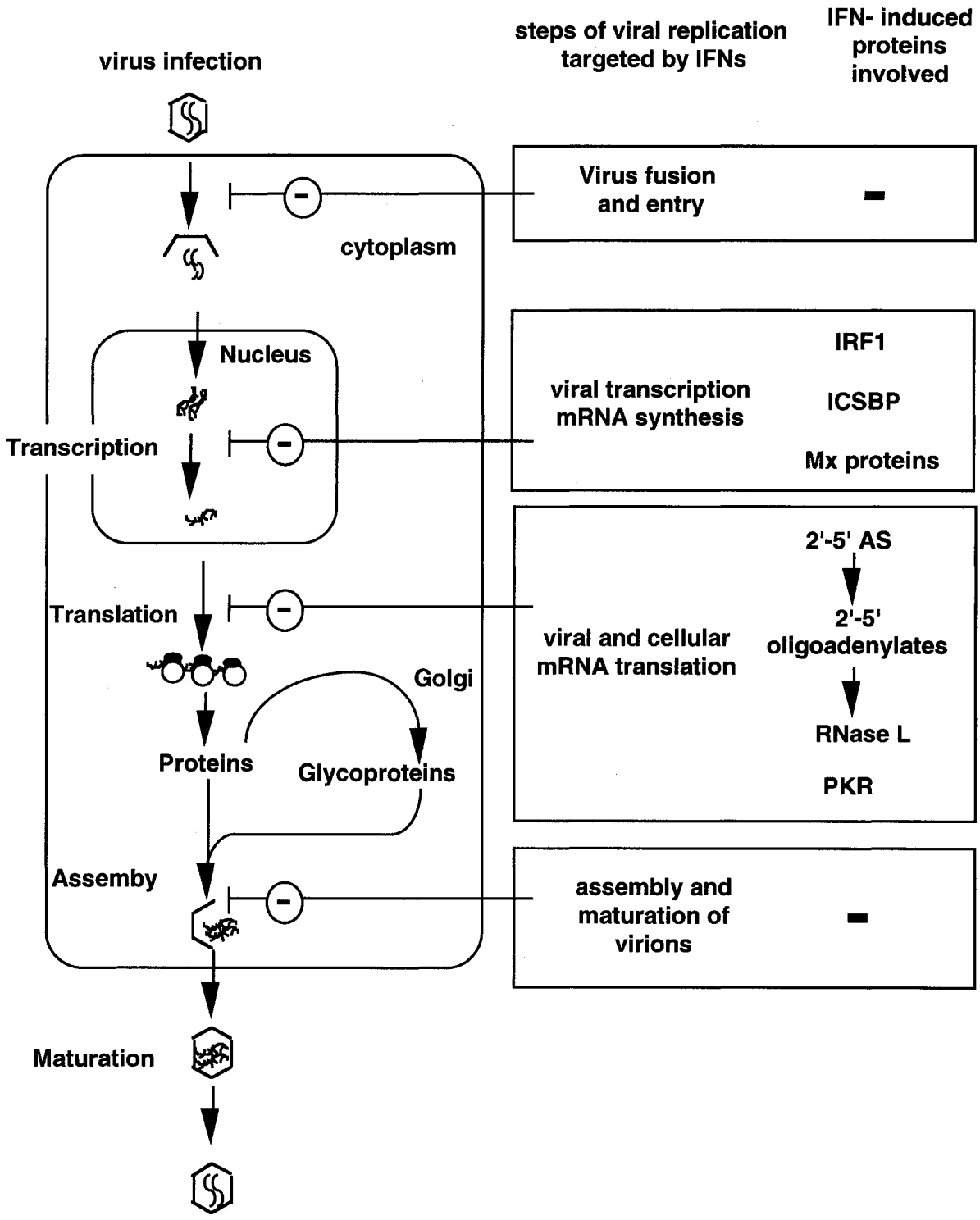
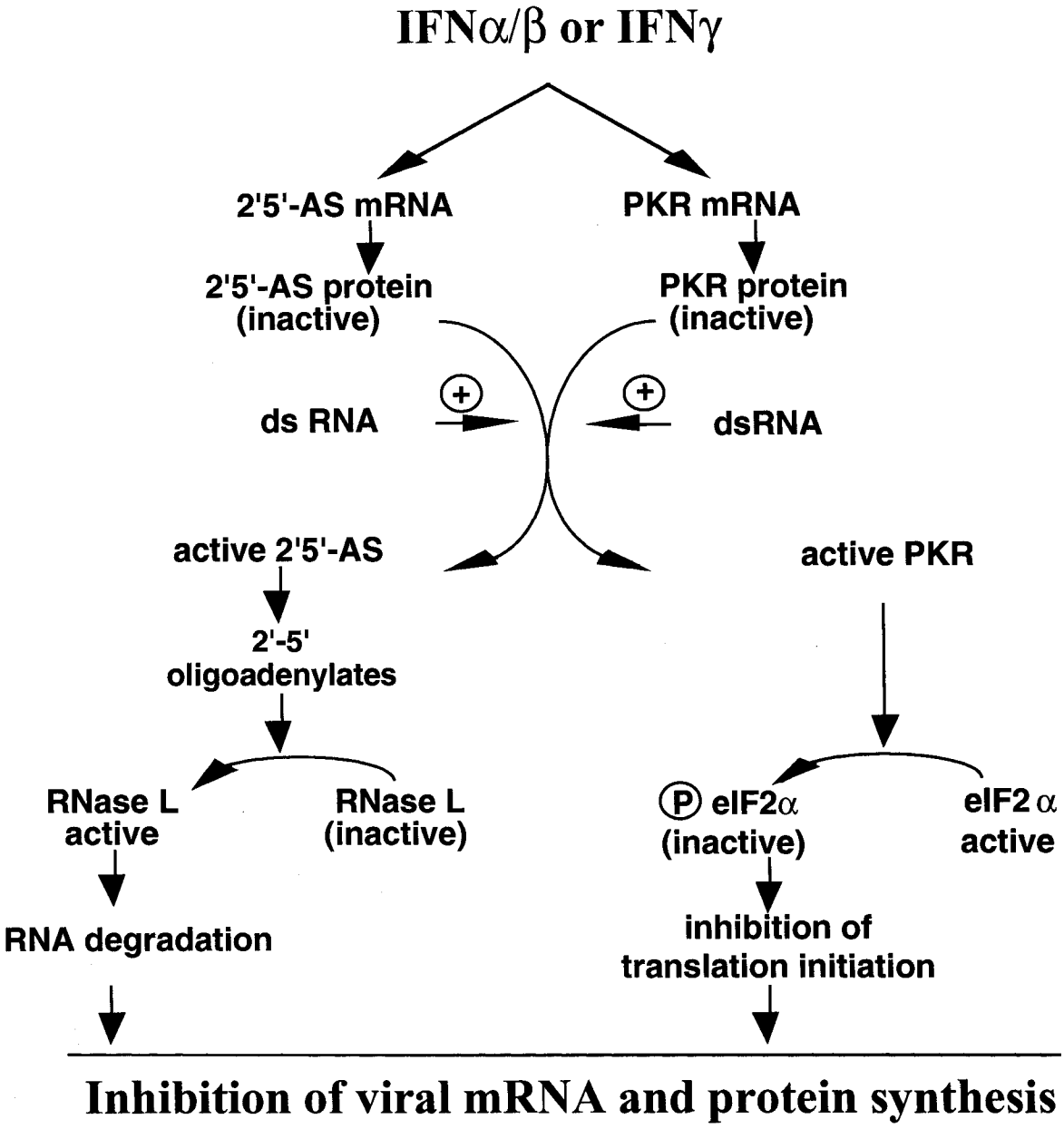

virus infection
steps of viral replication targeted by IFNs
IFN- induced proteins involved
cytoplasm
Virus fusion and entry
Nucleus
Transcription
viral transcription mRNA synthesis
IRF1
ICSBP
Mx proteins
Translation
viral and cellular mRNA translation
2'-5' AS
2'-5' oligoadenylates
RNase L
PKR
Golgi
Proteins
Glycoproteins
Assemby
assembly and maturation of virions
Maturation

Figure 18: Antiviral mechanisms of interferons. The best-characterized IFN-induced antiviral pathways utilize the dsRNA-dependent protein kinase (PKR) and the 2-5A system. 2-5A oligoadenylate synthetase produces 2',5'-oligoadenylates (2-5A) which bind to inactive RNase L and induce its enzymatic activity of RNA degradation. PKR is normally inactive, but is activated by dsRNA and subsequently phosphorylates host substrates. The antiviral effect of PKR is due to its phosphorylation of eIF2, which is a component of the translation initiation complex. Phosphorylation results in rapid inhibition of translation.


IFNα/β or IFNγ
2'5'-AS mRNA
PKR mRNA
2'5'-AS protein (inactive)
PKR protein (inactive)
ds RNA
dsRNA
active 2'5'-AS
active PKR
2'-5' oligoadenylates
RNase L active
RNase L (inactive)
P eIF2α (inactive)
eIF2 α active
RNA degradation
inhibition of translation initiation
Inhibition of viral mRNA and protein synthesis

Although PKR and Mx genes are induced preferentially by type I IFNs, 2-5A-synthetase and RNase L are induced by both types. Also, the activation of macrophages by IFN-γ inhibits the growth of vaccinia, HSV-1 and other viruses. Therefore, different antiviral pathways may be induced in different cell types, depending on the type of IFN involved. Many IFN-induced proteins are poorly characterized, and some of these are very likely to possess antiviral activity. For instance, expression of the IFN-inducible 9-27 protein led to a partial inhibition of VSV replication.

The Mx proteins

Mx proteins are IFN-inducible, 70- to 80-kDa GTPase proteins with the ability to hydrolyze GTP. Mx affects viral replication by interfering with the growth of influenza and other negative-strand RNA viruses at the level of viral transcription; murine Mx suppresses the growth of influenza viruses while human MxA inhibits influenza, VSV, measles and other virus types. Mx interferes with the primary transcription of influenza virus by inhibiting the trafficking or activity of viral polymerases and possibly at later steps in the viral life cycle.

Interestingly, viruses fight back against the host immune responses in general and in particular against the IFN system (Table 6). Some viruses produce proteins that interfere with the immune response or the IFN pathway. An important target for many viruses is the inhibition of PKR; at least four different mechanisms are used, including inhibitory viral RNA, inhibitory viral or cellular proteins, and proteolytic cleavage (156, 212).

Table 6: **Inhibition of the Interferon system by viruses**. Viruses fight back against the host immune responses in general and in particular against the IFN system. Some viruses produce proteins that interfere with the immune response or the IFN pathway. An important target for many viruses is the inhibition of PKR; at least four different mechanisms are used, including inhibitory viral RNA, inhibitory viral or cellular proteins, and proteolytic cleavage.

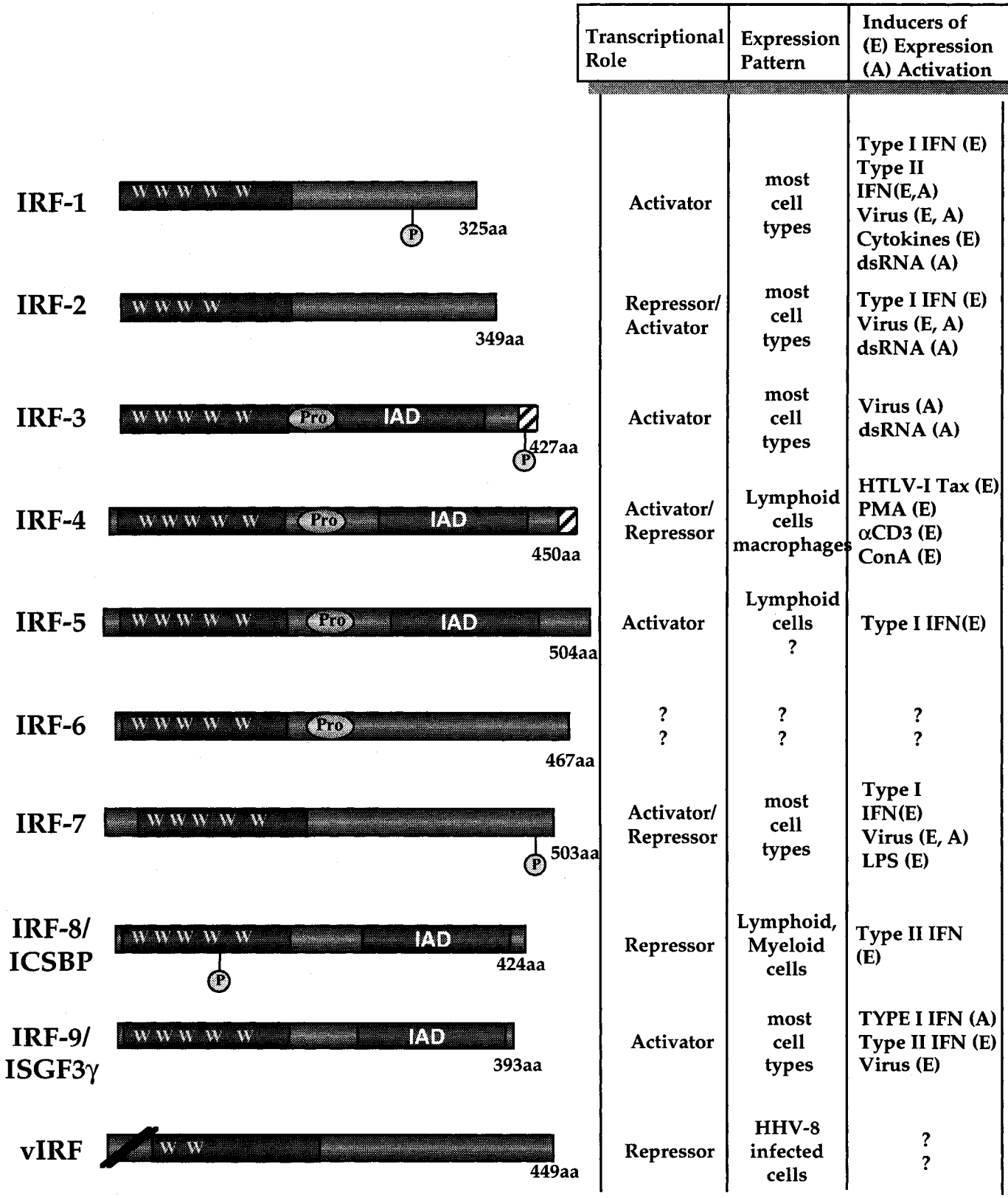
Virus	Viral gene product	Effect on the IFN system
Adenovirus	VAI RNA	Blocks PKR activation
	E1A	inhibits activation IFN induced factors (STAT)
B Hepatitis virus	TP domain of polymerase	inhibits transduction of IFN signal
Epstein-Barr virus	EBER (EBV small RNA)	Blocks PKR activation
	EBNA-2	inhibits activation IFN induced factors (STAT)
Herpes Simplex virus	2-5A analogs	Blocks RNase L activation
HIV-1	TAT	Blocks PKR activation
Kaposi Sarcoma associated Herpes Virus (HHV-8)	vIRF	homology with IRF family members
Myxoma virus	MT-7	Inhibits IFNγ signaling by soluble IFN-γ decoy
Reovirus	RNA binding protein	Blocks PKR activation
Vaccinia virus	SKI	Sequesters ds RNA
	K3L	substrate for PKR

3. Interferon Regulatory Factors

Prompt and regulated cellular response is essential to host defense and is coordinated by a genetic regulatory network in which a given transcription factor controls the expression of target genes depending on the cell type and/or the cellular stimuli. The functional diversity of such a transcription factor depends on its posttranslational modification and/or interaction with other transcription factors. Such regulatory networks are critical for host defense against extracellular pathogens. One such regulatory network is seen with the Interferon Regulatory Factor family of transcription factors.

The best characterized members of the IRF family, IRF-1 and IRF-2, were originally identified through transcriptional studies of the human IFN-β gene (48, 49, 71, 139); the family has now expanded to include seven additional members: IRF-3, IRF-4 (Pip/LSIRF/ICSAT), IRF-5, IRF-6, IRF-7, IRF-8/ICSBP, IRF-9/ISGF3γ/p48, (Figure 19) (125, 190, 212). All members of the family share homology in their first 115aa encompassing the DNA binding domain that contains a characteristic repeat of five tryptophan residues spaced by 10-18 aa. Through this DNA binding domain, IRF family members bind to similar DNA motifs termed Interferon Stimulated Response Element (ISRE; found in most IFN-inducible gene promoters, A/G NGAAANNGAAACT (30), Interferon Consensus Sequence (ICS: the ICSBP recognition site found in the MHC class I promoter, G/A G/C TTTC (36, 153, 232) or Interferon Regulatory Element (IRF-E or Positive Regulatory Domain (PRD) I and III in the IFN-β promoters, G(A)AAA G/C T/C GAAAG/C T/C (209). Recently, virally encoded forms of IRF proteins in the genome of the Human Herpes Virus 8/ Kaposi Sarcoma Herpes Virus (HHV-8/KHSV) were identified; four open reading frames encoding proteins showing homology to cellular IRFs were found in the viral genome (142, 185).

Figure 19: **Interferon Regulatory Factor family members**. The IRF family members; expression patterns and transcriptional roles. The conserved tryptophan repeats in the DNA binding domain (DBD) (black bar) are represented by W. Certain IRF family members possess a proline-rich domain shown by (Pro), an IRF Association domain (IAD), a C-terminal autoinhibition domain (hatched bars) and phosphorylation sites designated by Ⓟ .


Transcriptional Role
Expression Pattern
Inducers of (E) Expression (A) Activation
IRF-1
325aa
Activator
most cell types
Type I IFN (E)
Type II IFN(E,A)
Virus (E, A)
Cytokines (E)
dsRNA (A)
IRF-2
349aa
Repressor/ Activator
most cell types
Type I IFN (E)
Virus (E, A)
dsRNA (A)
IRF-3
Pro
IAD
427aa
Activator
most cell types
Virus (A)
dsRNA (A)
IRF-4
Pro
IAD
450aa
Activator/ Repressor
Lymphoid cells macrophages
HTLV-I Tax (E)
PMA (E)
αCD3 (E)
ConA (E)
IRF-5
Pro
IAD
504aa
Activator
Lymphoid cells
?
Type I IFN(E)
IRF-6
Pro
467aa
?
?
?
?
?
?
IRF-7
503aa
Activator/ Repressor
most cell types
Type I IFN(E)
Virus (E, A)
LPS (E)
IRF-8/ ICSBP
IAD
424aa
Repressor
Lymphoid, Myeloid cells
Type II IFN (E)
IRF-9/ ISGF3γ
IAD
393aa
Activator
most cell types
TYPE I IFN (A)
Type II IFN (E)
Virus (E)
vIRF
449aa
Repressor
HHV-8 infected cells
?
?

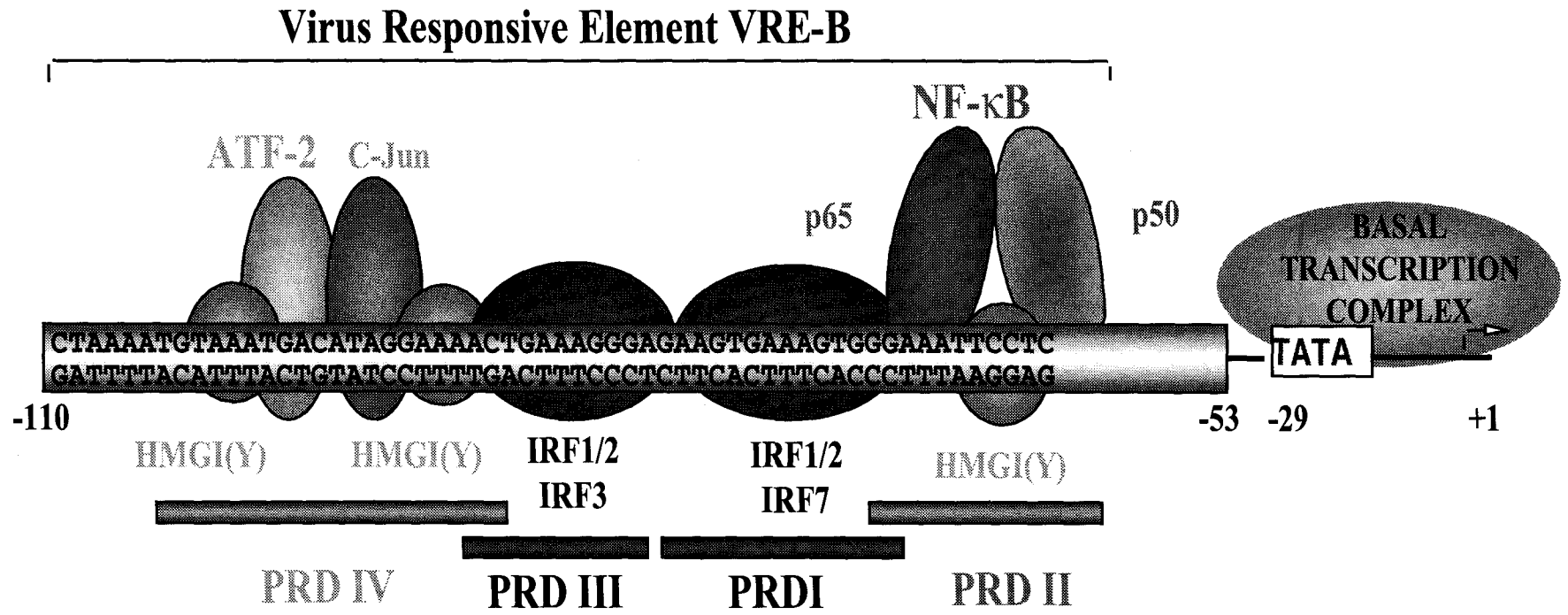
Structurally, the IRF family shares homology with the Myb oncoproteins that also display the tryptophan repeat motif in their DNA binding domain. The best-characterized member, c-Myb regulates differentiation and proliferation in immature hematopoietic and lymphoid cells (61, 113), although the relationship of the c-Myb family to the interferon system remains undefined.

The presence of IRF-like binding sites in the promoter region of IFNβ and IFNα genes implicated the IRF factors as direct regulators of IFN gene induction. As discussed above, IRF-1 was first cloned and described as a positive regulator of IFNβ gene induction while the IRF-2 factor suppressed IFN expression (Figure 20) (49, 73, 139). However, the essential role of IRF-1 in the regulation of IFNβ and IFNα genes became controversial with the observation that mice containing homozygous deletion of the IRF-1 gene, the cells derived from these mice were not impaired for IFNα and IFNβ gene expression following virus infection (132). In contrast, homozygous deletion of IRF-9 (ISGF3γ/p48) or STAT1, or IFN receptors abolished the sensitivity of these mice to the antiviral effect of IFNs, and virus infected macrophages from IRF-$9^{-/-}$ mice showed an impaired induction of IFNα and IFNβ genes, thus implicating the JAK-STAT pathway in the control of the IFNα and IFNβ gene expression (72, 114, 245).

The fact that targeted disruption of these IRF family members did not abolish primary IFN gene induction in response to virus infection led to the search of other IRF family members that would be involved in IFN gene induction. Two groups had also described high molecular weight DNA binding complexes, termed VIF and DRAF, binding to ISRE-like sequences that were specifically induced by virus infection or dsRNA and which had an IFN and protein synthesis independent activation mechanism (14, 29, 53). However, their precise molecular nature and their activation mechanism remained to be elucidated.

IRF-3 was recently characterized as a component of DRAF1 complex (230). Among the IRF family, IRF-3 and IRF-7 have recently been identified as key regulators for the induction of IFNs (117, 118, 190, 212) (Figure 20).

Figure 20: **Interferonβ promoter**. The regulatory sequences which control the transcription of the human IFNβ gene are located within a 110bp region immediately upstream from the transcription initiation site. Four distinct regions, designated PRDI to PRDIV (positive regulatory domains I to IV) are required for maximum induction of the IFNβ promoter. PRDI (-77 to-64) and PRDIII (-94 to -78) are recognition site for IRF-1, IRF-2, IRF-3 and IRF-7. The PRDII domain (-64 to -55) binds NF-κB p65 and p50 as well as HMGI(Y) factors. PRDIV interacts with the ATF-2, c-Jun and HMGI(Y). The IFNβ promoter also contains an 11bp negative regulatory element (NRE located at -60 to –50 which partially overlaps PRDII. The NRE is occupied by NRE-binding protein which represses IFNβ expression prior to and post-induction.


Virus Responsive Element VRE-B
NF-κB
ATF-2
C-Jun
p65
p50
BASAL TRANSCRIPTION COMPLEX
CTAAAATGTAAATGACATAGGAAAACTGAAAGGGAGAAGTGAAAGTGGGAAATTCCTC
GATTTTACATTTACTGTATCCTTTTGACTTTCCCTCTTCACTTTCACCCTTTAAGGAG
TATA
-110
-53
-29
+1
HMGI(Y)
HMGI(Y)
IRF1/2
IRF3
IRF1/2
IRF7
HMGI(Y)
PRD IV
PRD III
PRDI
PRD II

3.1 IRF-4

The birth of another member of the IRF family resulted from an effort to clone factors binding to the murine immunoglobulin light chain enhancer $E_{\lambda 2\text{-}4}$ (39). PU.1 interaction partner, Pip or IRF-4, was identified as a novel murine transcription factor with an IRF-like N-terminal domain. IRF-4 bound to DNA, but exclusively in association with PU.1, a member of the ETS family of transcription factors that, in their own right, contribute to lymphoid and myeloid lineage development (15). Serine phosphorylation at aa 148 of PU.1 was required for PU.1-Pip interaction and subsequent binding of the heterodimer to the ISRE-like λB site in the Ig enhancer region (Figure 19).

Independently, during the isolation of Pip, another group also cloned the same protein which they called lymphoid specific IRF (LSIRF) (131). In contrast to the previous group, Matsuyama et al. showed that IRF-4 was able to bind autonomously to DNA, specifically to the ISRE of the MHC class I promoter. Unlike most of the IRF family members, IFNs did not induce IRF-4 expression. In primary cultured lymphocytes, IRF-4 is expressed at low levels but can be dramatically induced by antigen-receptor mediated stimuli such as plant lectins, CD3 or IgM crosslinking.

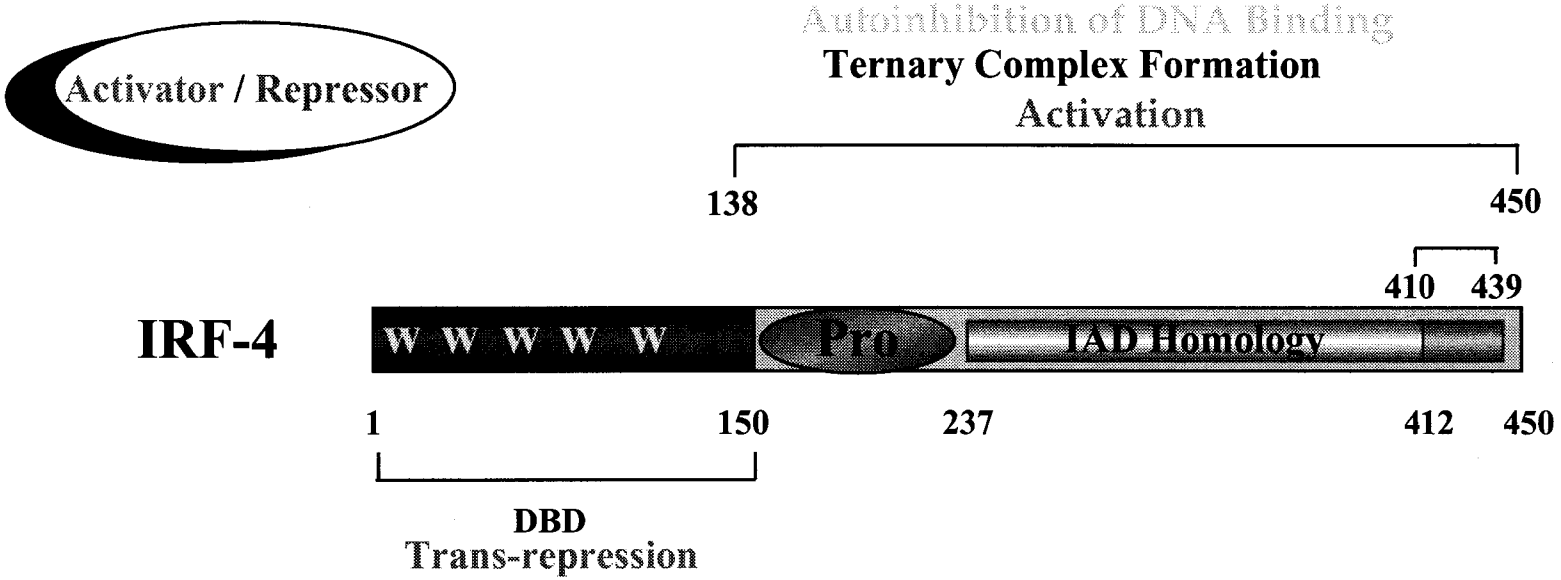
A third group, Yamagata et al. also isolated the human equivalent of Pip/LSIRF from an adult T-cell leukemia cell line, hence its name IFN consensus sequence-binding protein in adult T-cell leukemia cell lines or activated T-cells (ICSAT) (239). Like murine IRF-4, human IRF-4 is structurally most similar to IRF-8/ ICSBP, but its expression is not inducible by IFNs. However, ICSAT/IRF-4 possesses a very different function compared to its murine counterpart. While PU.1-Pip functioned as a transactivator complex, ISCAT/ IRF-4 exerted an IRF-2 and IRF-8/ICSBP-like repressive effect on IFN- and IRF-1-induced gene activation. ICSAT/IRF-4 is expressed exclusively in the lymphoid lineage but expression is restricted to a specific subset of lymphocytes: only T-cells treated with phorbol myristate acetate (PMA) or infected with the Human T-cell Leukemia Virus-1 (HTLV-I) express this IRF protein.

3.1.1 IRF-4 structure-function analysis

Structurally, IRF-4 most closely resembles IRF-8/ ICSBP, sharing 80% N-terminal homology and 48% homology over a 160 aa C-terminal region of IRF-4. The amino-terminal of IRF-4 is highly hydrophilic and is separated from the carboxy-terminal half by a potential PEST (a region rich in proline, glutamate, serine and threonine residues) whose presence in intracellular proteins correlates with short half-life (39). This 31-aa domain (aa208-238) contains 45% P, E, S, and T and is flanked by basic amino acids. The region of IRF-4 protein that contains this putative PEST domain is in general proline-rich (aa151-237, 20% proline), representing a potential transcriptional activation domain. In addition, there is a region in the C-terminus of IRF-4 that is rich in glutamine residues (aa354-419, 15% glutamine). Glutamine-rich domains have also been shown to function as transcriptional activation domains (Figure 21) (39).

As mentioned, IRF-4 was originally cloned by virtue of its ability to bind to the λB and the κ3' elements of the immunoglobulin light chain enhancer. Studies *in vitro* and *in vivo* have demonstrated that full-length IRF-4 can only bind to these elements in the presence of PU.1 phosphorylated at serine-148. The DBD of IRF-4 (aa1-150) alone was able to bind DNA in the absence of its partner, suggesting a potential inhibitory domain within IRF-4 which prevented DNA binding by the full-length protein; this domain may be masked by IRF-4 association with PU.1. The PU.1- IRF-4 dimer functions as a transactivator when bound to the enhancer but these factors display mutual co-dependence for activity. PU.1 is able to bind DNA on its own, but will not transactivate unless associated with IRF-4; therefore, PU.1 and IRF-4 function as mutually dependent transcription factors (Figure 22).

Figure 21: IRF-4 and PU.1 schematics. (A) IRF-4 is an IRF which is able to both activate and repress transcription of target genes. IRF-4 possesses the conserved tryptophan repeats in the N-terminal 115 amino acids which comprises the DNA binding domain (DBD; red). Homology extends to the IRF Association Domain (IAD; grey), a region responsible for interaction with IRF-8/ ICSBP. An activation domain is found between aa 138-450. IRF-4 contains a 31-aa domain (aa208-238) which possesses 45% P, E, S, and T and flanked by basic amino acids. The region of IRF-4 protein which contains this putative PEST domain is in general proline-rich (aa151-337, 20% proline; green), representing a potential transcriptional activation domain. Two regions of IRF-4 are responsible for autoinhibition of DNA binding and ternary complex formation: the domain found between aa 138-450 (blue) is a separate functional domain whose fusion to a heterologous IRF DBD prevents DNA binding and association with PU.1 and DNA, whereas aa410-439 (pink) is not a separable domain but is essential for this autoinhibition. **(B)** PU.1 has a C-terminal Ets domain (aa167-260; dark green) which is involved in DNA binding as well as protein-protein interaction with AP-1 family members. Immediately adjacent to the Ets domain is a proline-, glutamic acid-, serine-, and threonine-rich (PEST) region (aa108-167; blue) which is involved in protein-protein interaction with IRF-4. Phosphorylation of Serine-148 by CKII is critical for IRF-4 interaction and subsequent DNA binding. At the N-terminus of PU.1, resides a series of 3 independent transcriptional activation domains, including two acidic subdomains (aa33-74; light green) and one glutamine-rich domain (aa74-100; black). The N-terminus has also been shown to be involved in protein-protein interaction with Rb and TBP. Adapted from Rao, 1999.


Activator / Repressor
Autoinhibition of DNA Binding
Ternary Complex Formation
Activation
138
450
410
439
IRF-4
W W W W W
Pro
IAD Homology
1
150
237
412
450
DBD
Trans-repression

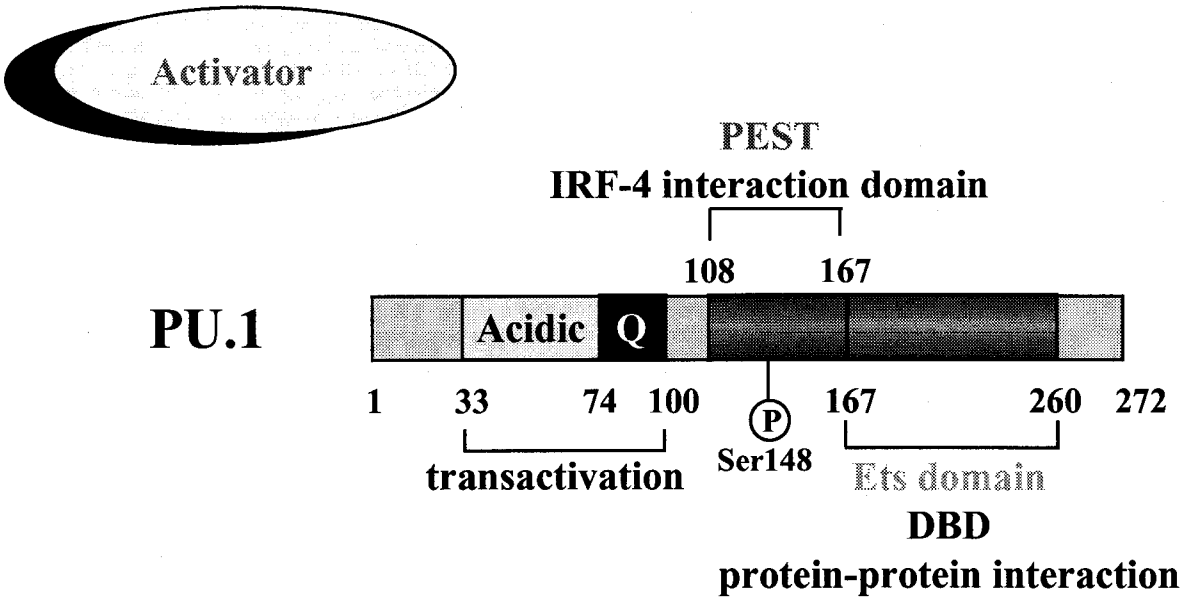
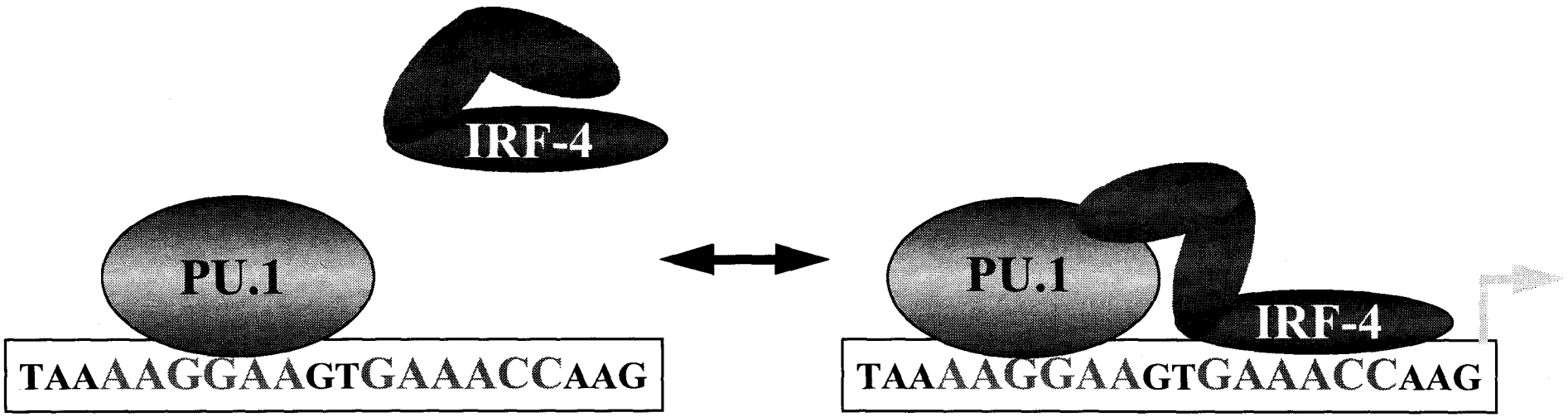

Activator
PEST
IRF-4 interaction domain
108
167
PU.1
Acidic
Q
1
33
74
100
P
167
260
272
transactivation
Ser148
Ets domain
DBD
protein-protein interaction

Figure 22: Ternary complex formation. Autoinhibition of the DNA binding activity of IRF-4 is indicated by an interaction between the N-terminal DBD and C-terminal autoinhibitory domain. This inhibition is alleviated by an interaction of the autoinhibitory region of IRF-4 and the PEST region of PU.1 containing phosphoserine-148. Following interaction of IRF-4 with PU.1 and DNA, IRF-4 undergoes a conformational change that swivels the regulatory domain (aa170-450) away from its own DBD (aa 1-150), and into direct contact with the PEST region of PU.1. Additional contact may occur between the DBDs of IRF-4 and PU.1. Adapted from Brass,1996.



λB sequence of the Immunoglobulin Light Chain gene enhancer

Further studies have mapped unique functional domains in the IRF-4 protein (15). IRF-4's closest sibling, IRF-8/ ICSBP, is also able to form a ternary complex with PU.1 and the λB DNA sequence. However, unlike PU.1- IRF-4, PU.1-IRF-8 (ICSBP) does not activate nor repress expression of a reporter CAT construct driven by λB sites, indicating that the transactivation observed with PU.1- IRF-4 must result from an activation domain present in IRF-4. Indeed, the C-terminal region of IRF-4 consisting of aa 150 to aa 450 exerted transcriptional activity when fused to the heterologous GAL4 DBD. Two regions within the aa 150-450 segment of IRF-4 may serve as activation domains: a pro-rich segment (aa151-237) and a C-terminal region (aa354-419) containing 15% glutamine residues. The functional difference between PU.1-IRF-4 and PU.1-IRF-8 complexes may be explained by the presence of this putative activation domain within IRF-4 (15).

Other IRFs such as IRF-8/ ICSBP, IRF-1, IRF-2 and IRF-9/ ISGF3γ are able to specifically bind to the λB site. IRF-1 and IRF-9/ ISGF3γ recognized λB and activated transcription of λB-CAT constructs. The DBD of IRF-4 (aa1-150) alone, but not the IRF-4 activation domain (aa150-450), was able to trans-repress IRF-1 and IRF-9/ISGF3γ-mediated transactivation in the absence of PU.1. IRF-8/ ICSBP, as well as IRF-2, had a similar effect as IRF-4 on IRF-1 and IRF-9/ISGF3γ-mediated transactivation of λB-CAT constructs (15). IRF-4wt, IRF-4 aa1-150 and IRF-8/ ICSBP also inhibited the activation of the λB-CAT construct in NIH3T3 cells stimulated with IFNα/β. Interferon stimulation of NIH3T3 cells resulted in inducible binding of IRF-1, IRF-2 and IRF-9/ISGF3γ to the λB site. This data suggests that IRF-4 is a "dichotomous regulator"; as it can block IRF-1- and IRF-9/ISGF3γ-mediated transactivation in the absence of PU.1, or activate transcription in conjunction with PU.1. The biological significance of the ability of IRF-4 to repress transcription is unclear. IRF-4 may function as a buffering agent in lymphoid cells (B cells, macrophages and activated T cells) exposed to IFNα/β, by attenuating expression of IFN-inducible genes and inhibiting the anti-proliferative effects of IFNs (15).

The λB element and the homologous element within enhancer κ3' are closely related to the ISRE consensus sequence to which IRF family members bind. The λB element sequence is TAAAAGGAAGTGAAACCAAG; PU.1 binds to the first underlined site and IRF-4 to the second one (15). The ISRE consensus is A/GNGAAANNGAAACT (30). It should noted that, whereas the λB element can function as an ISRE, the converse appears not to be the case. The following ISREs: ISG15, MHC I and ISG-54 do not support PU.1-IRF-4 ternary complex formation (15).

Interestingly, IRF-4 possesses a domain in its C-terminal end (aa 410 to aa 439) which prevents the protein from binding to DNA in the absence of PU.1 and is concomitantly important for high affinity ternary complex formation. When the region of IRF-4 containing the DNA autoinhibitory domain (aa 138 to aa 450) is fused to the DBD of IRF-9/ISGF3γ, it prevents IRF-9/ISGF3γ binding to DNA and also confers PU.1-IRF-9 ternary complex formation with λB. A model for the regulation of IRF-4 activity was proposed by Brass et al (15): The C-terminal DNA binding autoinhibition domain of IRF-4 exerts its repressive effects by folding over and interacting with the DBD of IRF-4. In the presence of a PU.1 protein whose serine 148 is phosphorylated, PU.1- IRF-4 interaction occurs which disrupts IRF-4 autoinhibition and subsequent high affinity ternary complex formation with DNA. Potential association between PU.1 and the DBD of IRF-4 may also contribute to their DNA binding activities.

Recent studies by Brass et al. have shown that the IRF-4/ PU.1 binding is cooperative and regulated by multiple interdependent DNA-protein and protein-protein interactions (16). A previously identified α-helical region within the IRF-4 carboxy-terminus (aa395-413) is critical for both ternary complex formation (IRF-4/ PU.1/ DNA) and for autoinhibition of DNA binding by maintaining IRF-4 in a closed conformation. The IRF-4 regulatory domain (aa170-450) is the region which interacted with the PEST region of PU.1 in binding to a λB element. Although much weaker, a cooperative interaction between the IRF-4 and PU.1 DNA binding domains was also detected (16).

A more detailed model of IRF-4/ PU.1 complex activation has since been proposed: following interaction of IRF-4 with PU.1 and DNA, IRF-4 undergoes a conformational change that swivels the regulatory domain (aa170-450) away from its own DBD (aa 1-150), and into direct contact with the PEST region of PU.1. Ortiz et al. also delimited the IRF-4 interaction domain to residues 245-422 (158). Site-directed mutagenesis of conserved amino acids within two predicted α-helices confirmed the importance of these residues for IRF-4-PU.1 DNA binding and transactivation. These two α-helices are also highly conserved amongst the other IRF family members and could therefore be involved in heterodimerization with different transcription factors (158).

Perkel et al. have also suggested a similar model for PU.1 recruitment of IRF-4 to DNA. PU.1 and IRF-4 can physically interact via the PU.1 ETS domain and IRF-4 DNA binding domain. This interaction does not lead to IRF-4 recruitment to the DNA. Phosphorylation of PU.1 on ser-148 (either before or after interaction with IRF-4) induces a conformational change in the IRF-4 autoinhibitory and DNA binding domains. IRF-4 is subsequently recruited to the λB DNA element (165). It has been suggested that the DNA binding domains of IRF-4 and PU.1 also mediate their cooperative interaction. Yee et al. determined that the minimal DNA-binding domain of IRF-4 was aa20-137, corresponding to the conserved DNA binding region of other IRFs. Fluorescence polarization of fluorescein-labelled DNA was used to shown the presence of aa 1-19 decreased the binding affinity of IRF-4 N-terminal constructs from two- to fivefold. However, all IRF-4 constructs bound better to the λB element in the presence of the DNA binding domain of PU.1 (aa160-272). This cooperative interaction was not dependent on phosphorylation of the PEST domain of PU.1 (ser-148), but was dependent on the proper spacing of the binding sites of IRF-4 and PU.1 (243). Within the IRF family, IRF-4 has the longest sequence (aa1-19) N-terminal to its DBD, suggesting a function specific to IRF-4. At least part of this function could be its DNA binding inhibitory properties since IRF-4 is a poor DNA binding protein compared to other IRFs such as IRF-1, IRF-2 and IRF-3. NMR

spectroscopy of 15N-labelled IRF-4 constructs indicates that aa1-19 may be unstructured (243). Gross et al. also determined the presence of cooperative interactions between the DBDs of IRF-4 and PU.1 by quantitative hydroxyl radical footprinting. The DBDs of both proteins bound to DNA in the major groove with potential protein-protein interactions near the intervening minor groove (65).

IRF-4 cellular localization to this date is still controversial. Initial studies using indirect immunofluorescence and GFP-tagged IRF-4 revealed it to be mostly nuclear (15) (YM unpublished data). IRF-4 is not restricted to compartments within the nucleus such as nuclear bodies but found in a diffuse pattern in all the nucleus except the nucleoli (43). Recent evidence suggests that IRF-4 is also present in the cytoplasm of primary effusion lymphoma (B cell non-Hodgkin's lymphomas)(21), multiple myeloma cells (43), mouse lens cells (116) and in macrophages (126).

Studies of IRF-9/ISGF3γ revealed the presence of a bipartite nuclear retention signal within its N-terminal DNA binding domain. This signal is conserved in two other IRF proteins, IRF-4 and IRF-8/ICSBP (107). Bipartite basic signals typically consists of two interdependent clusters of basic aa separated by a flexible linker of at least 10 amino acids. In agreement with this definition, the determined IRF DNA-binding domain crystal structure illustrated that 10 residues including the flexible loop, L2, separate residues 66-70 and 80-85. Mutations in either cluster of basic amino acids within IRF-4 (residues 78, 80, 82 or 94, 96, 98 were changed to alanine) resulted in the loss of nuclear retention. A model was also proposed where these IRFs, IRF-4, IRF-9/ISGF3γ and IRF-8/ICSBP could be retained in the cytoplasm by interaction with other proteins (IRF-4 and PU.1, IRF-9/ISGF3γ and STAT2) until the proper stimulus leads to nuclear translocation of the IRF in question (107).

3.1.2 IRF-4 in B cells

The expression of murine immunoglobulin heavy and light chain genes is tightly controlled in a cell-type- and developmental stage-specific fashion. This regulation occurs at two levels, transcription and recombination. Transcription of Ig genes is regulated by cell-type-specific promoter and enhancer elements (38). Cell-type-specific enhancers were first identified in the J-C introns and in the 3' constant region of both the heavy and κ light chain. The λ light-chain gene locus, which is organized differently also possesses two transcriptional enhancers, each located 3' of the J λ - C λ gene cluster. These two enhancers, designated E λ2–4 (15.5Kb 3' of C λ 2-C λ 4) and E λ3–[35 Kb 3' of C λ 3-C λ 1), are more than 90% homologous and are thought to have arisen by an evolutionary gene duplication event, and presumably function in a similar manner (39). The E λ2–4 contains two domains λA and λB, which are essential for enhancer activity. The λB element consists of two juxtaposed but distinct transcription factor binding sites. One site of the composite λB element is bound by PU.1, a member of the ETS family of transcription factors which contribute to lymphoid and myeloid lineage development (31, 127). The other site is likely occupied by a factor first called NF-EM5, which was later identified as Pip or IRF-4 (38, 39).

PU.1 This ETS factor is crucial for lymphoid and myeloid cell development (Figure 21). It regulates the expansion of progenitors by controlling the expression of important genes (31). In B cells, PU.1 binding is important for the transcriptional activity of the immunoglobulin heavy and light chain (κ and λ) enhancers and the J chain, mb-1, CD20 (B cell marker involved in B cell activation and regulation), CD45 (transmembrane tyrosine phosphatase) and CD72 (B cell marker important in B-T cell interactions) (59, 127). Similarly, PU.1 is necessary for promoter functions of a number of myeloid specific genes including CD11b (involved in adhesion and phagocytosis), CD18 (adhesion molecule), c-fes, granulocyte colony-stimulating (G-CSF) receptor, macrophage-CSF receptor, c-fms, macrophage scavenger receptor, FcγRIb (involved in macrophage activation and phagocytosis), FCγRIIIA , IL-1β, and IL-18 (99, 127, 128). PU.1 -/- mice are severely impaired in lymphoid (B cells) and

myeloid (macrophages and neutrophils) lineages. The block in the development of these lineages occurs at very early stages, the PU.1 -/- mice lack lymphoid and myeloid progenitor cells, rendering it difficult to determine the precise role of PU.1 in stage- and lineage-specific gene expression. Recently, Dekoter et al. demonstrated that reconstitution of PU.1 expression by retroviral transduction in PU.1-/- hematopoietic progenitor cells allowed them to differentiate into macrophages and B cells *in vitro*. Interestingly, the progenitor cells expressing high levels of PU.1 were committed to differentiate into macrophages, whereas the cells with low levels of PU.1 were committed to differentiate into pre-B cells (31). SPI-B is a B cell-specific ETS transcription factor that shares high degree of similarity with PU.1. Rao et al. demonstrated that SPI-B interacts with the PU.1 binding partner IRF-4 as well as TBP, cJun and NF-IL6 (174). PU.1 may therefore act also as a scaffolding protein which bind DNA and then allows other transcription factors to bind through protein-protein interaction.

IRF-4 and PU.1. The discovery of another member of the IRF family resulted from an effort to clone factors binding to the murine immunoglobulin light chain enhancer $E\lambda_{2\text{-}4}$ (39). PU.1 interaction partner, or Pip/IRF-4, was identified as a novel murine transcription factor with an IRF-like N-terminal domain (Figure 21). IRF-4 bound to DNA, but exclusively in association with PU.1, a member of the ETS family of transcription factors which contribute to lymphoid and myeloid lineage development (reviewed in (127)). Serine phosphorylation at aa148 of PU.1 was required for PU.1-IRF-4 interaction and subsequent binding of the heterodimer to the ISRE-like λB site in the Ig enhancer region (15, 39).

Expression of IRF-4 was detected in all stages of B cell differentiation (higher in the later stages) and activated T cells; however, PU.1 is exclusively expressed in B cells and myeloid cells, thus conferring B-cell specificity to the PU.1- IRF-4 heterodimer. In an attempt to characterize novel genes involved in B cell tolerance and activation, Glynne et al demonstrated by microarray analysis that IRF-4 expression was specifically increased during B cell activation (59, 60). In B cells, IRF-4 can be

upregulated by IL-4 and anti-CD40 costimulation (B cell activation stimuli) (69). The PU.1- IRF-4 dimer functions as a transactivator when bound to the enhancer but these factors display mutual co-dependence for activity. To analyze the function of the IRF-4 /PU.1 dimer *in vivo*, a chimeric repressor was engineered by fusing PU.1 and IRF-4 DNA binding domains through a flexible POU domain. This fused dimer strongly repressed expression of a rearranged immunoglobulin λ gene (IgLl) and therefore established the importance of the IRF-4/ PU.1 complex in B cell gene expression (16).

IRF-4-PU.1 heterodimer also regulate an other gene important in B cell development and activation; CD20 is a B-lineage-specific gene expressed at the pre-B cell stage of B cell development that disappears when B cells terminally differentiate into plasma cells (78). It is interesting to note that IRF-4-PU.1 regulate genes such as Ig kappa light chain, Ig lambda light chain and CD20 which are important in early B cell development and differentiation.

Previous studies have also demonstrated that PU.1 and IRF-4 function synergistically with c-Fos, c-Jun and E12/E47 to stimulate the κE3'-enhancer (immunoglobulin κ light chain). PU.1 participates in the assembly of this enhanceosome. A recent study by Rieske et al. demonstrated that the functional synergy between these factors is enhanced in response to mitogenic associated protein kinase (MAPK), MEKK1, in NIH3T3 cells, where the enhancer is inactive. MEKK1 was able to stimulate the activity of PU.1 but was unable to induce the κE3'-enhancer activity. The Ras-PI3K-dependent external regulated kinase, AKT, could however induce κE3'-enhancer activity both in pre-B and plasmacytoma cells. AKT stimulation of the κE3'-enhancer was primarily due to PU.1 induction. Activation of AKT had no effect on PU.1, IRF-4 expression levels or protein-protein interaction between the two factors. Through a series of deletions, they determined that the PU.1 acidic-rich (aa33-74) transactivation domain was necessary for AKT mediated induction (177). These results provide evidence that PU.1 is a target for phosphatidylinositol 3-kinase dependent signal where AKT is involved in the inducible or constitutive activation of

its target genes. AKT or MEKK1 stimulation of PU.1 suggests that PU.1 induction may be mediated through the activation of IKKs.

Potential PU.1/IRFcomposite sites have also been identified within the IL-1β, IL-18 and macrophage scavenger receptor promoters. PU.1 and IRF-4 could be involved in the regulation of the above mentioned genes (127).

IRF-4 and E47. PU.1 is no longer the only transcriptional activating partner of IRF-4. The role of IRF-4 as a transcriptionally activating partner in B cells is further reinforced by the observation that IRF-4 interacts with E47, a component of the E2A transcription factor. The ubiquitously expressed E2A is crucial for normal B cell development and is composed of E12 and E47, which are two splice variants of the same gene. E2A -/- mice fail to develop B cells past the pro-B-cell stage (149). IRF-4 was shown to bind together with E47 to the immunoglobulin κ 3' light chain enhancer region and to generate a 100-fold transcriptional synergy in a reporter gene assay. Meraro et al. demonstrated that the IAD of IRF-4 and of IRF-8/ ICSBP mediated the interaction and transcriptional synergy with non-IRF members such as PU.1 and E47. The IAD domain within IRF-1 and IRF-2 were also crucial for interaction with IRF-8/ICSBP. These interactions through the IAD seem necessary for regulation and modulation of the immune system (135).

IRF-4 and Stat6. Stat6 can also interact with IRF-4 and drive the expression of IL-4 -inducible genes such as CD23 (69). Stat6 is activated primarily by Jak1 and Jak3 bound to the IL-4R upon IL-4 stimulation (236). IL-13, being functionally similar to IL-4, is also capable of activating Stat6. Both these cytokines are important in proliferation, viability, gene expression and differentiation of lymphocytes (236). The IL-4-inducible gene CD23, is expressed on activated B cells and macrophages. It is a low affinity receptor for IgE and regulates IgE synthesis. The transactivating ability of IRF-4 on the CD23 promoter was also blocked by the repressor factor BCL-6 (B cell lymphoma 6). BCL-6 is a Kruppel zinc finger repressor, which is highly expressed in germinal B cells and is frequently altered in non-Hodgkin's lymphomas.

BCL-6 has been shown to bind to the GAS element of the CD23 promoter and repress Stat6-mediated promoter function. Through knockout analysis, the role of BCL-6 has been defined as a repressor of IL-4 signaling *in vivo* (32). Interestingly, the expression of IRF-4 in B cells increases with differentiation while the expression of BCL-6 decreases. IRF-4 expression in B cells can also be upregulated by IL-4 and anti-CD40 alone or in combination (69). This may be an important step in progression of B cells toward the terminal stages of differentiation. IRF-4 is therefore both a target and a modulator of the IL-4 signaling pathway.

IRF-4, BCL-6 and Blimp1. Gupta et al. previously demonstrated that IRF-4 was induced in response to B cell-activating stimuli and could act as a transactivator of the CD23 gene expression (69). IRF-4 function was blocked by B cell lymphoma 6 (BCL-6) a Krüppel-type zinc finger repressor normally expressed in germinal center B cells. However, CD23 expression is known to be downregulated in plasma cells despite the high level expression of IRF-4 and the lack of BCL-6. This suggests that in plasma cells, the IRF-4-mediated induction of CD23 is prevented by its interaction with another distinct repressor. IRF-4 was shown to interact with B lymphocyte-induced maturation protein (Blimp1) or positive regulatory domain I-binding factor 1 (PRD1-BF1). This Krüppel-type 88kDa protein containing five zinc-finger motif, is mainly detected in late B and plasma cells. Interestingly, this factor had been previously shown to target IRF binding site in the IFNβ enhancer and act as a potent repressor of PRDI-dependent transcription (97). Gupta et al. reported that Blimp1 could bind to the same functional element in the human CD23 promoter to which BCL-6 and IRF-4 had been previously shown to bind. Like BCL-6, Blimp1 repressed IRF-4 transactivating ability (68). IRF-4 function seems to be modulated in a stage-specific manner by its interaction with developmentally restricted sets of Krüppel-type zinc finger proteins.

IRF-4 -/- B cells. IRF-4 deficient mice were generated and like many other IRF-/- mice developed severe immunodeficiencies (137). A normal T- and B- cell distribution was observed at 4 to 5 weeks of age, but with time IRF-4-/- mice

gradually exhibited severe lymphadenopathy. At 10 to 15 weeks, although the thymus of IFR-4-/- mice was normal, the spleen was enlarged 3 to 5 times and lymph nodes were enlarged 10 times compared to normal mice. This was due to an increase in number of CD4+ and CD8+ T cells and B cells. Both B- and T-cell activation were profoundly affected. In B cells, serum immunoglobulin concentrations and antibody responses were decreased. Antibody secretion in response to LPS or α-CD40 was severely impaired in the IRF-4 -/- mice. As well the addition of IL-4, LPS, α-IgM alone or in combination could not restore B cell proliferation in these mice. IRF-4 has been shown to bind to immunoglobulin light chain gene enhancers. Together, these facts could explain the significant impairment of immunoglobulin production in IRF-4 -/- mice (137).

IRF-4 and B cell Lymphomas. The role of IRF-4 in B cell lymphomas and myelomas has also been investigated by many groups. Multiple myelomas, diffuse large B cell lymphomas, B cell chronic lymphocytic leukemias, small lymphocytic leukemias, Hodgkin's lymphomas and primary effusion lymphomas all express high levels of IRF-4 (21, 84, 217). The pathogenesis of multiple myeloma, an incurable tumour causing the deregulated proliferation of B cells is unclear. In some patients with multiple myeloma, a chromosomal translocation - t(6;14)(p25;q32) - juxtaposes the immunoglobulin heavy-chain (IgH) locus to MUM1 (multiple myeloma 1); the MUM1 locus at 6p25 is identical to IRF-4. IRF-4 is transcriptionally activated as a result of this chromosomal translocation (217). This translocation involving IRF-4 may thus contribute to leukemogenesis since MUM1/IRF-4 has oncogenic activity *in vitro* (84).

IRF-4 expression was also detected in primary effusion lymphoma (PEL). PEL is a peculiar B cell lymphoma characterized by infection by Human HerpesVirus-8/ Kaposi sarcoma-associated herpesvirus and preferentially grows in the serous body cavities. PELs exist predominantly as malignant effusions in the visceral cavities, usually without significant tumor mass or lymphadenopathy. These lymphomas occur predominantly in HIV-seropositive individuals with advanced stages of

immunosuppression (13). Carbone et al. demonstrated that 1) IRF-4 expression in PEL corroborated the notion that PEL originates from the post-germinal center, preferentially from differentiated B cells which are BCL-6 negative 2) IRF-4 may help in the differential diagnosis of PEL among other lymphomas involving serous body cavities and 3) IRF-4 may interact with the HHV-8 vIRF and thus contribute to PEL escape from interferon-mediated control of viral infection (21).

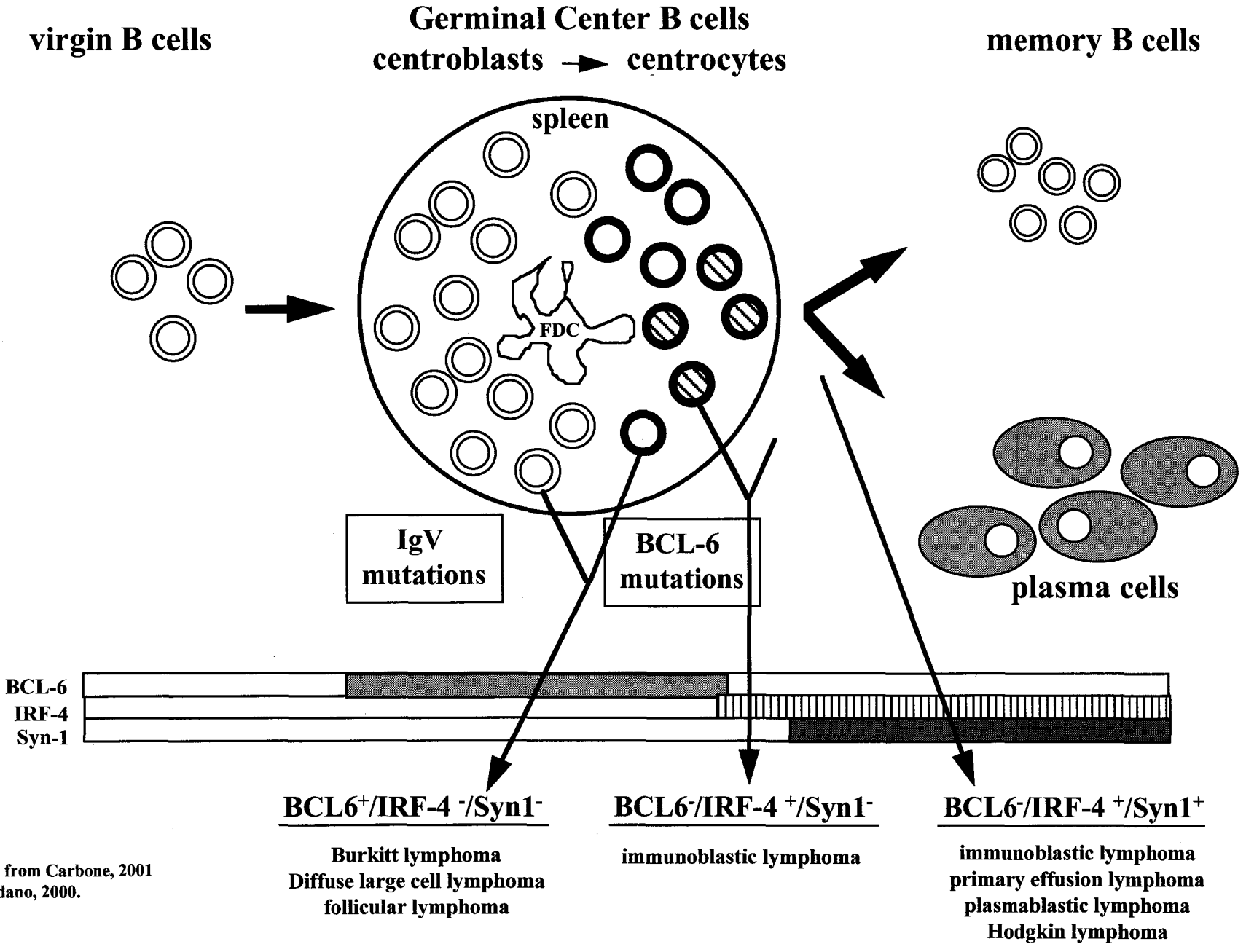
Recently the field of B cell lymphoma histogenesis has progressed rapidly due to the increasing availability of histogenic markers. Genotypic markers of B cell histogenesis are represented by mutations of IgV and BCL-6 genes, which are somatically acquired at the time of B cell transit through the germinal center (GC) of the spleen. Phenotypic markers such as BCL-6 and CD138/Syndecan-1 allow the distinction between GC and post-GC B cells (Figure 23). IRF-4 has now been added to the panel of phenotypic markers of B cell lymphoma histogenesis (50). The use of theses three markers: BCL-6 (expressed in centroblasts and centrocytes), IRF-4 (expressed in late centrocytes and post-germinal center B cells) and CD138/Syn-1 (expressed by post-GC B cells) now permits a better understanding of B cell lymphomas origins (22).

A recent study of HIV-related B cell lymphomas revealed a new classification pattern: 1) Burkitt, diffuse large cell and follicular lymphomas which originated from centroblasts and centrocytes were classified as BCL-6+/IRF-4-/Syn- 2) immunoblastic lymphomas (originated from centrocytes and post-germinal center B cells) were classified as BCL-6-/IRF-4+/Syn- and 3) primary effusion, plasmablastic and Hodgkin lymphomas (originated from post-GC B cells) were BCL-6-/IRF-4+/Syn+ (Figure 23) (22, 50). The expression pattern of these markers may contribute to differential diagnosis of B cell lymphomas, influence prognosis and treatment.

IRF-4 and cRel. The control of IRF-4 expression has important potential physiological consequences: Aberrant expression of IRF-4 could lead to dysregulation of immunoglobulin synthesis, B cell differentiation and activation, and

leukemogenesis. A recent study of cRel -/- B cells revealed that IRF-4 expression was dramatically decreased (66). Promoter analysis of the murine IRF-4 gene revealed two important κB element (-1733 and -686) involved in IRF-4 expression. The induction of IRF-4 by Tax was also shown to be Rel/NF-kB dependent. The finding that IRF-4 is a transcriptional target of Rel may have important implication for v-Rel (viral oncoprotein of the avian reticuloendotheliosis virus) mediated transformation. v-Rel -induced changes in IRF-4 expression could disrupt cell-growth and may help explain the basis of lymphoid-specific transformation by v-Rel. Recently, chicken IRF-4 was cloned and shown to increase v-Rel-induced transformation in fibroblasts (Pose, unpublished data, 2000).

Figure 23: A model for the histogenesis of B cell lymphomas. This model is based on the physiological stages of mature B cell-development identified by histogenetic markers, such as mutation pattern of immunoglobulin variable region genes (IgV) and BCL-6 gene. Virgin B cells do not display immunoglobulin or BCL-6 mutations and lack expression of BCL-6, IRF-4 and Syn-1/CD138. At the time of B cell transit through the germinal center (GC) of the spleen or lymph nodes, B cells acquire IgV and BCL-6 mutations, which are maintained during further differentiation, thus constituting markers of GC transit. Post-GC B cells undergoing maturation toward the plasma cell stage switch off BCL-6 and stain positive for Syn-1. IRF-4 seems expressed in the late stages of intra-GC differentiation (centrocytes) and by post-GC B cells undergoing plasma cell maturation. GC B cells are identified as follows; centroblasts: large open circle, centrocytes: circle with black outline, IRF-4+ centrocytes: stripped circle with black outline, FDC: follicular dendritic cells. The putative histogenic derivation of each lymphoma category is indicated by an arrow originating from the relevant B cell compartment. Adapted from Carbone, 2001 and Gaidano, 2000.



Adapted from Carbone, 2001
and Gaidano, 2000.

3.1.3 IRF-4 in T cells

Independently, another group cloned IRF-4 from murine spleen cells as a lymphoid specific IRF (LSIRF) (131), expressed at all stages of B cell development and in mature T cells. IRF-4 expression was also inducible in primary lymphocytes by antigen mimetic stimuli such as Concavalin A, CD3 crosslinking, anti-IgM, cycloheximide and PMA treatment whereas IFNs or TNFα did not induce expression. The induction of this unique IRF family member by T-cell activation signal such as PMA, Con A and CD3 crosslinking implies that IRF-4 functions in the transduction of proliferative signals in response to T-cell activation. Furthermore, ConA stimulated expression of IRF-4 was significantly downregulated by cyclosporin A treatment, an immunosuppressant which inhibits T cell activation. IRF-4 expression in activated T cells was also shown to parallel CD30 expression (Ki-1, found in activated T and B cells) (43). In contrast to B cells, IRF-4 could bind autonomously to the ISRE of the MHC class I promoter (131).

The murine IRF-4 promoter revealed several potential transcription factor binding sites such as NF-IL6, AP2, AP1, NF-κB, Ets-1, GATA-3 and PU.1. There were no obvious ISRE or GAS sites which support the finding that IRF-4 is not IFNα/β nor IFNγ inducible (131). A personal communication (Dr. L. Glimcher, Boston, USA) has provided insight in the regulation of IRF-4 expression. Double knockouts of NF-ATc2 and NF-ATc3 T and B cells have decreased IRF-4 expression. IRF-4 expression in the knockout T cell can longer be upregulated by stimuli. IRF-4 expression seems to be regulated by both the NF-AT and NF-κB/Rel families of transcription factors (66) (Sharma, 2001 in preparation)(Dr. L. Glimcher, personal communication).

IRF-4 -/- T cells. IRF-4 deficient mice were generated and, like many other IRF-/- mice, developed severe immunodeficiencies (137). A normal T- and B-cell distribution was observed at 4 to 5 weeks of age, but with time IRF-4-/- mice gradually exhibited severe lymphadenopathy. At 10 to 15 weeks, although the thymus of IFR-4-/- mice was normal, the spleen was enlarged 3 to 5 times and lymph nodes

were enlarged 10 times compared to normal mice. This was due to an increase in number of CD4+ and CD8+ T cells and B cells. Both B- and T-cell activation were profoundly affected: serum immunoglobulin concentrations and antibody responses were decreased and cytotoxic and antitumor responses were absent in IRF-4 knockout mice. Normal early T-cell events such as calcium influx and expression of the T-cell activation markers CD25 and CD69 in IRF-4-/- T-cells indicated that IRF-4 may function at later stages of T-cell activation, possibly at the level of IL-2 production and/or IL-2 response. This hypothesis was supported by the observation that the reduced T-cell proliferation in these mice was not reversed by exogenous IL-2 treatment; thus IRF-4 appears to be essential for the function and homeostasis of both mature B and mature T lymphocytes (137).

IRF-4 and Chronic Myeloid Leukemia. Chronic Myeloid Leukemia (CML) derives from the uncontrolled proliferation of myeloid progenitor cells. Much information actually points to an additional role for T cells in the disease. A new study by Schmidt et al. showed a significant downregulation of IRF-4 in T cells from CML patients. IFNα is frequently used to treat CML patients and has been shown to increase survival. Interestingly, a significant increase in IRF-4 expression was detected in CML patients as well as purified CML T cells during IFNα therapy. Although, IFNs could not upregulate IRF-4 in normal or CML T cells, an indirect mechanism involving STATs is believed to be involved in the upregulation of IRF-4 expression in the CML patients during IFNα treatment (191). A correlation was also established between a good response to IFNα therapy and the increase of IRF-4 expression in CML patients treated with IFNα. The higher the expression of IRF-4 in T cells of CML patients during IFNα treatment, the better the response of the patient to the therapy. These findings were not due to changes in the blood differential (191). IRF-4 expression could become a good marker for clinicians to determine the response to therapy in CML patients. A gene therapy approach, which would upregulate IRF-4 expression, could potentially be beneficial to CML patients.

IRF4 and Sarcoidosis. Sarcoidosis is characterized by granulomatous inflammation of organs. African Americans are more commonly and severely affected with this disease than Caucasians. Rybicki et al screened for associations between marker alleles in candidate gene regions and sarcoidosis in African Americans. One of the genetic marker looked at, F13A, is tightly linked to the gene that codes for IRF-4 on chromosome 6p23-25.3. For the F13A marker, a 188bp allele (F13A*188) was strongly associated with sarcoidosis making IRF-4 an attractive candidate gene for this disease (186).

3.1.4 IRF-4 in HTLV-I infected T cells

The human equivalent of IRF-4 was isolated by a third group from an adult T-cell leukemia cell line, as the "IFN consensus sequence-binding protein in adult T-cell leukemia cell lines or activated T-cells" (ICSAT) capable of binding to the human IL-5 promoter (239). Like murine IRF-4, human IRF-4 was structurally similar to IRF-8/ICSBP, and expression was not inducible by IFN. However, ICSAT/IRF-4 possessed a very different function compared to its murine counterpart; while PU.1-IRF-4 functioned as a transactivator complex, IRF-4 alone exerted an IRF-2 and IRF-8/ICSBP-like repressive effect on IFN- and IRF-1-induced gene activation. The repressive effects exerted by IRF-4 were different from those exerted by IRF-2 and IRF-8/ICSBP. While IRF-2 and IRF-8/ICSBP recognize and bind to the ISRE, resulting in the repression of IFN-α-mediated activation of ISRE-driven reporter constructs, IRF-4 associates with the ISRE but is unable to execute its repressive function; this effect may be due to its inability to compete with IFNα-induced ISGF3 (239). Furthermore, although IRF-4 repressed both IFN-α and IFN-γ-activated gene expression, repression occurred only through the ISRE, and not the gamma activated sequence (GAS), a similar interferon consensus sequence exclusively bound by IFNγ-stimulated proteins.

ICSAT/IRF-4 was expressed exclusively in a restricted subset of lymphocytes: only T-cells treated with phorbol myristate acetate (PMA) or infected with the human T-cell leukemia virus-1 (HTLV-I) produced IRF-4. Jurkat cells transiently transfected

with the HTLV-I Tax gene also expressed IRF-4 mRNA, indicating that Tax may activate the IRF-4 promoter (239). Since the oncogenic potential of HTLV-I resides in the viral Tax oncoprotein (reviewed in (79, 248)), induction of IRF-4 expression by Tax may be an important cellular target implicated in HTLV-I-induced leukemogenesis. Similar results were not observed in HIV-1 infected T cells or myeloid cells (YM, unpublished data). Therefore retroviral infection of cells is not sufficient to induce IRF-4 expression.

The relationship between IRF-4 expression and oncogenicity is further highlighted by the observation that in some patients with multiple myeloma, a chromosomal translocation - t(6;14)(p25;q32) - juxtaposes the immunoglobulin heavy-chain (IgH) locus to the IRF-4 locus at 6p25. This chromosomal translocation involving IRF-4 may thus contribute to leukemogenesis since IRF-4 has oncogenic activity *in vitro* (84).

IRF-4 transgenic mice. Transgenic mice overexpressing IRF-4/ICSAT were generated. Surprisingly, IRF-4 overexpression was not sufficient to cause Adult T cell Leukemia or multiple myeloma in these mice. It was observed that IL-2 production with ConA stimulation was significantly increased in the transgenic mice. IRF-4 may be regulating T cell activation and its overexpression may lead to leukemogenesis via controlling IL-2 production (189). These results also suggests that IRF-4 may require additional factors *in vivo* to promote its oncogenic activity (212).

The discovery of an IRF family member specifically expressed in HTLV-I infected cells has led to the convergence of two area of research - regulation of IRF-4 expression and the involvement of the Tax oncoprotein in HTLV-I induced leukemogenesis. IRF-4 may interfere with the antiviral effect of IFNs by disrupting the finely regulated IFN-regulated genes, leading to HTLV-I proliferation.

3.1.5 IRF-4 in macrophages

IRF-4 was thought to be expressed only in lymphoid cells: B cells and activated T cells. Marecki et al. provided new insight in the expression of IRF-4 in myeloid cells. Primary murine and human macrophages were shown to express IRF-4. The levels of IRF-4 expression were much lower than in B cells; 25-35% of that seen in B cells. IRF-8/ICSBP expression in macrophages was dramatically upregulated by IFNγ treatment but had no effect on IRF-4 expression, consistent with previous findings. In macrophages, IRF-4 was seen in both the cytoplasm and the nucleus. LPS treatment was able to induce nuclear translocation of IRF-4 in macrophages after 2 hours of treatment. By contrast, IRF-8/ICSBP or PU.1 levels were not significantly altered by LPS treatment (126).

Since PU.1 is also constitutively expressed in macrophages, protein-protein interaction and transactivation assays with IRF-4 and IRF-8/ICSBP were examined (126-128). EMSA analysis revealed that macrophages contained both IRF-4-PU.1 and IRF-8-PU.1 complexes, analogous to B cells. In macrophages and NIH3T3 cells, IRF-4 and IRF-8 alone were able to repress transcription of a MHC class I promoter. By contrast, IRF-4 only was able to activate the IL-1β promoter in a dose-dependent manner in these cells. IL-1β is a proinflammatory cytokine predominantly secreted by macrophages and monocytes induced by LPS. The IL-1β promoter has been previously shown to be regulated by LPS and IFNγ, and require a functional PU.1 binding site. Synergistic activation of this promoter was observed when cells were cotransfected with both IRF-4 and PU.1. Maximal increase of the human IL-1β reporter gene and the endogenous IL-1β gene was observed in cells coexpressing PU.1, IRF-4 (or IRF-8), IRF-1 and IRF-2. These factors may function as an enhanceosome of the IL-1β promoter in macrophages (128). As in B cells, IRF-4 and PU.1 act as mutually dependent transactivating complexes. Although the role of IRF-4 in B cells is partially known i.e. immunoglobulin light chain synthesis, its function in macrophages is still very much unclear (126, 127).

IRF-8/ICSBP and IRF-4 were also shown to form specific complexes that bind to the ISG15 gene in B cells and in macrophages. IRF-4 and IRF-8 were able to interact with each other in the absence of DNA. Both IRFs downregulated the expression of the ISG15 promoter in macrophages. Synergistic downregulation was also observed in the presence of both IRF-4 and IRF-8. ISG15 has been described as an IFNα/β-stimulated cytokine secreted by macrophages and lymphocytes that increases IFNγ production in these cells. IFNγ is a key regulator of inflammatory responses, and its uncontrolled activity may lead to harmful pathological changes. A possible mechanism for terminating IFNγ responses is the IRF-8/ICSBP and the IRF-4-mediated downregulation of ISG15 (180).

3.1.6 IRF-4 in non-hematopoietic cells

Recently, IRF-4, IRF-8/ICSBP, IRF-1 and IRF-2 were shown to be constitutively expressed in mouse lens cells (116). This is the first time that expression of IRF-4 and IRF-8/ICSBP has been demonstrated in a tissue that is not directly involved in regulating immune responses. Both IRF-1 and IRF-2 are expressed at high levels in differentiated lens fiber cells but not in undifferentiated lens epithelial cells. IRF-8/ICSBP levels are low but increased upon IFNγ treatment. All these IRFs are present in both the cytoplasm and the nuclei of lens cells (116). These results suggests that the expression of IRFs is spatially regulated in the lens and that distinct IRFs may contribute to differential gene regulation in the epithelial and fiber compartments of vertebrate lens.

3.2 IRF-8/ ICSBP

3.2.1 IRF-8/ ICSBP structure-function analysis

Interferon consensus sequence binding protein or IRF-8 was originally isolated as the protein recognizing the ISRE motif in the promoter region of the MHC class I, H-2L^D gene (36, 232). IRF-8, IRF-5 and IRF-4 - are the only IRFs expressed in a tissue-specific manner; IRF-8 expression is restricted to cells of the lymphocyte and monocyte/macrophage lineages (Figure 19). Its function is important in the development of these lineages through interaction with other transcription factors

such as IRF-1 and IRF-2 (196). The DNA binding activity of IRF-8 alone is quite weak but is dramatically increase by interaction with IRF-1 and IRF-2.

The interaction domain of IRF-8/ICSBP, localized between aa 200-377, is conserved among IRF members such as IRF-3, IRF-4, IRF-5 and IRF-9/ISGF3γ and has been termed the IRF Association Domain (IAD). The IAD domain was found to be important in mediating IRF-8/ICSBP-repressive activity as well as interaction with other IRFs. *In vitro* studies have also shown that direct binding of IRF-8 to DNA is negatively regulated by tyrosine phosphorylation within its DBD (196). Furthermore, tyrosine phosphorylated IRF-8 bound to its recognition site in DNA only in association with IRF-1 or IRF-2; hence tyrosine phosphorylation appears to be essential for heterodimer formation. The fact that several tyrosines within the DBD of IRF-8 are conserved in other IRF members suggests that tyrosine phosphorylation may modulate the biological activities of IRFs in a fashion similar to the STATs (196).

3.2.2 IRF-8/ ICSBP and the immune system

Heterocomplexes containing IRF-8 function predominantly as activating transcriptional complexes. Cooperative interactions between IRF-8, PU.1 and IRF-1 were shown to increase expression of the gp91phox gene, which encodes a subunit of the phagocyte respiratory burst oxidase catalytic subunit. The transcription of CYBB - the gene encoding gp91phox - is regulated in a lineage and differentiation state-specific fashion through cooperativity with IRF-8, PU.1 and IRF-1 (40, 212). IRF-8 and PU.1 are also involved in the expression of IL-18 and CD11b in macrophages (99, 127). In B cells, the IRF-8-PU.1 complex seems to be less transcriptionally active than the IRF-4-PU.1 complex. IRF-8 by itself acts as a repressor of genes containing ISRE element such as MHC-I, 2'5'-ologo-adenylate synthetase and ISG54 (reviewed in (127)).

IRF-8 is also involved in immune regulation and homeostasis. IRF-8-/- mice were selectively sensitive to particular viral and parasitic infections. These mice were defective in regulating TH1 cytokine production in antigen presenting cells and in naive T cells. They failed to produce IL-12 and IFNγ after stimulation, thus contributing to their increased susceptibility to viral and parasitic infections such as *Toxoplasma Gondii* and *Leishmania major* infections (235). Thus, IRF-8 is essential for Th1 cell development through its role in regulating IL-12 and IFNγ gene expression.

IRF-8-/- macrophages were also defective in iNOS and FcγR1. The enzyme encoded by iNOS gene catalyses the production of nitric oxide, a short-lived volatile gas that plays a major role in the effector phase of Th1 immune response i.e. macrophage cytotoxicity against tumor cells, bacteria and other target. FcγR1 or CD64 is a high-affinity Fcγ receptor involved in phagocytosis, antibody-dependent cell-mediated cytotoxicity and macrophage activation (212). Although IRF-8 is known to affect the activity of MHC class I and β2-microglobulin promoters, no significant alterations were detected in mRNA levels or the surface expression of MHC class I on IRF-8-/- lymphoid cells, pointing to an alternative pathway of expression (212).

Perhaps most importantly, IRF-8-/- mice were afflicted with a pathological syndrome similar to human chronic myelogenous leukemia (81). These results implicated IRF-8 in the development and proliferation of myeloid progenitor cells. Interestingly, Schmidt et al. also found a correlation between low levels of IRF-8 mRNA and human myeloid leukemia (192).

3.3 IRF-9/ ISGF3γ/ p48.

Interferon-stimulated gene factor-3gamma (ISGF3γ), p48 or IRF-9 (Figure 19), usually exerts its transcriptional effects exclusively in association with signal transducer and activator of transcription-1 (STAT1 or p84/p91) and -2 (STAT2 or p113) proteins (the latter two are collectively termed ISGF3α) activated through specific phosphorylation events by type-I IFNs (10, 114). This trimolecular complex,

termed ISGF3, is formed within minutes of IFN treatment and participates in the transcriptional activation of a large number of IFN-inducible genes by binding to the ISRE; in this regard, unlike the other IRFs, ISGF3γ functions as an immediate early protein. Like IRF-1 and IRF-2, IRF-9 is expressed in a variety of tissues and shown to be essential for the antiviral response by type I and type II IFNs (Figure 19) (212).

IRF-9 is found both in the cytoplasm and the nucleus of cells regardless of IFN stimulation. IRF-9 was shown to be efficiently and constitutively targeted to the nucleus. IRF-9, as well as IRF-4 and IRF-8 contain a bipartite nuclear retention signal within their DNA binding domain. Disruption of this bipartite nuclear retention signal dramatically disrupted their nuclear accumulation but had no effect on DNA binding (107). Nuclear distribution of IRF-9 was also dramatically altered by coexpression of Stat2, indicating that Stat2 formed a cytoplasmic complex with IRF-9, overriding IRF9 nuclear targeting. Retention by Stat2 may serve to regulate the activity of free IRF-9 and guarantee that cytoplasmic pools of preassociated Stat2-IRF-9 be available for rapid activation of the IFN response (107).

Although the role of IRF-9 in the IFN system has been extensively studied, its role in oncogenesis has not yet been elucidated. The importance of IRF-9 in cancer has been analyzed in the context of IFNα/β -mediated suppression of tumor cell growth. Retinoic acid induced Stat1, Stat2 and IRF-9 expression in myeloid leukemia cells and enhanced their responsiveness to IFNs (130). Retinoic acids have been successfully used in the treatment of acute promyelocytic leukemia by inducing cellular proliferation which is blocked in these cancer cells and by degrading the oncogenic PML-RAR (Promyelocytic gene fused the retinoic acid receptor gene) fusion protein. (92). Induction of Stat1, Stat2 and IRF-9 could be involved in this increase in cellular proliferation. In contrast, it was reported that IRF-9 is transcriptionally activated by c-myc, and that cells lacking IRF-9 expression are highly susceptible to the cytocidal action of anticancer drugs (231). These findings suggest an involvement of IRF-9 in cell cycle regulation, although this field requires further clarification (212).

4. Immunophilins - Peptidyl-Prolyl Isomerases

The activity of transcription factors in eukaryotic cells can be controlled by posttranslational modification, interaction with inhibitory molecules and cytoplasmic retention. A number of different enzymes can regulate transcription factor function such as kinases and phosphatases through phosphorylation and dephosphorylation, protein lysine acetylases and protein methylases. Recently, a new class of enzymes called peptidyl-prolyl isomerases (PPIases) have been added to the list of enzymes which can regulate transcription factor through posttranslational modification (83). Leverson and Ness reported that the DNA-binding activity of the c-Myb transcription factor (61, 62) was negatively regulated by a stable interaction with Cyp40, a member of the immunophilin family of PPIases. The human Cyp-40 was shown to regulate the transcriptional activity of c-Myb by interfering with the capacity of c-Myb to bind DNA; interestingly, the oncogenic form of v-Myb (viral oncoprotein of the avian myeloblastosis virus) evaded this inhibition of DNA binding (113). Point mutations and deletions in v-Myb that contribute to its oncogenic potential also abrogate the cyclophilin-mediated inhibition of DNA binding of v-Myb (61, 120) (Figure 24).

There are three families of PPIases or immunophilins: the cyclophilins, which bind the immunosuppressant cyclosporin A (CsA), the FK506-binding proteins (FKBPs), which bind the immunosuppressant FK506, and the parvulin family (Table 7) (83). A large number of cyclophilins and FKBPs have been isolated varying primarily by molecular weight and tissue distribution. They are all characterized by a highly conserved ligand -binding domain.

Figure 24: **Cyp40-cMyb interaction**. cMyb is composed of a DNA binding domain followed by an activation domain (AD) and a negative regulatory domain (NRD). The human Cyp-40 was shown to regulate the transcriptional activity of c-Myb transcription factor by interfering with the capacity of c-Myb to bind DNA; This effect was dependent on the functional PPIase activity of Cyp40 (blue circle). Interestingly, the oncogenic form of v-Myb evaded this inhibition of DNA binding. Point mutations and deletions in v-Myb that contribute to its oncogenic potential also abrogate the cyclophilin-mediated inhibition of DNA binding of v-Myb. Adapted from Leverson, 1998 and Hunter, 1998.

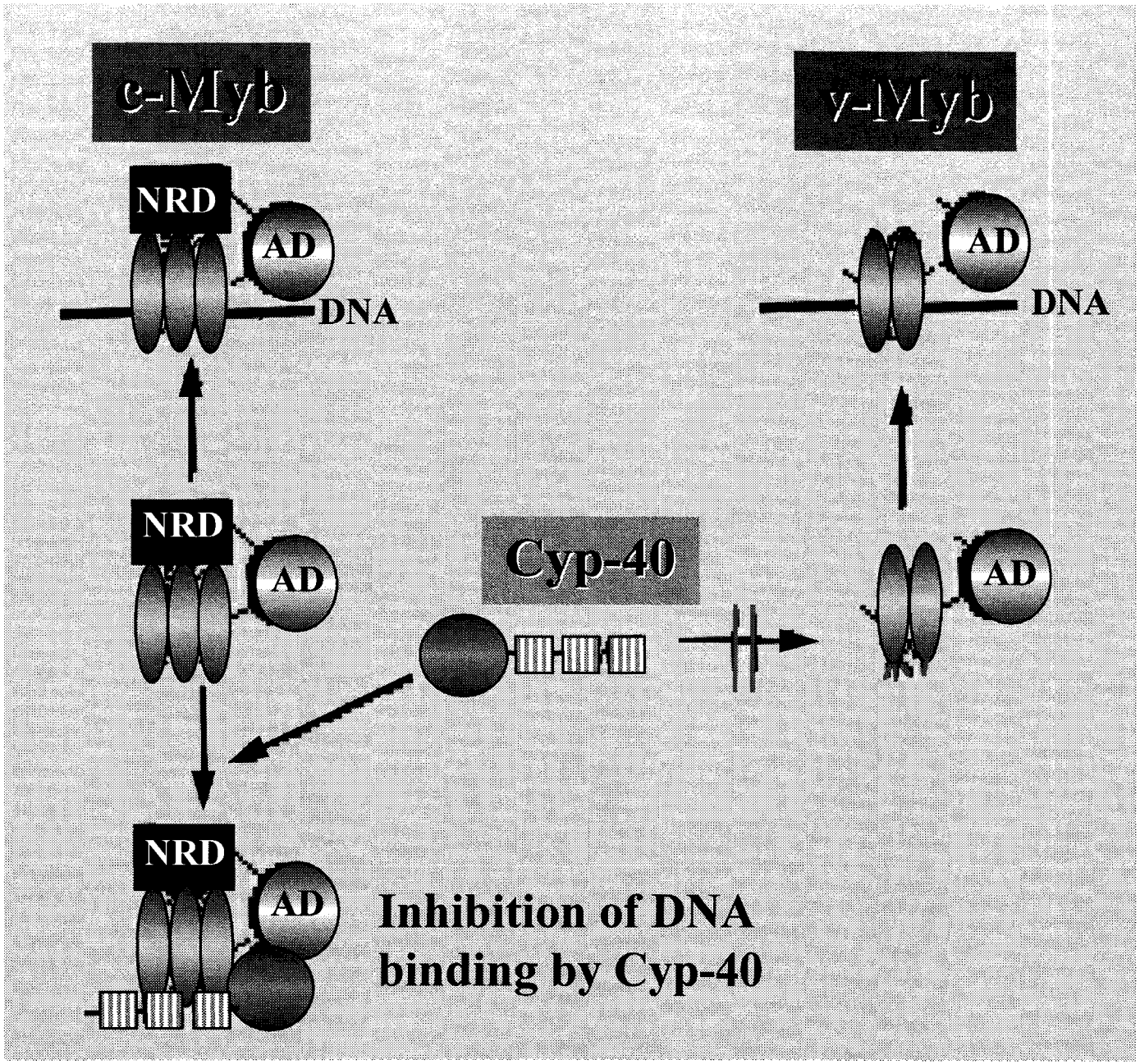

c-MYb
v-MYb
NRD
AD
DNA
NRD
AD
Cyp-40
AD
DNA
NRD
AD
Inhibition of DNA
binding by Cyp-40

Table 7: Immunophilin characteristics. Immunophilins are divided into 3 families: Cyclophilins which bind cyclosporin, FKBPs which bind to macrolides and parvulins. Their expression is quite conserved throughout evolution and therefore they are believed to possess fundamental cellular functions. Adapted from Kay, 1996.

	FKBP	Cyclophilin	Parvulin
PPIase activity	- to ++	+ to +++	? to +++
Distribution	Mammals Plants Lower Eukaryotes Prokaryotes	Mammals Plants Lower Eukaryotes Prokaryotes	? ? Lower Eukaryotes Prokaryotes
Natural Inhibitors	Macolides: FK506 Ascomycin Rapamycin Meridamycin	Peptides: Cyclosporin	?
Known in Humans	7	7	?
Domains found within larger proteins	Yes	Yes	Yes
Multiple domains found	Yes	No	Yes

The high abundance of immunophilins has led to growing studies looking at their role in cellular biochemistry such as protein folding, stabilization of Ca2+ release channels, protein tyrosine kinase receptors, and inactive steroid receptors (85). Interestingly, complexes of immunophilins with both immunosuppressive and nonimmunosuppressive ligands have been shown to possess neuroregenerative properties. Cyclophilin A has also been implicated in the formation of the viral capsid of HIV-1 (83, 85). While the significance of these activities in cells still remains controversial, there is growing evidence that immunophilins perform important cellular functions.

These highly conserved enzymes have been found in prokaryotes, lower eukaryotes such as yeast, plants and primates. They are also extremely abundant accounting for approximately 1% of total cellular proteins (133). Most of them are cytoplasmic but several of them have dual nuclear and cytoplasmic localization (Table 8) (83). Several physiological roles have been ascribed to immunophilins: 1) binding and sequestration of calcineurin; 2) protein folding and assembly; 3) protein trafficking; 4) direct regulation of protein activity; and 5) chaperone-like activity (96).

4.1 Cis-trans isomerization

Immunophilins are first peptidyl-prolyl isomerases (PPIases) involved in catalyzing cis-trans isomerization of proline residues only, within proteins. Cis prolines are important to protein structural integrity by introducing bends within proteins. During protein synthesis, the peptide bonds on the amino side of proline residues are found in the open trans-conformation. When three-dimensional structures of proteins were determined, 15% of these bonds were in the alternate cis-conformation (96, 129). Polypeptides with prolines bond all in the trans conformation have a very linear and plan configuration. Cis-bonds within proteins introduce a kink or bend in the protein conformation (Figure 25).

Table 8: Human Immunophilins. Only cyclophilins and FKBPs have been found in humans. Parvulin have yet to be identified. They are quite different in structure and expression patterns as well as cellular functions. Adapted from Kay, 1996 and Marks, 1996.

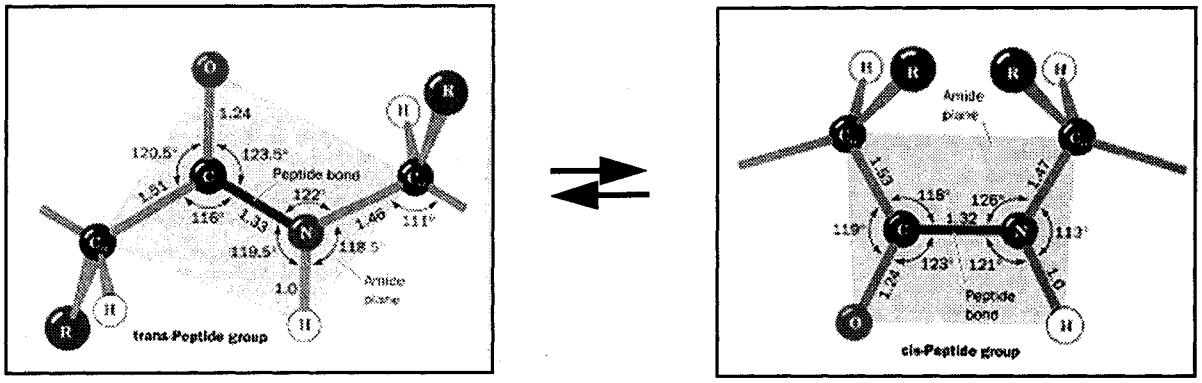
Human Immunophilins

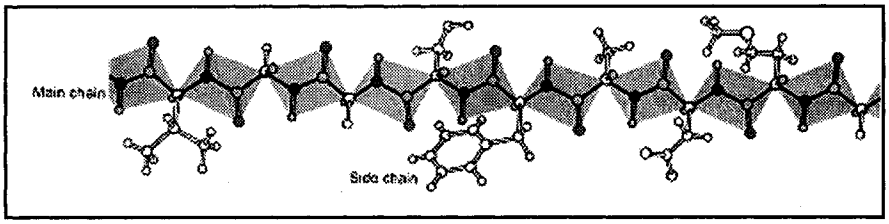
Name	Size (kDa)	Remarks
Cyclophilins		
CypA	18	Cytosolic; interacts with HIV-I Gag; catalyses ribonuclease1 folding
CypB	21	Localized to the ER; secreted in human milk
CypC	23	Localized to the ER
CypD	22	Mitochondrial signal sequence
Cyp40	40	Estrogen receptor associated; TPR motifs
CypNK/ NKTR	150	Surface protein on NK cells
IL-8	58-67	Cytokine; glycosylated; no PPIase activity
FKBPs		
FKBP12	12	Modulates function of Ca2+ release channels; associated with TGFβ receptor and with calcineurin; released from mast cells after anti-IgE stimulation; associated with the ryanodine receptor
FKBP12.6	12.6	Associated with cardiac Ca2+ release channels
FKBP13	13	Localized to the ER
FKBP25	25	Complexed to nucleolin and CKII; higher affinity for rapamycin than FK506
FKBP38	38	Contains leucine zipper and TPR
FKBP51	54	Only FKBP expressed primarily in T cells; associated with progesterone receptor and HSP90
FKBP52	59	Associated with progesterone-, estogen-, androgen- and glucorcorticoid receptors with HSP90; binds ATP, GTP and calmodulin; TPR motifs

Figure 25: **Cis-trans isomerization catalyzed by immunophilins. (A)** PPIase catalyzes cis-trans isomerization at specific proline residues. Peptide bonds on the amino side of proline residues are found mainly in the trans-conformation which give a polypeptide a very plan and linear confirmation. The appearance of the cis-conformation is catalyzed by PPIases. Amino acids preceding and following the proline residue are linked to polypeptide chains. Adapted from Voet et Voet, 1995. **(B)** Chemical structures of the cis-proline and trans-proline provided by Dr. Ivery (Ivery, 2000).

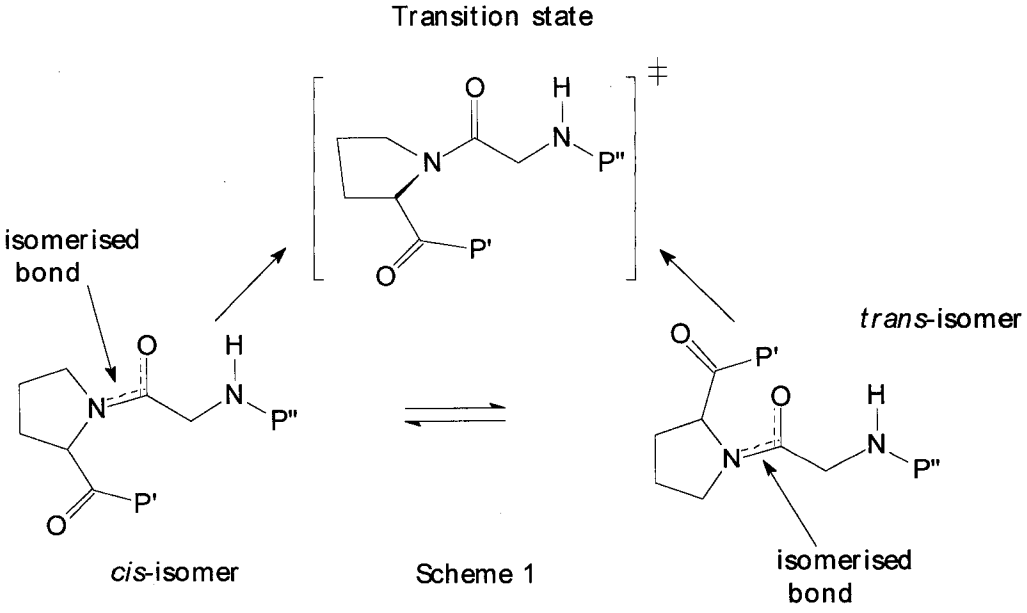
A

TRANS **CIS**


1.24
120.5°
123.5°
Peptide bond
122°
1.51
116°
1.33
1.46
111°
119.5°
118.5
1.0
Amide plane
trans-Peptide group
Amide plane
1.53
116°
126°
1.47
119°
1.32
113°
123°
121°
1.24
1.0
Peptide bond
cis-Peptide group


Main chain
Side chain

B


Transition state
isomerised bond
cis-isomer
trans-isomer
isomerised bond
Scheme 1

Enzymes with peptidyl-prolyl isomerase activity such as immunophilins catalyze the cis-trans interconversion, a modification that would otherwise occur slowly. Biochemical isomerization reactions involve the intra-molecular shift of a hydrogen atom so as to change the location of a double bond. Therefore, cis-trans isomerization does not require an external source of energy, the energy needed to break bonds is supplied by the formation of new bonds. This reaction also shows no pH dependency (85).

Immunophilins do show specificity, they target specific proline residues: the FKBPs and parvulins prefer a Pro preceded by a bulky hydrophobic residue at the position -1, whereas cyclophilins show little preference at this position for cis-trans isomerization (83, 96, 129). PPIase activity is normally assayed by using a coupled spectrophometric assay developed by Fisher (45). This assay exploits the high conformational selectivity of chymotrypsin towards substrates of the type Xaa-Pro-Phe-pnitroanilide where Xaa is any amino acid. The cleavage of p-nitroanilide group by chymotrypsin occurs only with the substrate being in the trans Xaa-Pro conformation. The presence of immunophilins accelerate the cleavage p-nitroanilide by converting Xaa-Pro bond into the trans conformation (45, 85). The limitation of this assay is that in normal aqueous solution about 85% of the Xaa-Pro bond is already in the trans conformation. Attempts have been made to increase the sensitivity of this assay (87).

4.2. Calcineurin inhibition

It was also discovered that despite their lack of structural similarities, the cyclophilins and FKBPs have another common property beside PPIase activity, namely that they interact stably with the calcium-regulated protein-phosphatase calcineurin. This interaction with calcineurin only occurs when the immunophilin is bound to its respective immunosuppressant: cyclophilins with CsA, and FKBP with FK506. Immunophilins alone do not bind to calcineurin. It was found that some immunophilin/immunosuppressant complexes such as CypA-CsA and FKBP12-FK506, could sequester and inhibit calcineurin in lymphocytes which prevented these

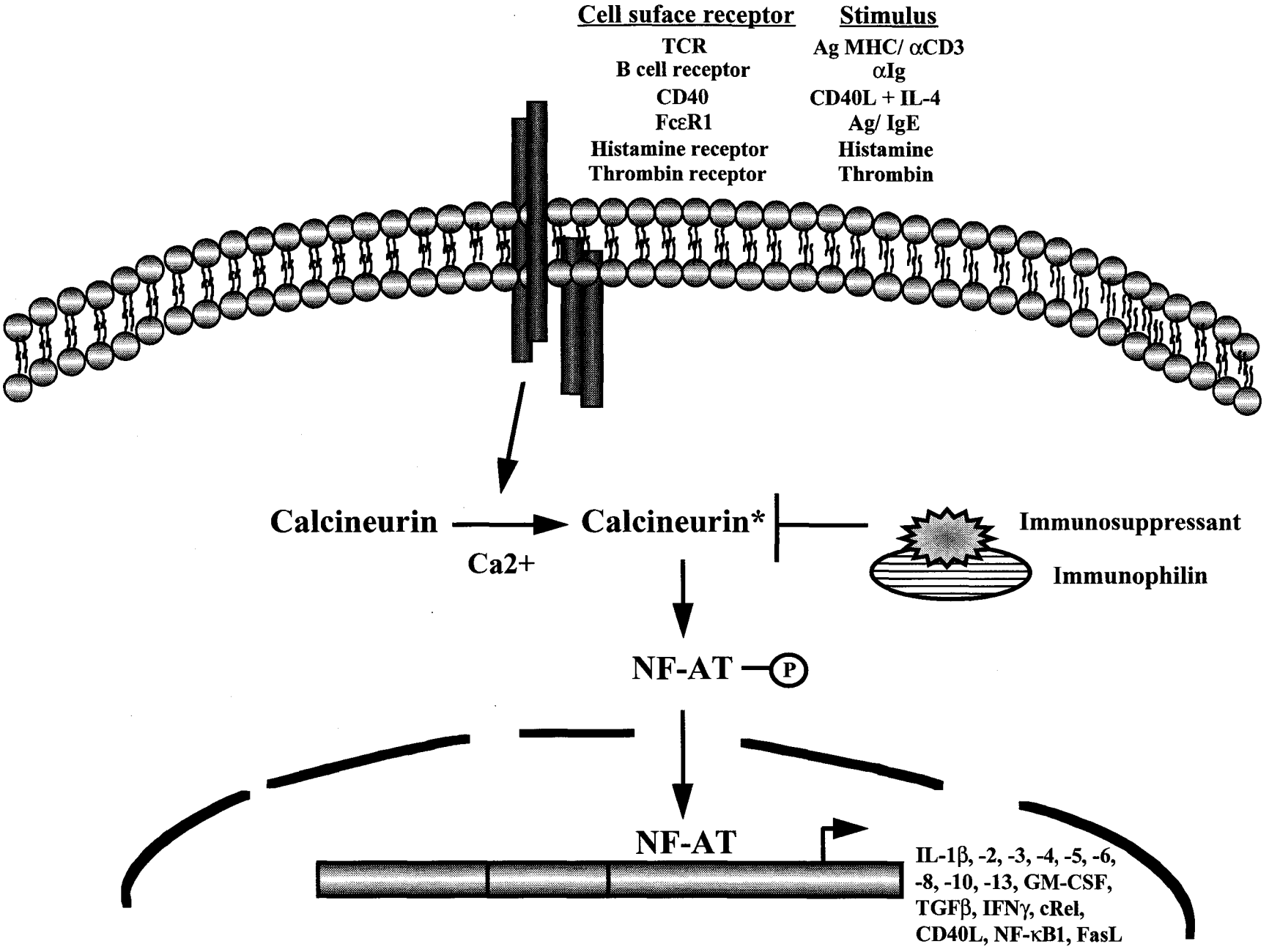
cells from responding to antigen-induced mitogenic signals, thus resulting in immunosuppression. Indeed, one of the first described members of the immunophilin family - cyclophilin A - was shown to be the primary target of cyclosporin A, a clinically used immunosuppressive drug that blocks the calcineurin dependent activation of NF-AT in T cells (42, 133, 134, 146).

The NF-AT (nuclear factor of activated T cells) family of transcription factors play a pivotal role in inducible gene expression during the course of an immune response. There are at least five structurally related subsets of NF-AT proteins differentially expressed in various classes of immune cells that have been implicated in the regulation of cytokines (IL-2, IL-4, TNF-α), cell surface receptors (IL-2Rα, FasL) and transcription factors (Oct-2, c-Rel). NF-AT proteins are activated through stimuli that elicit calcium mobilization, such as engagement of the antigen receptors on B and T cells, the Fcγ receptor on NK cells and macrophages or treatment with calcium ionophore or inhibitors of the endoplasmic reticulum Ca^{2+}-ATPase. In unstimulated cells, it is believed that phosphorylated NF-AT proteins exist in the cytoplasm in a closed conformation, which masks the nuclear localization signal. A rise in intracellular calcium activates a number of cellular enzymes, including the calcium/calmodulin-dependent phosphatase calcineurin, which is a major upstream regulator of NF-AT. Activation of calcineurin results in the rapid dephosphorylation and subsequent nuclear translocation of NF-AT. In the nucleus, dephosphorylated NF-AT shows an increased affinity for DNA. Each step in the early activation of NF-AT proteins can be inhibited by the immunosuppressive drugs cyclosporin A and FK506 (98) (Figure 26).

NF-AT was initially described as a transcriptional complex involved in the induction of the IL-2 gene in the context of early T cell activation. The NF-AT family members NF-ATc (NF-ATc1/NF-AT2) and NF-ATp (NF-Atc2/NF-AT1) was shown to bind with cellular factors such as AP-1, Oct-1 and NF-κB to specific enhancer sequences on the IL-2 promoter. During the course of HTLV-I infection, constitutive activation of NF-AT proteins is also associated with upregulation of the IL-2

promoter, through NF-AT consensus sites and a Tax-inducible element termed the CD28RE (63).

Figure 26: NF-AT signaling, calcineurin and the role of immunophilins. NF-AT proteins are activated through stimuli that elicit calcium mobilization, such as engagement of the antigen receptors on B and T cells, the Fcγ receptor on NK cells and macrophages, or treatment with calcium ionophore, inhibitors of the endoplasmic reticulum Ca^{2+}-ATPase. In unstimulated cells, it is believed that phosphorylated NF-AT proteins exist in the cytoplasm in a closed conformation, which masks the nuclear localization signal. A rise in intracellular calcium activates a number of cellular enzymes, including the calcium/calmodulin-dependent phosphatase calcineurin, which is a major upstream regulator of NF-AT. Activation of calcineurin results in the rapid dephosphorylation and subsequent nuclear translocation of NF-AT. In the nucleus, dephosphorylated NF-AT shows an increased affinity for DNA. Each step in the early activation of NF-AT proteins can be inhibited by the immunosuppressive drugs cyclosporin A and FK506 binding to their respective immunophilins. Only the immunophilin-immunosuppressant complexes will sequester calcineurin which will inhibit NF-AT dephosphorylation and translocation to the nucleus. Adapted from Ho, 1996 and Rao, 1997.


Cell suface receptor
Stimulus
TCR
Ag MHC/ αCD3
B cell receptor
αIg
CD40
CD40L + IL-4
FcεR1
Ag/ IgE
Histamine receptor
Histamine
Thrombin receptor
Thrombin
Calcineurin
Ca2+
Calcineurin*
Immunosuppressant
Immunophilin
NF-AT
P
NF-AT
IL-1β, -2, -3, -4, -5, -6,
-8, -10, -13, GM-CSF,
TGFβ, IFNγ, cRel,
CD40L, NF-κB1, FasL

4.3 FK506 Binding Protein 52- FKBP52

The human immunophilin FK506 Binding Protein 52 (FKBP52, HBI, p59, HSP56) is a member of the FKBP family of proteins with PPIase activity and chaperone-like functions (Figure 27) (27, 166, 173). FKBP52 was originally discovered in association with unliganded steroid receptor complexed with heat shock proteins HSP70 and HSP90 and involved in cytoplasmic to nuclear shuttling (109, 164, 166). FKBP52 was also shown to associate with microtubules and during mitosis FKBP52 mainly localizes with the mitotic apparatus (centrosome, spindle and the interzone separating the chromosomes) (166). Immunolocalization studies have shown that FKBP52 is distributed in both the nucleus and the cytoplasm, with a fibrillar staining pattern in the cytoplasm which suggests again an association with the cytoskeleton (27, 183).

FKBP52 contains 3 "FKBP-like" domains: domain 1 possesses features required for PPIase activity and FK506 binding, domain 2 contains GTP and ATP binding sites and domain 3 the tetratricopeptide repeats (TPR) which are responsible for protein-protein interaction such as its association with Heat Shock Protein 90 (HSP90) and a calmodulin binding site (Figure 28) (96, 173). Unlike FKBP12-FK506, the FKBP52-FK506 does not inhibit calcineurin activity and thus does not function in immunosuppression. Like all immunophilins, the PPIases activity of FKBP52 is inhibited when bound to the immunosuppressant (96, 141).

The three tetratricopeptide repeats in FKBP52 are found between aa 273-306, 322-355 and 356-389, each 34-aa motif possesses two short amphipathic α-helices (96). Only another FKBP, FKBP51 was shown to possess these three tetratricopeptide repeats.

Figure 27: Human FK506 Binding Proteins. All human FKBPs possess one or more FKBP domain. Some FKBP possess protein-protein interaction modules called tetratricopeptide repeats as well as calmodulin binding site. FKBP13 is the only one which possesses a signal sequence which is involved in endoplasmic reticulum localization. Adapted from Kay, 1996.

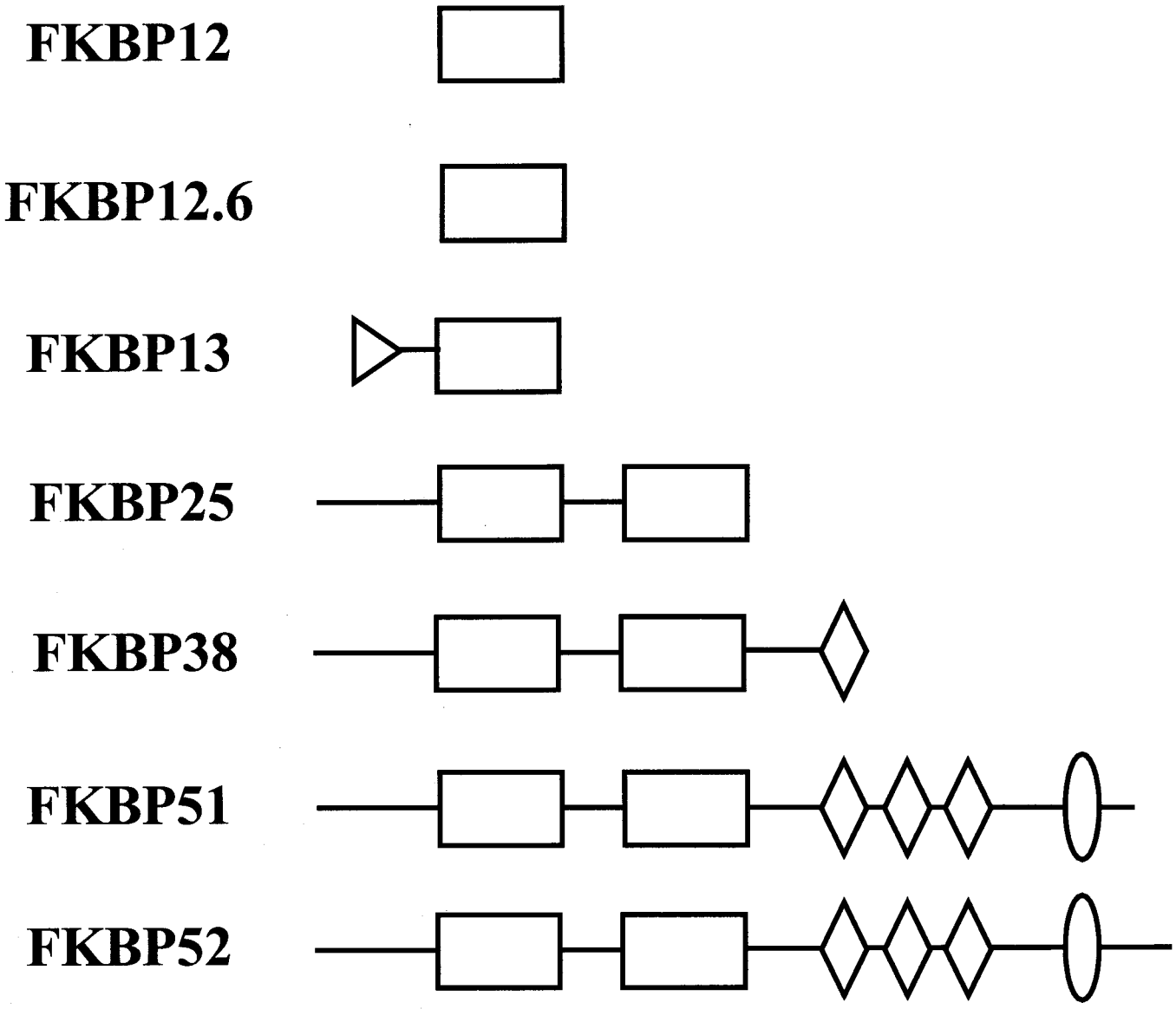
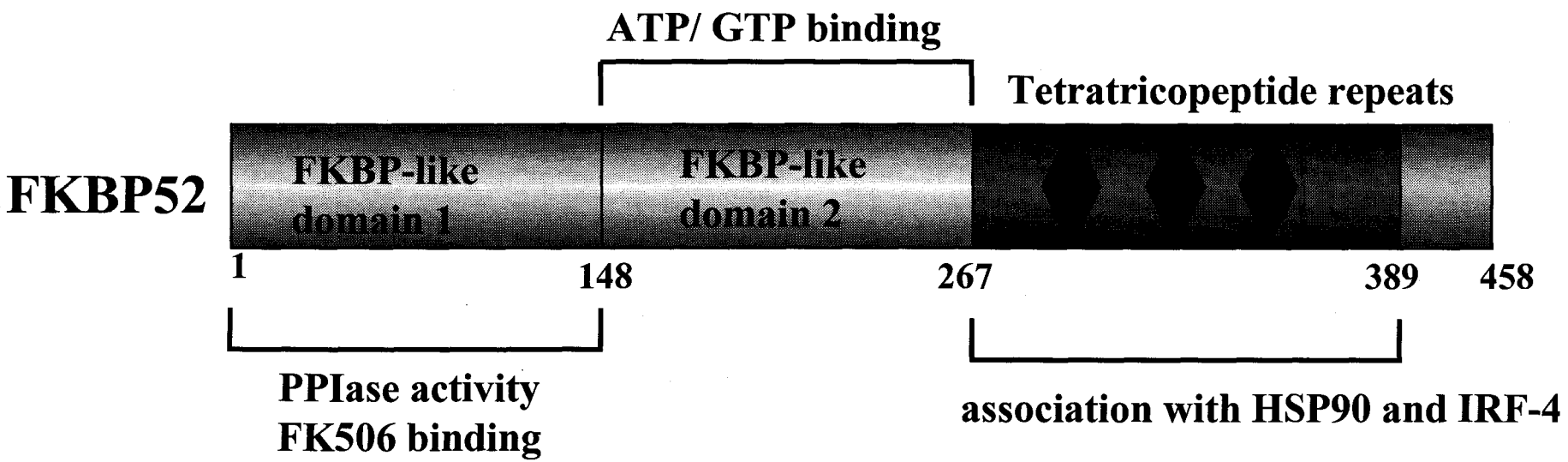

FKBP12
FKBP12.6
FKBP13
FKBP25
FKBP38
FKBP51
FKBP52
FKBP domain
Signal sequence
Tetratricopeptide repeat
Calmodulin binding site

Figure 28: Schematic representation of FKBP52. FKBP52 possesses three functional domains (brackets): FKBP-like domain 1 with the PPIase activity and immunosuppressant binding site (aa1-148), FKBP-like domain 2 with an ATP/GTP binding site (aa148-267) and 3 tetratricopeptide repeats (aa267-389) which mediate protein-protein interactions.


ATP/ GTP binding
Tetratricopeptide repeats
FKBP52
FKBP-like domain 1
FKBP-like domain 2
1
148
267
389
458
PPIase activity
FK506 binding
association with HSP90 and IRF-4

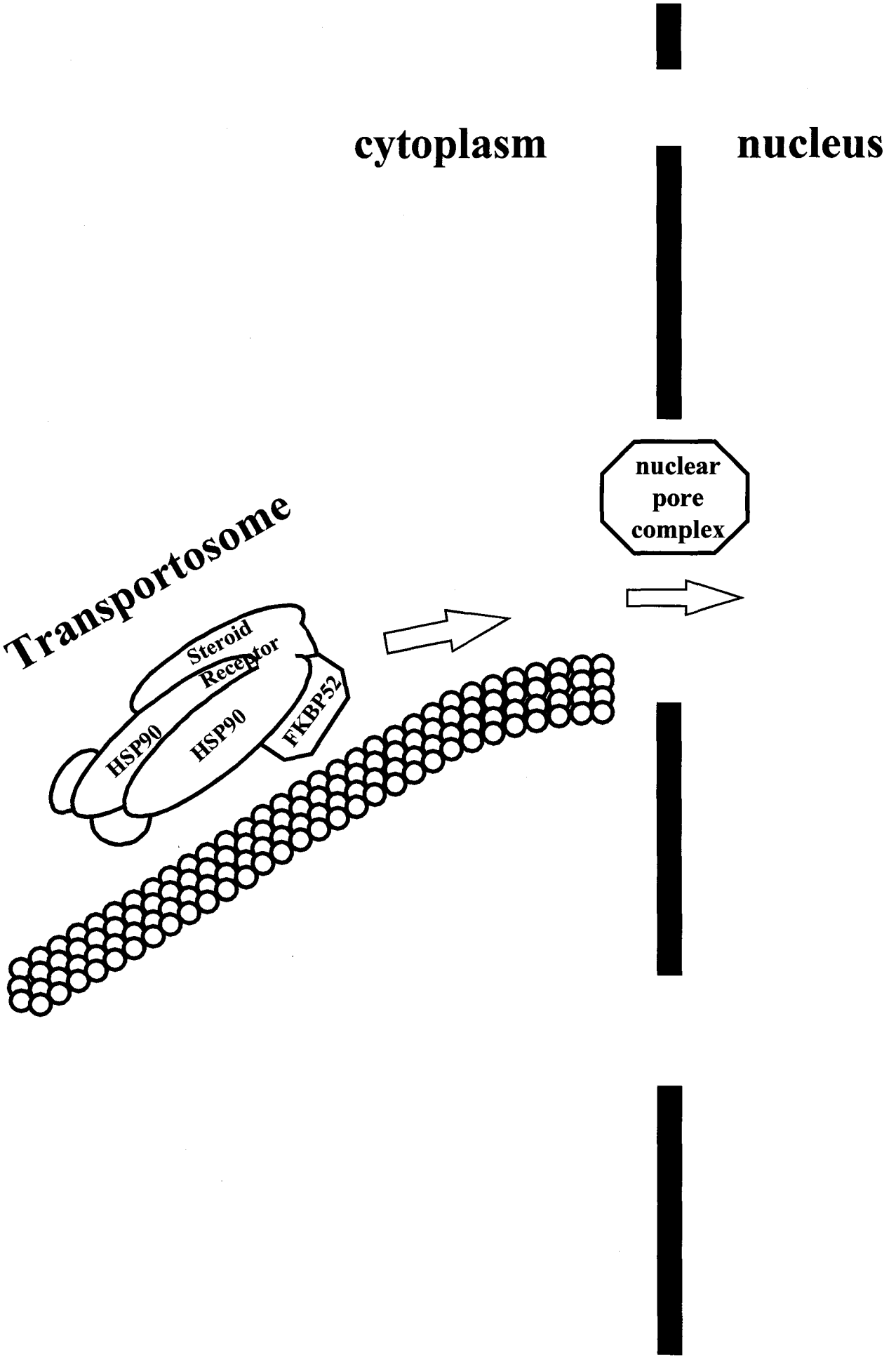
It has also been shown that the TPR containing immunophilins such as FKBP52, FKBP51, Cyp40, and Protein Phosphatase5 interact with HSP90 bound to the glucocorticoid receptor though these repeats (198). Other steroid receptors such as estrogen, progesterone and androgen receptors interact with HSP90 and FKBP52 (150, 175, 199, 201).

The role of the immunophilins in the steroid receptor complexes is unknown but is believed to be involved in targeting cytoplasmic-nuclear trafficking of receptors (198, 199) (Figure 29). FKBP52 interaction with HSP90 was shown to be regulated by phosphorylation. Casein kinase II (CKII) -phosphorylated FKBP52 no longer interacted with HSP90. Miyata et al. proposed that phosphorylation of HSP90-associated proteins by CKII may be one of the mechanisms that determine the molecular composition of HSP90-containing complexes (141).

4.4 FK506 Binding Protein 51- FKBP51

FKBP51 is the other TPR-containing FKBP. It possesses 53% overall identity with FKBP52. Unlike FKBP52 which is ubiquitous, FKBP51 is largely restricted to T cells. FKBP51 has a higher affinity for the immunosuppressant rapamycin than FK506. Unlike FKBP52-FK506, FKBP51-FK506 complexes are capable of binding and inhibiting calcineurin. FKBP51 is also associated with HSP90 and steroid receptors (5, 6, 150). Although very similar in structure and function, this particular study has shown that the effects seen on IRF-4 are specific to FKBP52 only. Therefor immunophilins possess overlapping roles as well as very specific and well-defined roles in protein regulation.

Figure 29: FKBP52, HSP90 and steroid receptor transport. FKBP52 is believed to be involved in the cytoplasmic to nuclear translocation of steroid (estrogen, progesterone, androgen and glucocorticoid) receptors with Heat Shock Protein 90 (HSP90). This big complex of FKBP52-HSP90-steroid receptor is termed the transportosome which migrates from the cytoplasm to the nucleus through the use of the cytoskeleton and nuclear pore complex. Adapted from DeFranco, 1998 and Pratt, 1999.

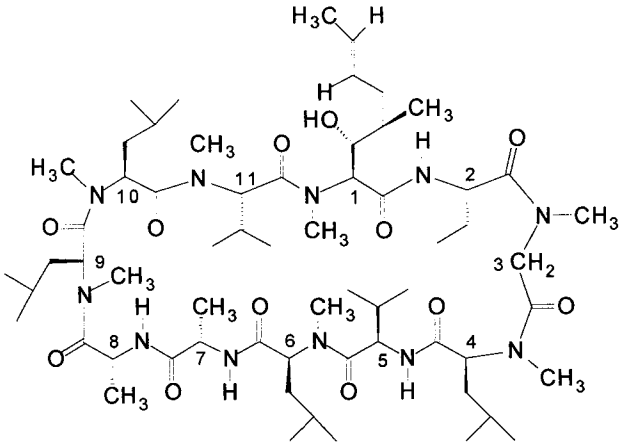

cytoplasm
nucleus
nuclear pore complex
Transportosome
Steroid Receptor
HSP90
HSP90
FKBP52

4.5 Immunosuppressants

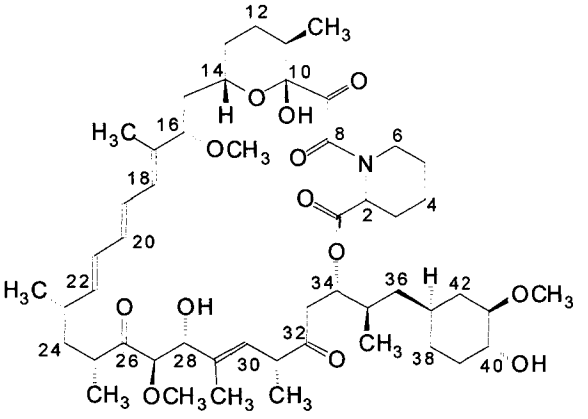
In the last two decades, the success rates experienced by transplantation surgery have been radically altered by the clinical use of the natural immunosuppressants such as cyclosporin A and FK506. The first to be used was CsA which was isolated from the fungi *Tolypocladium inflatum* in 1976 (12). CsA has subsequently been used for both treatment and prophylaxis of kidney and liver allograft rejection (85). In 1987, another potent immunosuppressant, the macrolide FK506 was isolated from *Streptomyces tsukubaensis* (85, 100, 101). Other very similar immunosuppressants such as ascomycin were as isolated and shown to have practically identical properties as FK506 (76, 159). Finally, rapamycin, another macrolide structurally related to FK506 has also been recently approved in the USA for use in kidney transplant patients (85).

Although structurally different, CsA and FK506 act in a very similar fashion, while FK506 and rapamycin, which are structurally similar, act in different manners (Figure 30). Both CsA and FK506 inhibit Ca2+ dependent signal transduction pathways in a range of cell types including transcription of early response cytokines, in particular IL-2 in antigen-activated T lymphocytes. The immunosuppressant-immunophilin complexes bind and sequester calcineurin which no longer dephosphorylates substrates such as the transcription factor NF-AT. Phosphorylated NF-ATs remain in the cytoplasm unable to activate genes such as IL-2. This results in immunosuppression by blocking the early proliferative signal within helper T-cells initiated by IL-2. Inhibition of the PPIase activity of immunophilins by the binding of the immunosuppressant is not involved in immunosuppression as previously believed. Rapamycin by contrast, has been shown to inhibit both Ca2+ dependent and independent pathways. It specifically acts at a much later stage of the immune response after activation of the IL-2 receptor by blocking the G1 to S cell cycle transition that initiates T-cell proliferation (Figure 10) (85, 184).

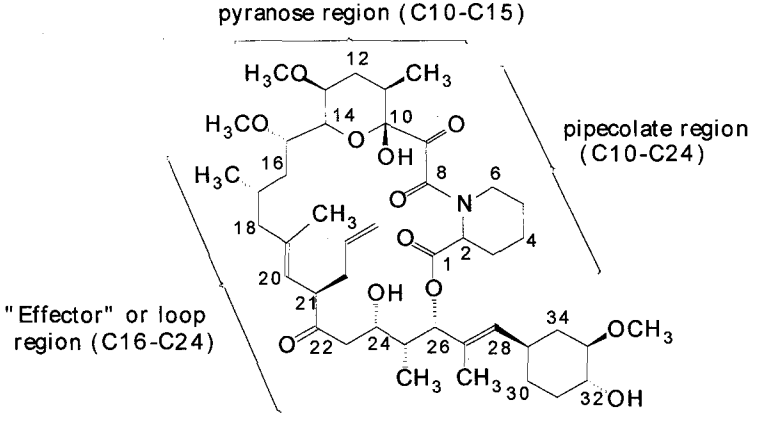
Figure 30: Structures of immunosuppressants. Macrolides such as FK506, ascomycin (ethyl analogue of FK506; ethyl group in rectangle) and rapamycin share similar structures as well as inhibit the PPIase activity of FKBPs. Cyclosporin A significantly varies from the macrolides structurally and inhibits the PPIase activity of cyclophilins. All of these compounds inhibit PPIase activity in nM concentration and in a similar manner through binding to immunophilins (Ivery, 2000, Pahl et al, 1992).



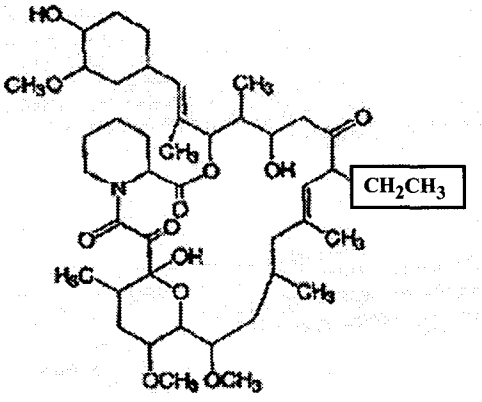
Cyclosporin A



Rapamycin (Sirolimus)



FK506 (Tacrolimus)



Ascomycin

Inhibit PPIase activity in nM concentration.

The effects of these immunosuppressants are much broader than just the inhibition of NF-AT-mediated gene activation. The inhibition of calcineurin also affects several other signaling pathways such as NF-κB, Nitric Oxide, Protein Kinase A, G-protein coupled receptor, integrin and JNK. Calcineurin has a growing number of substrates such as IκB, Nitric Oxide Synthase, Na2+ channels, Bcl2, dynamin, tau (184). Below is listed a series of genes downregulated or upregulated by CsA or FK506 treatment (Table 9).

Table 9: Genes affected by Cyclosporin A and by FK506 treatment. These immunosuppressants affect gene transcription in a similar manner, mostly by inhibiting cytokine, cell cycle and inflammatory signaling pathways. Adapted from Ruhlmann, 1997.

Genes Affected by Cyclosporin or FK506

+	−
collagenase	bcl2
erg-1	bcl-xl
ICAM-1	cyclin D2, D3
junaB	cypA
L-selectin	egr-2, -3
TGFβ	c-fos
	fyn
	glucagon
	GM-CSF
	hsp70
	hsc70
	lck
	IFNγ
	insulin
	IL-1β, -2, -3, -4, -5, -6, -8,
	IL-2R, -4R
	IP-10
	c-Jun
	MCP-1
	MIP2
	cMyb
	cMyc
	NF-κB p105
	NOS
	ras
	TCRα
	telomerase
	TNFα

RESEARCH OBJECTIVE AND SPECIFIC AIMS

The objective of this research was to investigate the activation and the regulation of IRF-4 in lymphoid cells and more specifically in HTLV-I leukemic T cells. One of the new members of the IRF family, IRF-4 was cloned as a lymphoid specific IRF which was found to be expressed in B cells, activated T cells and T cells infected with HTLV-I. The first specific aim was therefore to analyze the regulation of IRF-4 expression in these lymphoid cells. Promoter analyses using genomic footprinting and electrophoretic mobility shift assay revealed the importance of the NF-κB and NF-AT family members in regulating IRF-4 expression. The second specific aim was to investigate new IRF-4 interacting partners. A first two-hybrid screen using IRF-4 as bait, revealed an interaction with the immunophilin, FKBP52. Further investigation of the interaction demonstrated the importance of FKBP52's peptidyl-prolyl isomerase activity in regulating IRF-4 conformation and activity. FKBP52 could inhibit IRF-4 DNA binding and transactivation through a novel posttranslational mechanism involving its PPIase activity. A second two-hybrid screen using an HTLV-I transformed T cell cDNA library also revealed interesting IRF-4 interacting partners potentially involved in leukemogenesis. The third specific aim was to identify novel IRF-4 target genes and characterize their role in HTLV-I T cell-induced leukemogenesis. Using a stably expressing IRF-4 Jurkat cell line and cDNA array technology, many genes, most of them repressed were identified. These IRF-4 regulated genes are involved in cellular processes which are often deregulated in transformation such as DNA repair, cellular proliferation and apoptosis.

CHAPTER II

MATERIALS AND METHODS

1. Plasmid construction

1.1 IRF-4 and FKBP constructs

Murine IRF-4 FL as well as HA-tagged IRF-4 aa1-439, 1-419, 1-410, 1-380, 1-150 and Δ150-410 were a kind gift of Dr. Harinder Singh (University of Chicago). IRF-4 aa1-340, 1-237, 150-237, 150-340 and 150-410 were generated by PCR using the Vent polymerase after 30 cycles of amplification. PCR products were cut using XhoI and BamH1 and subcloned in frame into pCMV2-Flag (SalI and BamH1) and sequenced. The sequence of the primers used are listed below with the restriction enzyme site underlined:

IRF-4 -5': ATCACTCGAGCGCGTCATGAACTTG

IRF-4-5'- 150: ATCACTCGAGATTGTTCCAGAGGGAGCC

IRF-4-3'- 237: ATCATCTAGAGGATCCACAAGCATAAAAGGTTCC

IRF-4-3'- 340: ATCATCTAGAGGATCCATAAAGCCCATCTGGAGC

IRF-4-3'- 410: ATCATCTAGAGGATCCAAACTCCTCACCAAAGCA

The full-length cDNA of FKBP52 was obtained from the yeast two-hybrid screen and subcloned into pCDNA3Myc XhoI site (Dr. Lin) by cutting out the FL cDNA of FKBP52 from PACT-FKBP52 with XhoI. FKBP52 deletion mutants aa1-243 and 233-459 were constructed by PCR, subcloned in frame into pCDNA3Myc into XhoI/ XbaI and sequenced. The sequence of the primers used is listed below with the restriction enzyme (RE) sites underlined:

FKBP52 -5': ATCATCTCGAGTCGACCGCGCGGAGATGACAGCC

FKBP52-5'- 233: ATCATCTCGAGTCGACAAGTTCCAAATC

CCACCAAAT

FKBP52-3'-243: ATCATCTAGAGGGCCCTTTCAGCTCAGCATTTGG

FKBP52-3'- 459: ATCATCTAGAGGGCCCGGGCTATGCTTCTGTCTC

FKBP12 FL (gift from Dr. Steve Michnick) was subcloned in frame into pCDNA3Myc tagged vector by PCR using primers with RE sites underlined: XhoI / XbaI.

5': ATCATCTCGAGCGATGGGAGTGCAGGTG

3': ATCAGGATCCTCTAGATCATTCCAGTTTTAGAAG

FKBP51 FL (gift from Dr. David Smith) was subcloned in frame into pCDNA3Myc tagged vector by PCR using primers with RE sites underlined: XhoI/ XbaI.

5': ATCATCTCGAGCGATGACTACTGATGAAGGTG

3': ATCATCTAGATCATACGTGGCCCTCAG

All constructs were verified for expression in Cos7 by transfection with Lipofectamine (GIBCO-BRL Inc.) and Western blot analysis. See below for further instructions.

1.2 Yeast two hybrid plasmids

The fragment containing the proline rich and the IRF Association domains (aa150-410) of IRF-4 was PCR amplified with Vent (New England Biolab Inc.) and subcloned into the pAS2-1 vector SmaI/ BamHI (GAL4 $_{1-147}$ DNA binding domain,

TRP1; Clontech Inc.) in frame with the DNA binding domain of GAL4 and sequenced. The RE sites are underlined

5': ATCAGAATTCCCGGGAGTGTACAGGATTGTTCC

3': ATCAGTCGACGGATCCTGAGGGTCTGGAAACTCC

c-src cDNA (Genbank#NM_004383) was obtained Dr. R. Lefkowitz (Duke Medical Center, Durham, NC, USA) cloned into pCDNA3HA tagged (HindIII and XhoI). EIF4γ1 (aa142-1560) cDNA (Genbank#NM_004953) was obtained Dr. N. Sonnenberg (McGill University, Canada) cloned into pCDNA3HA tagged (EcoRI). PP2A catalytic cDNA (Genbank#J03804) was obtained Dr. A.H. Schonthal (University of Southern California, LA, California, USA) cloned into pCMVHA tagged (HindIII).

Rat Gi3 cDNA (Genbank#J03219) was obtained Dr. H. Itoh (University of Tokyo, Japan) cloned into pCMV (EcoRI). Gi3 was PCR amplified with PFU (Amersham Inc.) and subcloned into the pCDNA3-HIS version B vector (EcoR5/ XhoI) (Invitrogen Inc.) in frame with the His-Xpress tags and sequenced. The RE sites are underlined in the primer sequences below:

5': ATCAGATATCATGGGCTGCACGTTGAGC

3': ATCACTCGAGTCAGTAAAGCCCACATTCCTT

RhoANEF cDNA (Genbank#MN_014784) was obtained Dr. N. Kusuhara (Kazusa DNA Research Institute, Chiba, Japan) cloned into pBlueScript IISK+ (SalI/NotI). RhoANEF was PCR amplified with PFU Turbo (Amersham Inc.) and subcloned into the pCDNA3-HIS version B vector (EcoR5/ XbaI) (Invitrogen Inc.) in frame with the His-Xpress tags and sequenced. The RE sites are underlined in the primer sequences below:

5': ATCAGATATCATGAGTGTAAGGTTACCCCAGAGTAT

3': ATCATCTAGATTATGGTCCTGGTGACGCGGCTG

RhoGDI cDNA (Genbank#NM_004309) was obtained Dr.M-C. Dagher (Laboratoires DBMS/BBSI, Grenoble, France) cloned into pGEX-2T (BamHI/EcoRI). RhoGDI was subcloned into the pCDNA3-HIS version C vector (BamHI/EcoRI) (Invitrogen Inc.) in frame with the His-Xpress tags.

1.3 Recombinant protein plasmids

IRF-4 FL was subcloned in frame into pET-His tagged vector (Promega Inc.) by PCR using primers with RE sites underlined: XhoI/ BamHI.

5': ATCACTCGAGCGCGTCATGAACTTG

3': ATCAGGATCCGCCCTGTCAGAGTAT

FKBP52 FL was subcloned in frame into pGEX4T-2 GST-tagged vector (Pharmacia Inc.) by cutting out the FL cDNA of FKBP52 from PACT-FKBP52 with XhoI.

FKBP12 FL (gift from Dr. Steve Michnick) was subcloned in frame into pET-His tagged vector by PCR using primers with RE sites underlined: XhoI / BamHI.

5': ATCACTCGAGATGGGAGTGCAGGTG

3': ATCAGGATCCTCTAGATCATTCCAGTTTTAGAAG

FKBP51 FL (gift from Dr. David Smith) was subcloned in frame into pGEX4T-2 GST-tagged vector by PCR using primers with RE sites underlined: EcoRI/ XhoI.

5': ATCAGGAATTCCCATGACTACTGATGAAGGTG

3': ATCACTCGAGTCATACGTGGCCCTCAG

Both FKBP12 and FKBP51 were used as controls: Neither of them interacted nor affected IRF-4 DNA-binding, transactivation or migration as seen with FKBP52. It was therefor concluded that FKBP52 was acting on IRF-4 in a specific manner.

All constructs were verify by producing recombinant proteins loading them on SDS-PAGE, 1) staining with coomassie blue and 2) transferring to Western membrane to probe with the appropriate antibody. See below for further instructions.

1.4 MSCV retroviral system plasmid

IRF-4 FL was subcloned into pMSCV-neo vector (Clontech Inc.) by Vent PCR using primers with RE sites underlined: EcoRI/ XhoI.

5': ATCAGAATTCATGAACTTGGAGACGGG

3': ATCACTCGAGTTCTCACTCTTGGATGGA

1.5 IRF-4 promoter plasmids

IRF4PRO-PGL3B luciferase was generated by cloning the human promoter of IRF-4 into SacI/BglII digested luciferase reporter plasmid using specific primers (upstream primer 5'-ATGAAAATCCCTGGTCCAC-3' and downstream primer 5'-TGAGGGCAGCGGTGGGTCCC-3') to amplify the 1219 nucleotide upstream regulatory element based on the GenBank sequence #U52683. 5' deletion mutants of IRF4PRO-pGL3B were generated by digest with NotI/SmaI, NotI/HindIII and NotI/PstI. We also received a longer 5.0kB promoter fragment from Dr. Tak Mak (OCI/Amgen Inst., Toronto). The correct sequences of the constructs presented were confirmed by DNA sequence analysis.

2. Purification of recombinant proteins

The GST-FKBP52 fusion protein was expressed and isolated from *E.coli* BL21 following a 3h induction with 1mM IPTG (Pharmacia Inc.) at 30°C. Bacterial extracts in PBS containing 1% Triton X-100 were incubated with glutathione sepharose beads (Pharmacia Inc.) for 20min at room temperature and the fusion protein was eluted as previously described (118) The batches were 80-90% pure with a concentration of 33-100 ng/μl. The IRF-4 fusion protein was expressed as a poly-Histidine tagged protein, isolated from *E.coli* BL21 following a 3h induction with 1mM IPTG (Pharmacia Inc.) at 30°C. Bacterial extracts in PBS containing 1% Triton X-100 were incubated with His-Bind resin (Novagen Inc.) for 20 min at room temperature. High

purity (95%) and concentration (100 ng/μl) were obtained. GST-FKBP51 and His-FKBP12 were produced as described above.

3. Antibodies used and Companies

α-IRF-4/ ICSAT/ Pip:	Santa Cruz Inc., Zymed Inc.
α-PU.1:	Santa Cruz Inc.
α-FKBP52:	Biomol Inc.
α-FKBP12:	Biomol Inc.
α-FKBP51:	ABR Inc.
α-HA:	Boehringer Manheim Inc.
α-Flag:	Sigma Inc.
α-Myc 9E10	Sigma Inc.
α-Tax	National Institute of Health, Dr. W. Greene
α-IKKalpha	Santa Cruz Inc.
α-HSC70	Calbiochem Inc.
α-RhoA	Santa Cruz Inc., Dr. R. Desrosiers
α-RP-A	Dr. T. Kelly, Dr. W. Taylor
α-ERBB3	Santa Cruz Inc.
α-14-3-3η	Transduction Laboratories Inc.
α-Cyclin B1	Dr. J. Lee, Dr. W. Taylor
α-EB1	Transduction Laboratories Inc.
α-PCNA	Transduction Laboratories Inc.
α-MAPKK3	New England Biolabs Inc.
α-NIP3	Dr.R.Bruick

4. Cell culture and generation of cell lines

4.1 Transient transfections and reporter gene assays

All CAT assays were carried in monkey embryonic kidney COS-7 grown in Dulbecco's MEM media (GIBCO-BRL Inc.) supplemented with 10% fetal bovine serum and antibiotics. Subconfluent cells were transfected with 2-15 μg of CsCl purified plasmid, by lipofectamine (GIBCO-BRL Inc.) method. The reporter plasmids contained the IRF-4/PU.1 responsive elements (B4TK-CAT) (39) or the Interferon Stimulated Gene 15 (ISG15) promoter (117) linked to CAT reporter gene as well as the IRF-4, PU.1, ΔN-FKBP52 (aa233-459) and FKBP52 expressing plasmids. Sixteen hours post-transfection, transfected COS-7 cells were treated with ascomycin (2 μM) or DMSO. 48h posttransfection, total protein extracts were prepared and 10μg were used for CAT assay (4 hours at 37°C) CAT activities represent an average of CAT values obtained from 3 to 6 independent experiments.

Transient expression assays using luciferase constructs were carried out in 293T cells. Cells were seeded at 50% confluency and transfected with 500ng of reporter plasmid (HTLV-ILTR-luc or IRF4PRO-luc) and 1.5ug of the appropriate expression vector (pCMV-Tax, pCMV-M22 or pCMV-M47) by the calcium phosphate method. Cells were harvested 48h post-transfection and luciferase activity was measured by the luciferase assay system (Promega Inc.). Luciferase activities represent an average obtained from 3 to 6 independent experiments.

4.2 Cell lines

The HTLV-I infected cell lines C8166, MT-2 and MT-4 were cultured in RPMI 1640 media (Wisent Inc.) and the HTLV-II infected cell line, MO-T, in Iscove's media (Wisent Inc.) supplemented with 10% fetal bovine serum and antibiotics. The B cell lines J558, Namalwa, S194, KR-12, 70Z/3 were purchased from ATCC and cultured according to their directives. The A20 cell line was cultured in RPMI 1640/ 10% fetal bovine serum to express high amounts of surface immunoglobulins (ATCC, Manassas, VA). Jurkat, T4 and CEM T cell lines were cultured in RPMI 1640 media and 10% fetal bovine serum (Wisent Inc.).

4.3 Primary T lymphocyte preparation

Peripheral blood mononuclear cells were obtained from leukophoresed blood (YM). Primary T lymphocytes were rosetted to 2-aminoethylisothiouronium (AET; Sigma Inc.)-treated sheep red blood cells (Institute Armand Frappier, Laval, Que) and the resulting RBC-T lymphocyte complexes were isolated by Ficoll-density centrifugation. Purified RBC-T lymphocyte complexes were washed 3-4 times in ammonium chloride to lyse sheep RBC and the purified T lymphocytes were maintained in RPMI 1640 (CLT) supplemented with 20% non-heat inactivated fetal bovine serum and antibiotics. For treatment of primary T lymphocytes, PMA and ionomycin (Calbiochem Inc.) were used at a concentration of 20 ng/ml and 200nM, respectively. Concanavalin A (Sigma) was used at a concentration of 10 mg/ml and α-CD3 antibody (Beckman Coulter) at a concentration of 2 mg/ml.

4.4 Generation of IRF-4 stably-expressing cell line

The packaging cell line Phoenix Amphotropic (transformed human embryonic kidney cells) were purchased from ATCC with the written permission from Dr. Gary Nolan (Stanford University). These cells were cultured in DMEM with sodium pyruvate and 10% FBS. The packaging cell lines were transfected as described by Dr. Nolan's protocols (www.stanford.edu/group/nolan/phx_helper_free.html) with two vectors: pMSCVneo wt (no insert) and pMSCVneo-IRF-4. 48 post-transfection, virus was harvested from the transfected packaging cell line and used to infect Jurkat T cells. They were infected twice, each 24-hour infection in the presence of 16μg/ml of polybrene (Sigma Inc.). 48 hours post-infection, the transduced Jurkat T cells were put under drug selection, G418/ neo at a final concentration of 1200 mg/l. After two weeks of selection by changing the media every 3 days, a polyclonal population resistant to neomycin emerged. Jurkat-MSCV-wt and Jurkat-MSCV-IRF-4 cells (20 millions each) were lysed in TNN (50mM Tris-HCl, 200mM NaCl, 0.5%NP-40) for 10 minutes on ice. Protein supernatants were recuperated after 10 minute spin at 14000 g. Protein concentration were determined using the Bradford reagent (Biorad Inc.) and a spectrophotometric assay. Protein extracts were loaded on a SDS-PAGE on transferred to a nitrocellulose membrane which was probed with α-IRF-4 antibody. The presence of high levels of IRF-4 protein was detected in Jurkat-MSCV-IRF-4 cells but not in Jurkat-MSCV-wt. A polyclonal population of these cells was used for RNA extraction needed for cDNA array analysis.

5. Western blot analysis and immunoprecipitation assays

Whole cell extracts (50-60μg) were fractionated by 10-15% SDS-PAGE and transferred to Hybond transfer membrane (Biorad Inc.). The membrane was blocked in 5% milk for one hour and probed overnight with at 4°C with the appropriate antibody at dilutions between 1:200 to 1:2000. The signal was detected with secondary antibodies conjugated to horseradish peroxidase at a dilution of 1:1000 and developed with chemiluminescence substrate (Amersham Inc.).

For co-immunoprecipitations, COS-7 cells (15 cm plate at 70-80% confluency) were transfected by the lipofectamine method (GIBCO-BRL Inc.) with 15 μg expression plasmids. Whole cell extracts from COS-7, HTLV-transformed cells and B cell lines (500 μg) were lysed with TNN (50mM Tris-HCl, 200mM NaCl, 0.5%NP-40). Immunoprecipitating antibodies such as α-MYC 9E10 or α-FKBP52 were crosslinked to 300ul Sepharose G or Sepharose A beads with dymethyl pimelimidate (Sigma Inc.). The extracts were incubated with 30ul of protein sepharose beads crosslinked with the particular antibody for 4 hours at 4°C. Immunocomplexes were eluted by boiling the beads and separated by SDS-PAGE.

6. Electrophorectic Mobility Shift Assay

6.1 Extract preparation

Cells were washed in Buffer A [10 mM HEPES, pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl; 0.5 mM dithiothreitol (DTT); and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] and were resuspended in Buffer A containing 0.1% NP-40. Cells were then

chilled on ice for 10 min before centrifugation at 10,000 g. Pellets were then resuspended in Buffer B (20mM HEPES, pH 7.9; 25% glycerol; 0.42 M NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA; 0.5 mM DTT; 0.5 mM PMSF; 5 μg/ml leupeptin; 5 μg/ml pepstatin; 0.5 mM spermidine; 0.15 mM spermine; and 5 μg/ml aprotinin). Samples were incubated on ice for 15 min before being centrifuged at 10,000 g. Nuclear extract supernatants were diluted with Buffer C (20 mM HEPES, pH 7.9; 20% glycerol; 0.2 mM EDTA; 50 mM KCl; 0.5 mM DTT; and 0.5 mM PMSF).

6.2 Oligonucleotide probes and complex analysis

Nuclear extracts from C8166 or COS-7 transfected cells (3 μg) or 200 ng recombinant proteins were assayed for IRF-4 binding in gel shift analysis using ^{32}P-labeled oligonucleotide corresponding to the ISRE of the ISG-15 gene (5'-GATCGGGAAAGGGAAACCGAAACTGAAGCC-3') and the λB element (GAAAAAGAGAAATAAAAGGAAGTGAAACCAAG) (39). Binding reactions and electrophoresis were performed as previously described (118). The resulting protein-DNA complexes were resolved by 5% polyacrylamide gel and exposed to X-ray film. To demonstrate the specificity of protein-DNA complex formation, 125-fold molar excess of the unlabeled oligonucleotide was added to the nuclear extract before adding labeled probe. The nature of the complexes was also analyzed by supershifting using α -IRF-4 or α-PU.1 antibodies (1μg). In some experiments, whole cell extracts and recombinant protein were incubated with 10μM of Ascomycin (Calbiochem Inc.) diluted in DMSO, an ethyl analogue of FK506. DMSO or ascomycin had no effect on IRF-4 DNA binding (data not shown).

7 Partial proteolysis of IRF-4

In vitro translated IRF-4 was produced using the T7 polymerase TnT system (Promega Inc.) with S35-labeled mix containing cysteine and methionine (ProRedivue, Amersham Inc.). Purity of labeled protein was verified by SDS-PAGE and autoradiography. Labeled IRF-4 (1µl per reaction) was preincubated with GST-FKBP52, ascomycin (1 µM) or BSA (200 ng) for 1 hour at room temperature. Chymotrypsin was added at different time points and the proteolytic reactions were stopped by the addition of 1X SDS-loading buffer. The control with no chymotrypsin was incubated for 30 min at room temperature. Ascomycin was also preincubated with IRF-4 alone and had no effect on proteolysis (data not shown). Samples were loaded on 15% SDS gels, enhanced with Amplify solution (Dupont Inc.), dried and exposed to film.

8 Yeast two-hybrid assays

Protocols concerning yeast two-hybrid were obtained from Clontech Inc. and followed as listed. The yeast strain Y190 was cotransformed by the lithium acetate permeabilization method with the pAS2-1-IRF-4 (150-410) and with an EBV-transformed B-cell cDNA library or an HTLV-I T cell cDNA library cloned into the pACT and PACT2 vectors, respectively (Clontech Inc.). Positive yeast clones were selected for prototrophy for leucine, tryptophan and histidine and screened for β–galactosidase expression. Subsequent two-hybrid assays were carried out by a mating assay between the Y187 and Y190. Diploid cells from the mating assay were

selected for Trp- Leu- His- selective medium and screened by replica plating for their ability to produce for β–galactosidase. Plasmid DNA was isolated from yeast and transformed in *E.coli* DH5α by electroporation. The constructs were sequenced using 5'PACT primer (5'-TACCACTACAATGGATG-3').

9 RNA detection

9.1 RNA isolation for RT-PCR

Total cellular RNA was isolated by using the RNA extraction kit from Qiagen Inc. Primary T cells RNA isolation was performed by using the Trizol reagent (GIBCO CLT). RNA concentration and purity was assessed by spectrophotometric analysis (260/280 nm). RNA integrity was also analyzed by electrophoresis on 1% agarose gel (1X TAE).

9.2 Reverse transcriptase-PCR assays

Total RNA isolated from 70Z/3, A20 cells and primary T cells was treated with 1 unit of RNase-free DNase (RQ1 DNase, Promega Biotech Inc., Madison, WI) for 30 min at 37°C, phenol-chloroformed and ethanol-precipitated. Reverse transcription was performed with 2 μg of total RNA and 0.2 pmol of random hexamers or oligo dT using 200 units of Moloney murine leukemia virus reverse transcriptase (GIBCO, CLT) in buffer containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10mM dithiothreitol, 3 mM $MgCl_2$, 500 nM dNTP, 0.1 mg/ml bovine serum albumin, 272.5 units/ml RNase inhibitors (Amersham Pharmacia Biotech Inc.). PCR assays were performed using 5 μl and 1 μl of RT product for IgL λ (undiluted) and GAPDH (100

fold diluted) as well as IRF-4 (10 fold diluted) mRNA, in 100 mM Tris-HCl, pH 8.4, 500 mM KCl, 15 mM $MgCl_2$, 200 mM dNTP, 15 pmol of and γ^{32}P-labeled primers, and 1.25 units of *Taq* DNA polymerase (Amersham Pharmacia Biotech.). Nucleotide sequence of primers used for IgL λ were as follows: 5' –ATGGCCTGGACTTCACTTA– 3' and 5' –TCTTGGGCTGGCTTAGGA– 3' and for IRF-4: 5'-CGCTCTTCAAGGCTTGGG-3' and 5'-GGTCGAGGGGTGGCATCA-3' The PCR reaction mixture was subjected to 25 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 62°C, and polymerization for 1 min at 72°C. PCR products were analyzed on a 5% denaturing polyacrylamide gel. Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as described previously (2, 117).

RT-PCR was performed to confirm cDNA array analysis results with the use of the Clontech Inc. kit (K1402-2). Primer sequences for the potential IRF-4 regulated genes were purchased from Clontech Inc and synthesized by CLT GIBCO Inc.

9.3 RNA isolation for cDNA arrays

Cells (50 million) from the stably expressing cell line, Jurkat-MSCVneo-IRF-4 as well as the control cell line Jurkat MSCVneo-wt were collected and lysed using the denaturation solution supplied by the Clontech kit. Total RNA isolation was performed as described in the "Atlas Pure Total RNA Labeling System" (#PT3231-1, Clontech Inc.). RNA purity and integrity was assessed as described in the manual provided.

9.4 cDNA arrays analysis

Probe synthesis, hybridization and detection were performed as described in the "Atlas Pure Total RNA Labeling System" (#PT3231-1, Clontech Inc.). Analysis of the membranes was possible through the use of the software "AtlasImage 1.5" purchased from Clontech (#PT3381-1). We wish to thank the Labs of Drs. Batist and Jamali for the use of their PC computers during cDNA array analyses. A series of three separate experiments (RNA extraction, probe synthesis and hybridization) were performed in order to determine the genes regulated by IRF-4.

10 Genomic footprinting

For in vivo methylation by dimethyl sulfate (DMS; Aldrich Chemical), exponentially growing MT-2 or Jurkat cells (10^8) were harvested and resuspended in RPMI 1640-10% FBS supplemented with 20 mM HEPES (pH 7.3). The methylation reaction was performed in the presence of 10 µl/ml of concentrated DMS for 1 minute at room temperature. The reaction was stopped by two 1ml washes with ice-cold PBS containing 2% β-mercaptoethanol. Genomic DNA extraction was performed as previously described (2). To obtain naked DNA, cells were first lysed to extract genomic DNA and then submitted to DMS treatment for 30 seconds. For each DNA sample, genomic DNA resuspended in 200 µl H_2O was treated with 20 µl of piperidine (Aldrich) for 30 minutes at 90 °C to cleave methylated G (or A) residues. For each sample, 5 µg of DMS-treated, piperidine-cleaved genomic DNA was used for ligation-mediated PCR (LM-PCR) using Vent DNA polymerase (New England

Biolabs), as described elsewhere (51). To ensure elongation of different fragment sizes, PCR amplification step was 2 minutes for the first cycle and was progressively increased to 10 minutes in the last cycle, with a total of 18 cycles. A third primer was radiolabeled by end labeling using T4 polynucleotide kinase (Amersham Pharmacia biotech) and [γ-^{32}P]ATP (ICN Pharmaceuticals). Two more PCR cycles were performed to labeled elongated DNA. Each reaction product was then phenol-chloroform extracted and ethanol precipitated prior to electrophoresis on a 7.5 % Explorer sequencing gel (Baker, Phillipsburg, NJ) in 1X TBE at 65 W. Reactions were visualized by autoradiography using Biomax MR films (Kodak). For the LM-PCR, several sets of primers were used. For analysis of the noncoding strand of the κB1/Sp1 region of the IκBα promoter, primer 1, 5'-CTCATCGCAGGGAGTTTCT-3', Tm 55 °C; primer 2, 5'-CCCAGCTCAGGGTTTAGGCTTCTTT-3', Tm 63°C; primer 3, 5'-GGGTTTAGGCTTCTTTTTCCCCCTAGCAG-3', Tm 66°C. For analysis of the noncoding strand of the –600 to –400 region of the IRF4 promoter, primer 1, 5'-GTCACTTCAATTCACCAGC-3', Tm 58°C; primer 2, 5'-GCAAAAGGATGTAAGCATGTCAGACACG-3', Tm 63°C; primer 3: 5'-GTAAGCATGTCAGACACGCAGAGACAGTATTTG-3', Tm 65 °C. For analysis of the noncoding strand of the –460 to –260 region of the IRF4 promoter, primer 4, 5'-GTGATGGCCTTGCCGA-3', Tm 60°C; primer 5, 5'-GCAACCTCCACCTCCAGTTCTCTT-3', Tm 63 °C; primer 6, 5'-ACCTCCAGTTCTCTTTGGACCATTCCTCC-3', Tm 66 °C.

11. Kinase assay

Cell lysates from C8166, MT2, T4 and Jurkat cells were immunoprecipitated with rabbit polyclonal antibody directed against IKKα (Santa Cruz Biotech., Santa Cruz, CA). The immunoprecipitates were incubated in kinase reaction buffer consisting of 10μCi [γ-^{32}P] ATP, 1mM ATP, 5 mM $MgCl_2$, 1 mM dithiothreitol, 100 mM NaCl and 50 mM TRIS-HCL (pH 8.0) at 30°C for 15 minutes. Substrates for the kinase reactions were GST-IκBα wild type or mutant (2ND4). Each kinase reaction was incubated in the presence of 1μg of substrate.

CHAPTER III

THE REGULATION OF IRF-4 EXPRESSION IN LYMPHOID CELLS AND IN HTLV-I INFECTED T CELLS

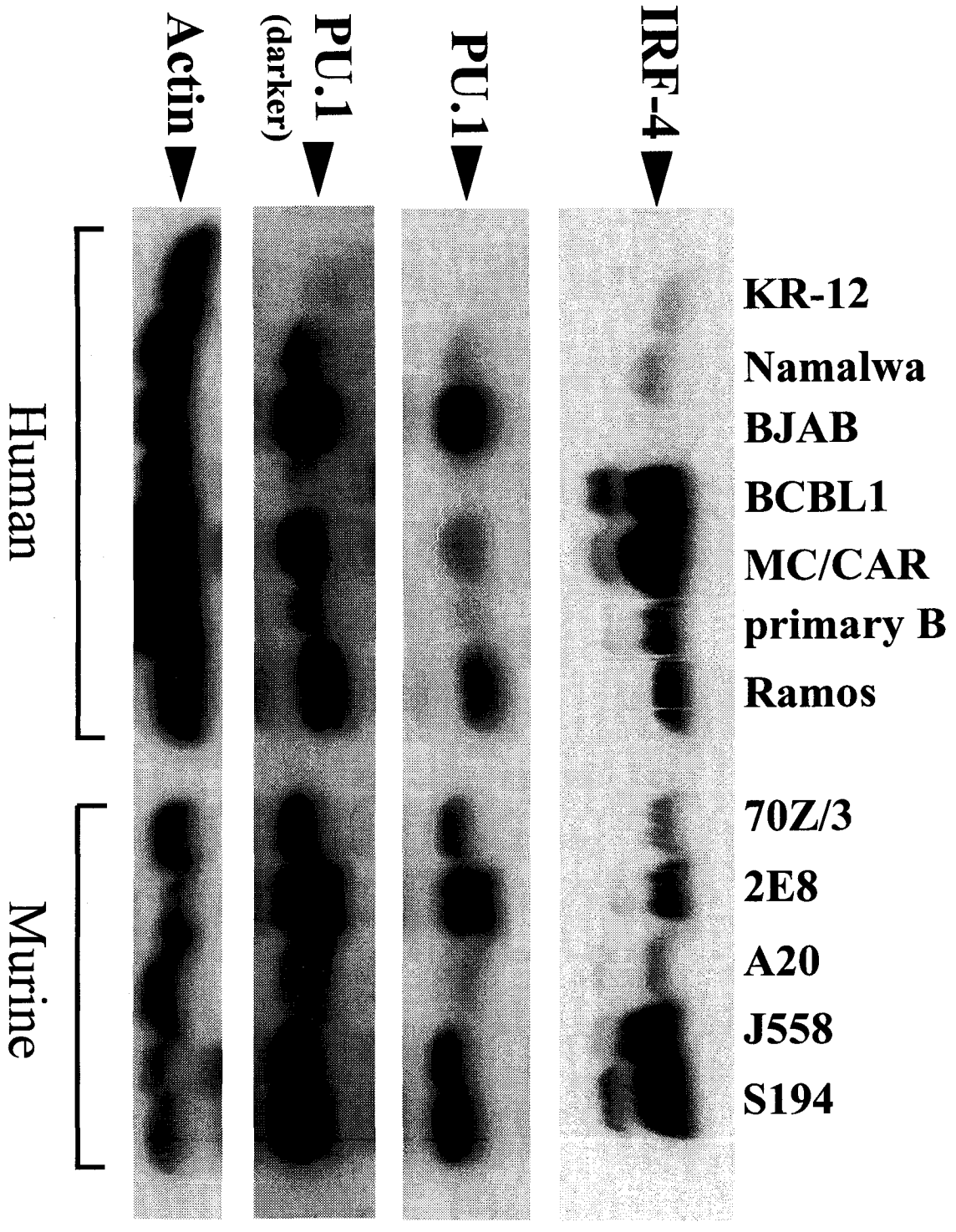
IRF-4 expression in B cell lineages.

The discovery of another member of the IRF family resulted from an effort to clone factors binding to the murine immunoglobulin light chain enhancer $E_{\lambda 2\text{-}4}$ in B cells (39). PU.1 interaction partner, or Pip/IRF-4, was identified as a novel murine transcription factor with an IRF-like N-terminal domain. IRF-4 bound to DNA but exclusively in association with PU.1, a member of the ETS family of transcription factors which contribute to lymphoid and myeloid lineage development (31, 127). IRF-4 expression as well as and the expression of its transcriptional partner, PU.1, was analyzed in murine and human B cells (Figure 31). Whole cell extracts prepared from different cell lines and primary B cells were loaded on SDS-PAGE and transferred to nitrocellulose membrane. The same membrane was successively blotted with an α-IRF-4, α-PU.1 and α-actin (loading control).

Both IRF-4 and PU.1 proteins were expressed in most B cell developmental stages: in pre-B cell (70Z/3; Figure 31, upper and middle panels, lane 8), immature B cells (KR-12, 2E8; Figure 31, upper and middle panels, lanes 1 and 9), mature B cells (Namalwa, BCBL1, Ramos, A20; Figure 31, upper and middle panels, lanes 2, 4, 7 and 10), plasmacytomas (MC/CAR, J558, S194; Figure 31, upper and middle panels, lanes 5, 11 and 12) and in primary B cells from blood (Figure 31, upper and middle panels lane 6). IRF-4 is present in the BJAB B cell line but in much lower levels than most B cells (Figure 31, upper panel, lane 3, data not shown). Interestingly IRF-4 was highly expressed in B cells infected with Herpes Virus 8 (HHV8) but PU.1 levels were virtually absent (Figure 31, upper and middle panels lane 4). The actin blot

revealed relatively equal loading of protein extracts (Figure 31, lower panel, lanes 1-12).

Figure 31: IRF-4 expression in B cells. Whole cell extracts (50μg) from murine (70Z/3, 2E8, A20, J558 and S194) and human B cell lines (KR-12, Namalwa, BJAB, BCBL1, MC/CAR, Ramos) as well as primary B cells were run on 10% SDS-PAGE and immunoblotted using αIRF-4 antibody (upper panel), αPU.1 antibody (two middle panels) and αactin antibody (loading control).


Actin
PU.1 (darker)
PU.1
IRF-4
Human
Murine
KR-12
Namalwa
BJAB
BCBL1
MC/CAR
primary B
Ramos
70Z/3
2E8
A20
J558
S194

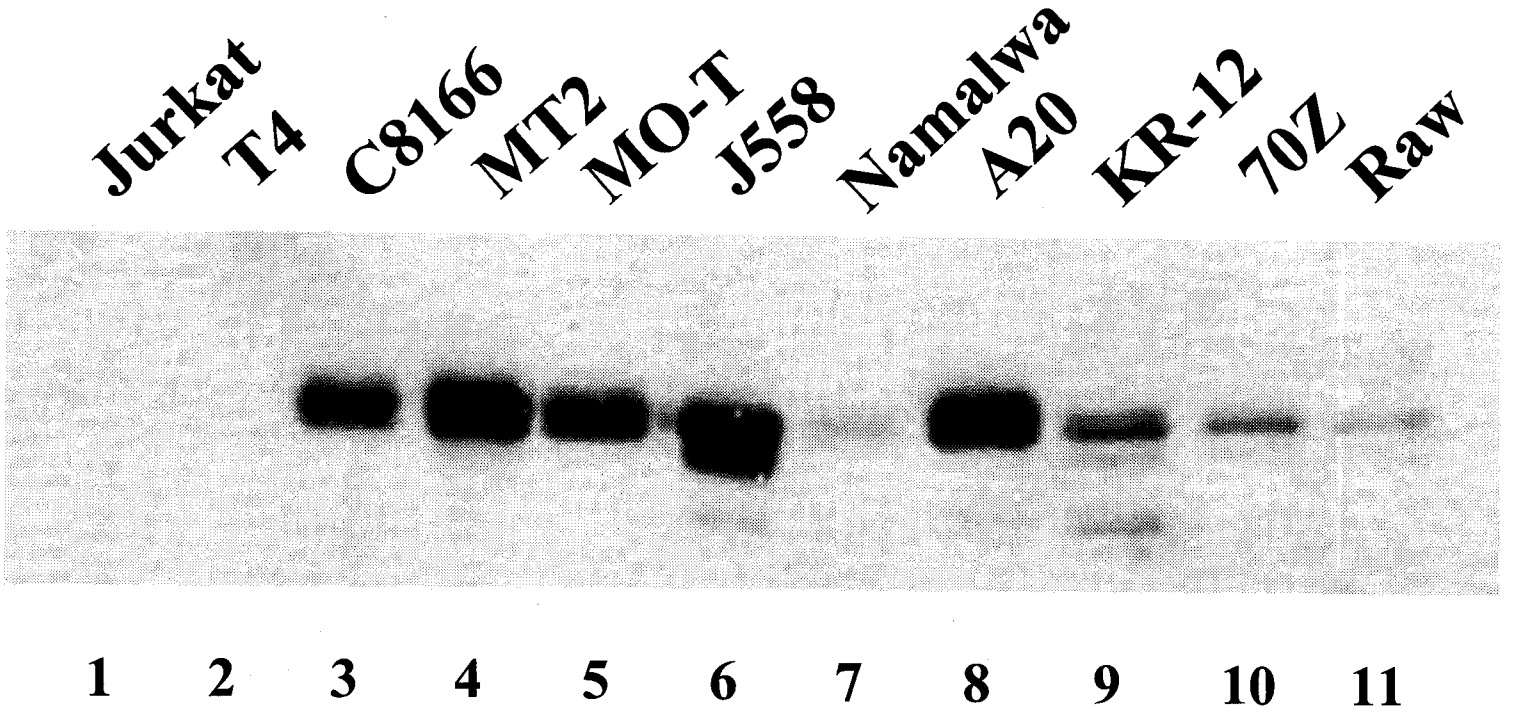
IRF-4 expression is upregulated in HTLV-I and HTLV-II infected cell lines.

IRF-4, a lymphoid/macrophage-specific transcription factor, is constitutively expressed in B cells and macrophages, but not in resting T lymphocytes. Initially, immunoblot analysis was used to evaluate IRF-4 expression in different HTLV-I or -II transformed T cells, B cells, macrophages and T cell lines (Figure 32). Whole cell extracts prepared from HTLV-I transformed cells - C8166 and MT2 (Figure 32, lanes 3 and 4), HTLV-II transformed cells - MoT (Figure 32, lane 5), B cells - J558, Namalwa, A20, KR-12 and 70Z/3 (Figure 32, lanes 6-10), macrophages - Raw264.7 (Figure 32, lane 11) as well as two T cell clones- Jurkat and T4 (Figure 32, lanes 1 and 2) were analyzed using a non-cross-reactive antibody against IRF-4. As shown in Figure 32, a 51-kD IRF-4 protein was detected in all B cells, macrophages and HTLV-I or II transformed cell lines. Whereas no IRF-4 was produced in non-HTLV infected T cell lines (Figure 32, lanes 1 and 2)

Tax expression is not sufficient to induce IRF-4 in T cells.

The human equivalent of IRF-4 was initially isolated from an adult T-cell leukemia cell line, as the "IFN consensus sequence-binding protein in adult T-cell leukemia cell lines or activated T-cells" (ICSAT) capable of binding to the human IL-5 promoter (239). We further analyzed IRF-4 and Tax expression in HTLV-I infected T cells. Two HTLV-I infected cell line C8166 and MT2, as well as two uninfected T cell lines, T4 and Jurkat were analyzed for IRF-4, Tax and FKBP52 (loading control) expression levels (Figure 33).

Figure 32: IRF-4 is expressed in HTLV-I and HTLV-II infected cells. Whole cell extracts (50μg) were prepared from C8166 and MT2 cells (HTLV-I infected), MoT cells (HTLV-II infected), J558, Namalwa, A20, KR-12 and 70Z cells (B cells), Raw264.7 cells (macrophages) and Jurkat and T4 cells (T cells). Extracts were fractionated by 10% SDS-PAGE and probed with anti-IRF-4 antibody. A 51kDa band corresponding to IRF-4 was detected in all HTLV-I and HTLV-II transformed cell lines, as well as in all B cell and macrophage cell lines.


Jurkat
T4
C8166
MT2
MO-T
J558
Namalwa
A20
KR-12
70Z
Raw
1
2
3
4
5
6
7
8
9
10
11

Immunoprecipitations were performed on whole cell extracts using an α-IRF-4, α-Tax and α-FKBP52 antibodies. Immunoprecipitated complexes were analyzed by Western blot using the same antibodies mentioned above. In HTLV-I infected T cells, IRF-4 protein levels seemed to parallel Tax expression levels. The HTLV-I infected cell line, C8166 expressed the most Tax as well as possessed the highest IRF-4 protein levels (Figure 33, upper and middle panel, lane 1). The MT2 cell line produced moderate levels of Tax and of IRF-4 (Figure 33, upper and middle panel, lane 2). IRF-4 expression was absent in the non-Tax expressing cell lines, T4 and Jurkat (Figure 33, upper and middle panel, lanes 3 and 4). FKBP52 was used as a control for the immunoprecipitation efficiency (Figure 33, bottom panel).

Although Tax has been previously documented to be involved in IRF-4 expression, it is not sufficient for IRF-4 expression in non-HTLV infected T cells. A Tax-inducible Jurkat cell line, JPX9, provided by Dr. K. Sugamura (Japan), was used to analyze IRF-4 expression. Tax expression in these cells was induced over a period of 3 days by using zinc chloride. Whole cell extracts from the JPX9 and C8166 cells were analyzed by Western Blot with an α-IRF-4 and an α-Tax antibody (Figure 34). Tax expression was seen after one day of ZnCl2 induction and its expression was sustained over the three days of zinc incubation (Figure 34, upper panel, lanes 3-5). No Tax protein was seen in uninduced JPX9 cells (Figure 34, upper panel, lane 2).

Figure 33: IRF-4 and Tax expression in HTLV-I infected cell lines. C8166, MT2, T4 and Jurkat WCE (500 μg) were immunoprecipitated with an αRF-4, αTax and an αFKBP52 antibody. Complexes were run on 10% SDS-PAGE and immunoblotted using an αIRF-4 antibody (upper panel), an αTax antibody (middle panel) and an αFKBP52 (lower panel). The control IP was performed by incubating WCE (500 μg) with normal precleared mouse serum (data not shown).

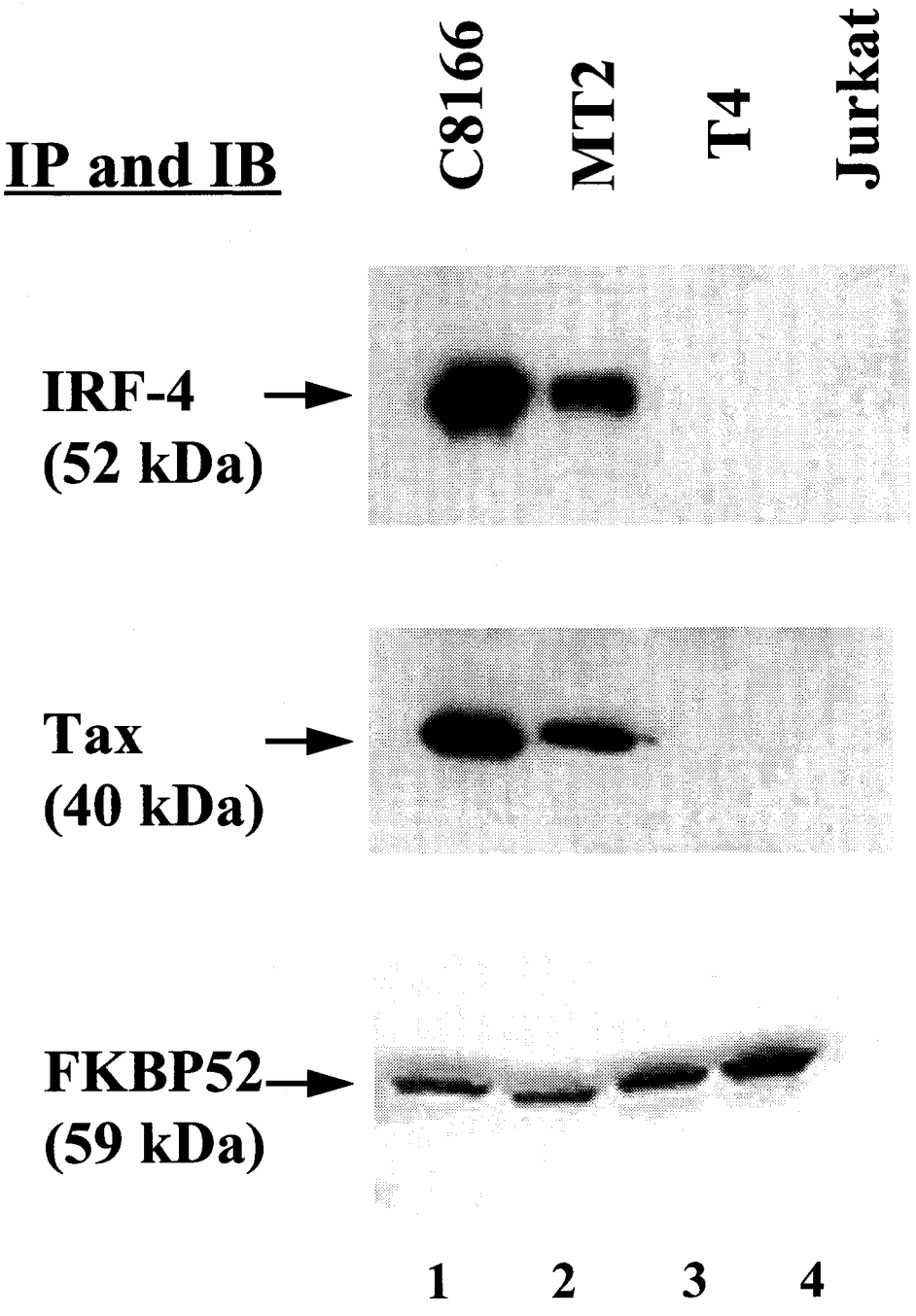
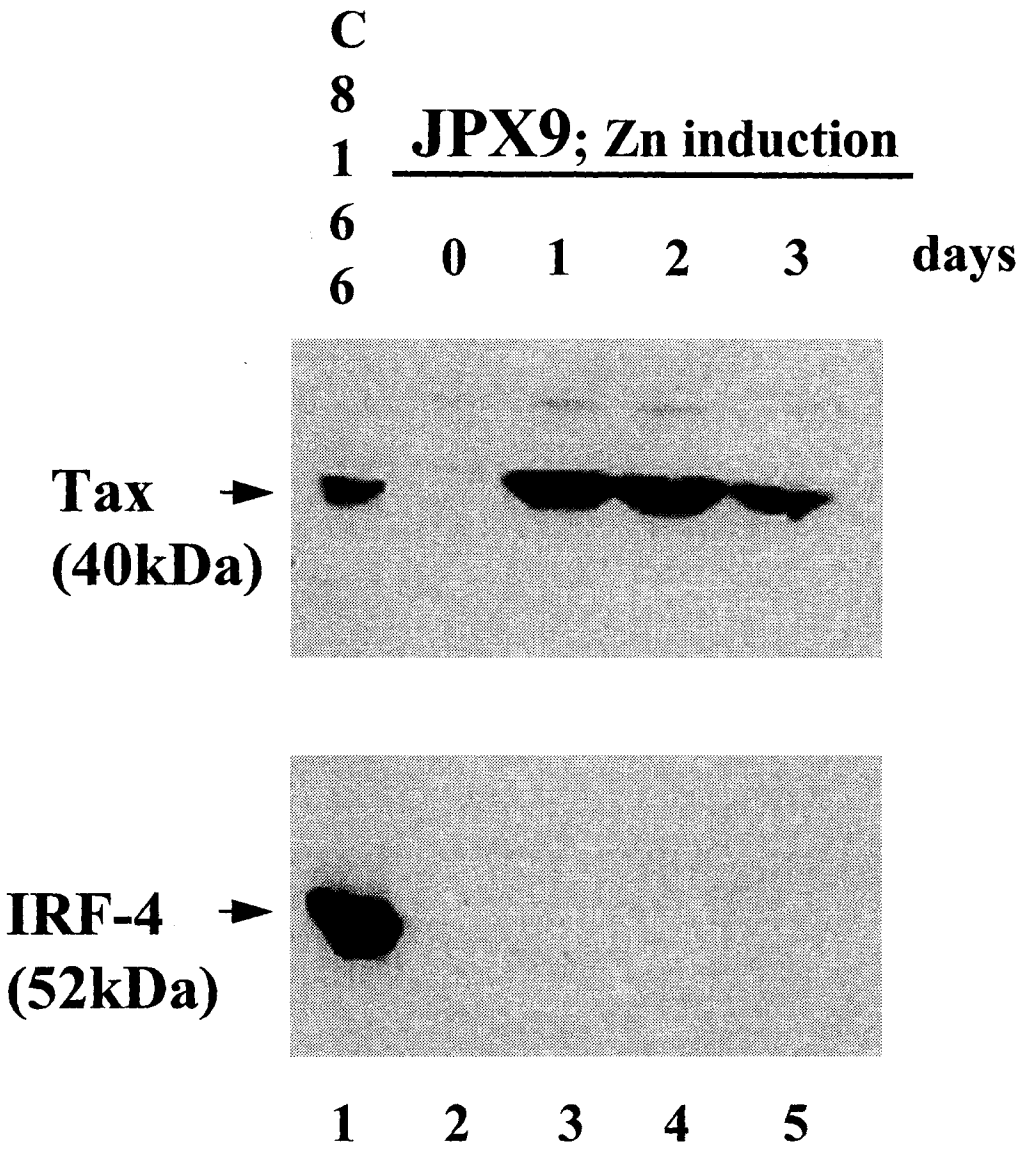

IP and IB
C8166
MT2
T4
Jurkat
IRF-4
(52 kDa)
Tax
(40 kDa)
FKBP52
(59 kDa)
1
2
3
4

Figure 34: IRF-4 expression in Tax-inducible cell line, JPX9. Whole cell extracts (50 μg) from C8166 cells and JPX9 cells (induced with zinc chloride for three days) were run on 10% SDS-PAGE and immunoblotted using an αTax antibody (upper panel) and an αIRF-4 antibody (lower panel).


C
8
1
6
6
JPX9; Zn induction
0 1 2 3 days
Tax
(40kDa)
IRF-4
(52kDa)
1 2 3 4 5

The same blot was reprobed with an α-IRF-4 antibody and no IRF-4 protein was detected over the same 3 day-period of Tax induction in these cells (Figure 34, lower panel, lanes 2-5). Both Tax and IRF-4 proteins were detected in the positive control, HTLV-I infected C8166 cells (Figure 34, upper and lower panels, lane 1).

IRF-4 is inducible in activated primary T lymphocytes.

Analysis of IRF-4 function in knockout mice demonstrated that IRF-4 was involved in late stage activation responses in B and T cells, such as cellular proliferation and cytokine production (137). To determine whether IRF-4 production could be induced in primary T lymphocytes after treatment with antigen-mimetic stimuli, sequence-specific oligonucleotide primers were used to perform semi-quantitative RT-PCR with RNA from primary T lymphocytes. IRF-4 mRNA was induced in primary T lymphocytes after 6h of treatment with anti-CD3 antibody, PMA/ionomycin or concanavalin A (Figure 35, lanes 5, 8 and 11); IRF-4 mRNA levels remained detectable after 24 hours. In contrast, treatment with TNFα did not induce IRF-4 mRNA production (Figure 35, lanes 1-4). In order to confirm that IRF-4 mRNA levels in activated primary T lymphocytes correlated with the expression of IRF-4 protein, immunoblot analysis was carried out using extracts from the primary T lymphocytes. IRF-4 protein expression peaked 24h after induction with anti-CD3 antibody, PMA/ionomycin or concanavalin A (Figure 36, lanes 5-7) but was not detected in unstimulated, TNF-α or LPS-treated T lymphocytes (Figure 36, lanes 2, 3 and 4). HTLV-I transformed MT2 cell extracts were used as a positive control for IRF-4 expression (Figure 36, lane 1).

Figure 35: IRF-4 mRNA is upregulated in primary activated T lymphocytes. 2μg of total RNA isolated from primary T lymphocytes stimulated with various activators for 6, 10 and 24h was reverse transcribed to cDNA. PCR was performed with sequence-specific oligonucleotide primers to detect the presence of IRF-4 mRNA. A 320-bp band corresponding to IRF-4 appeared after 6h of treatment with monoclonal antibodies to CD3, PMA/Ionomycin and concanavalin A (lanes 5, 8 and 11). IRF-4 mRNA was not detected in unstimulated T lymphocytes or in cells treated with TNF-α (lanes 1-4). GAPDH amplification was performed on each sample and used as a loading control.

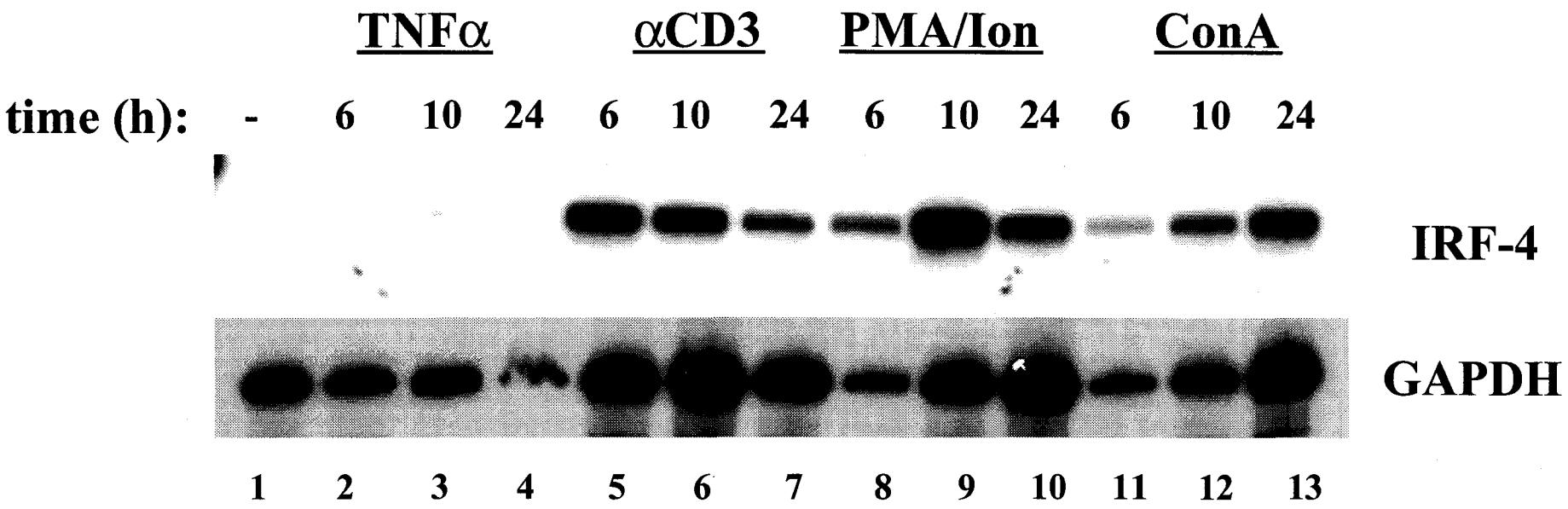
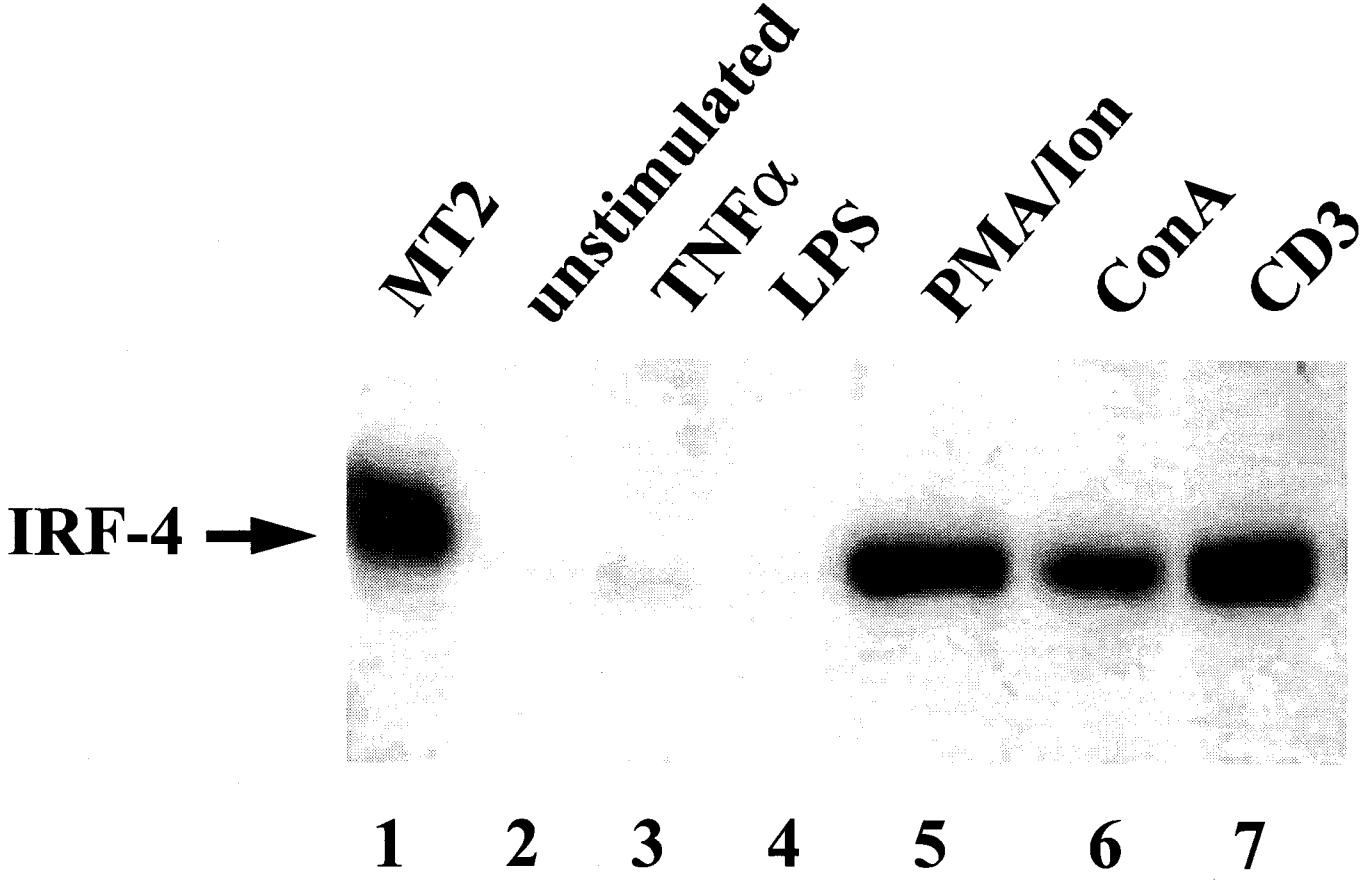

TNFα
αCD3
PMA/Ion
ConA
time (h): - 6 10 24 6 10 24 6 10 24 6 10 24
IRF-4
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 36: IRF-4 protein is expressed in primary activated T lymphocytes. 50μg of whole cell extract from primary T lymphocytes treated with different inducers for 24h were analyzed by Western blot using an αIRF-4 antibody. A 51kDa band corresponding to IRF-4 was detected in cells treated with anti-CD3, PMA/Ionomycin and concanavalin A (lanes 5-7). MT2 cells were used as a positive control for IRF-4 expression


MT2
unstimulated
TNFα
LPS
PMA/Ion
ConA
CD3
IRF-4
1
2
3
4
5
6
7

HTLV-I Tax is a positive regulator of the IRF-4 promoter.

Preliminary experiments were conducted to determine whether Tax could transactivate the 1.2kB human IRF-4 promoter in transient co-expression assays in 293T cells (Figure 37). As shown in Figure 38A, over-expression of Tax resulted in a 7-fold enhancement of the IRF-4 promoter activity. In addition, the Tax mutants M22 and M47, which are defective in activation of the NF-κB and CREB/ATF pathways, respectively, maintained the ability to transactivate the full length IRF-4 promoter approximately 4-fold. The NF-κB pathway seems involved in part in the IRF-4 promoter regulation. Preliminary promoter analysis revealed that a 0.4kB fragment of the IRF-4 promoter was activated 15-fold by Tax co-expression, indicating that the upstream IRF-4 region may negatively affect promoter (Figure 38B). The HTLV-I LTR, which is activated by Tax through the CREB/ATF pathway, was used as a positive control for Tax, M47 and M22-mediated gene activation in 293T cells (Figure 38C). As expected, wt Tax and the M22 mutant transactivated the HTLV-I LTR. The M47 mutant which is defective in the CREB/ATF pathway, no longer transactivated the HTLV-I LTR as previously described (221).

Figure 37: Schematic representation of the IRF 4 promoter. Sequence of the proximal –200 to –700 region of the IRF4 promoter, major consensus binding sites for NF-κB (κB1 and κB2), NF-AT (NF-1, NF-2 and NF-3), AP-1 and Ets-1 transcription factors and CD28-response element (CD28-RE) are shown. Transcription factor binding sites were determined by searching against the TRANSFAC database using MatInspector algorithm. Sequences that were used as probes for EMSA experiments are indicated in bold. Arrows correspond to primers used in genomic footprinting experiments. Primers 1, 2 and 3 and 4, 5 and 6 were designed to analyze the –600 to –400 and –460 to –260 regions of the promoter, respectively.

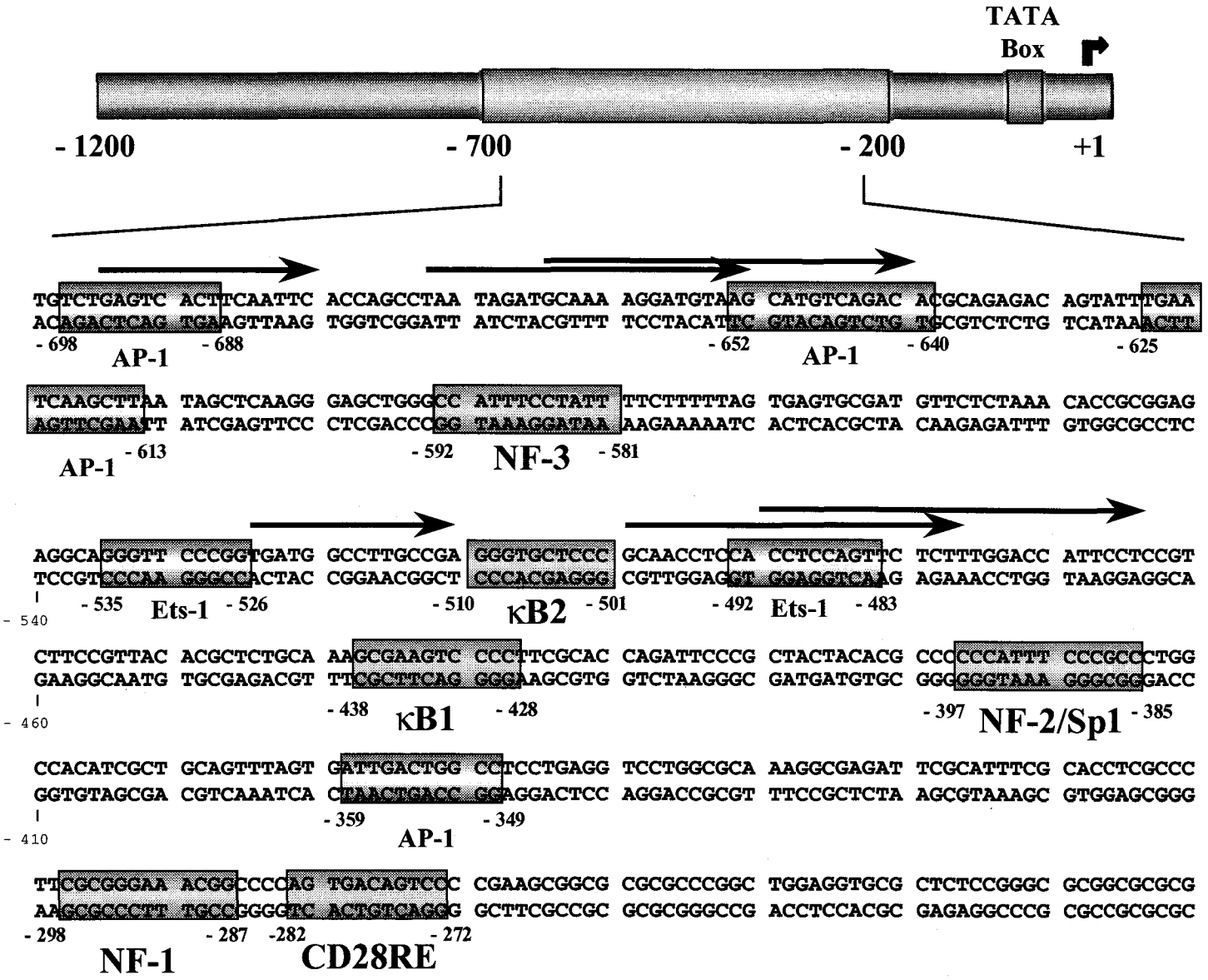
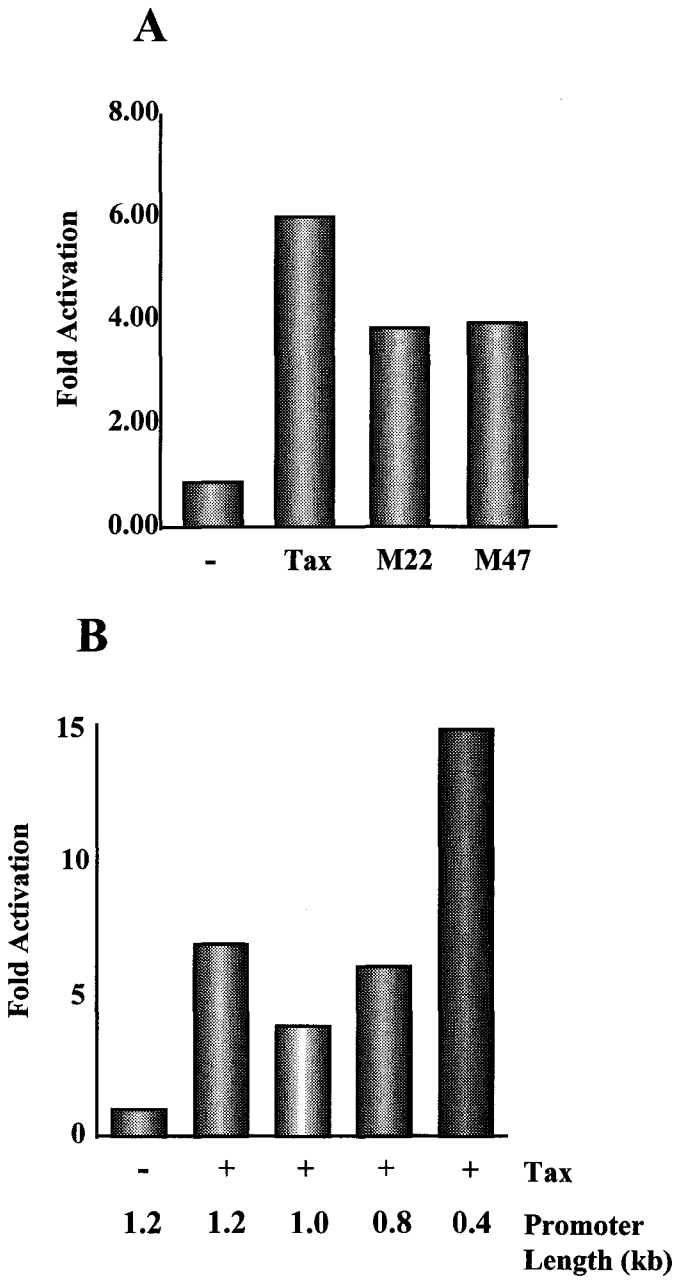

TATA
Box
- 1200
- 700
- 200
+1
TGTCTGAGTC ACTTCAATTC ACCAGCCTAA TAGATGCAAA AGGATGTAAG CATGTCAGAC ACGCAGAGAC AGTATTTGAA
ACAGACTCAG TGAAGTTAAG TGGTCGGATT ATCTACGTTT TCCTACATTC GTACAGTCTG TGCGTCTCTG TCATAAACTT
- 698
AP-1
- 688
- 652
AP-1
- 640
- 625
TCAAGCTTAA TAGCTCAAGG GAGCTGGGCC ATTTCCTATT TTCTTTTTAG TGAGTGCGAT GTTCTCTAAA CACCGCGGAG
AGTTCGAATT ATCGAGTTCC CTCGACCCGG TAAAGGATAA AAGAAAAATC ACTCACGCTA CAAGAGATTT GTGGCGCCTC
AP-1
- 613
- 592
NF-3
- 581
AGGCAGGGTT CCCGGTGATG GCCTTGCCGA GGGTGCTCCC GCAACCTCCA CCTCCAGTTC TCTTTGGACC ATTCCTCCGT
TCCGTCCCAA GGGCCACTAC CGGAACGGCT CCCACGAGGG CGTTGGAGGT GGAGGTCAAG AGAAACCTGG TAAGGAGGCA
- 535
Ets-1
- 526
- 510
κB2
- 501
- 492
Ets-1
- 483
- 540
CTTCCGTTAC ACGCTCTGCA AAGCGAAGTC CCCTTCGCAC CAGATTCCCG CTACTACACG CCCCCCATTT CCCGCCCTGG
GAAGGCAATG TGCGAGACGT TTCGCTTCAG GGGAAGCGTG GTCTAAGGGC GATGATGTGC GGGGGGTAAA GGGCGGGACC
- 460
- 438
κB1
- 428
- 397
NF-2/Sp1
- 385
CCACATCGCT GCAGTTTAGT GATTGACTGG CCTCCTGAGG TCCTGGCGCA AAGGCGAGAT TCGCATTTCG CACCTCGCCC
GGTGTAGCGA CGTCAAATCA CTAACTGACC GGAGGACTCC AGGACCGCGT TTCCGCTCTA AGCGTAAAGC GTGGAGCGGG
- 410
- 359
AP-1
- 349
TTCGCGGGAA ACGGCCCCAG TGACAGTCCC CGAAGCGGCG CGCGCCCGGC TGGAGGTGCG CTCTCCGGGC GCGGCGCGCG
AAGCGCCCTT TGCCGGGGTC ACTGTCAGGG GCTTCGCCGC GCGCGGGCCG ACCTCCACGC GAGAGGCCCG CGCCGCGCGC
- 298
NF-1
- 287
-282
CD28RE
- 272

Figure 38: Tax mediates the transactivation of the IRF-4 promoter. (A) 293T cells were transfected with 0.5μg of the full length IRF4 promoter reporter plasmid and 1.5μg of the appropriate expression vector (pCMV-Tax, pCMV-M22 or pCMV-M47). **(B)** Promoter deletion mutants of the IRF-4 promoter were transfected into 293T cells in the presence of wild type Tax expression plasmid. **(C)** The HTLV-I LTR, Tax, M22 and M47 were transfected into 293 T cells. The HTLV-I LTR is transactivated by wild type and M22 Tax (CREB+/NF-κB-) but not M47 Tax (CREB-/NF–κB+) mutants.


A
8.00
6.00
4.00
2.00
0.00
Fold Activation
-
Tax
M22
M47
B
15
10
5
0
Fold Activation
-
+
+
+
+
Tax
1.2
1.2
1.0
0.8
0.4
Promoter
Length (kb)
C
25
20
15
10
5
0
Fold Activation
HTLV-1
LTR
Tax
M22
M47

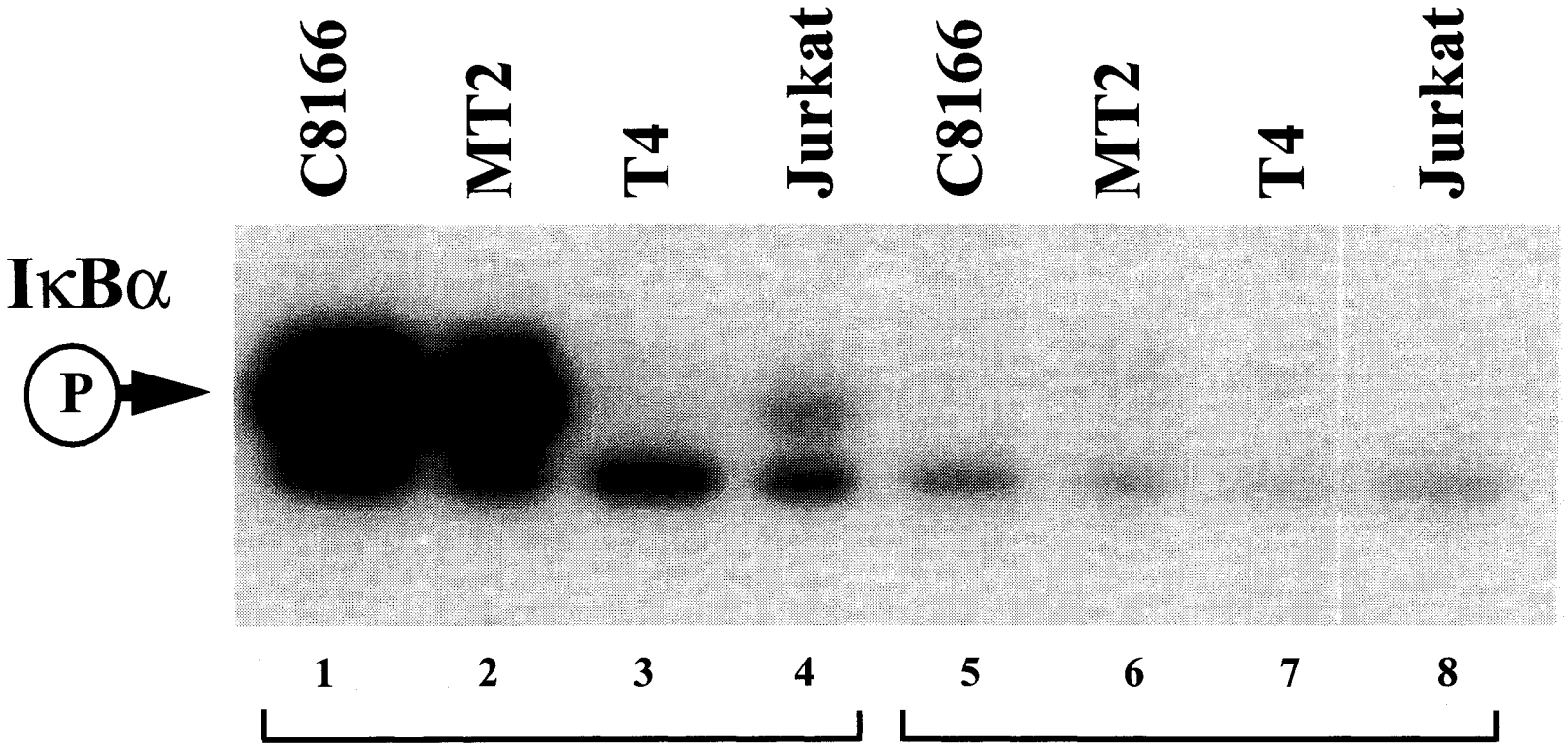
Constitutive NF-κB activation in HTLV-I infected cell lines.

Preliminary data indicated a role for NF-κB proteins in the regulation of IRF-4 in HTLV-I infected cells (67). These factors are constitutively activated in HTLV-I infected T cells (79). A kinase assay was performed in two HTLV-I infected cell lines, C8166 and MT2 and two uninfected T cell lines, T4 and Jurkat (Figure 39). The substrate GST-IκBα 1-55aa was used to determine the activation state of the IκB kinase in these cells. The IKK complex was immunoprecipitated from these cells using an α-IKKα antibody. The immunoprecipitated complexes were incubated with the substrate GST-IκBα 1-55aa or GST-2NIκBα 1-55aa and radiolabeled 32P-ATP (Figure 39, lanes 1-4 and lanes 5-8, respectively). Constitutive IκBα phosphorylation and IKK activation was seen in the HTLV-I infected T cells (Figure 39, lanes 1-2) but not in the uninfected ones (Figure 39, lanes 3-4). The control substrate GST-2NIκBα 1-55aa (Ser32ala and Ser36Ala) which could not be phosphorylated by IKK was used to confirm phosphorylation specificity. No phosphorylation of the mutated substrate, GST-2NIκBα 1-55aa, was seen in the infected or uninfected cells (Figure 39, lanes 5-8).

Genomic footprinting of the IRF-4 promoter in HTLV-I infected cell line; Involvement of NF-κB and NF-AT transcription factors .

The potential role of NF-κB sites in the regulation of IRF4 expression was further studied *in vivo*.

Figure 39: **Constitutive IκBα phosphorylation in HTLV-I infected T cells.** Whole cell lysates (500μg) from C8166, MT2, T4 and Jurkat cells were immunoprecipitated with rabbit polyclonal antibody directed against IKKα. The immunoprecipitates were incubated in kinase reaction buffer with 10μCi [γ-^{32}P] ATP at 30°C for 15 minutes. Substrates for the kinase reactions were GST-IκBα wild type (lanes 1-4) or mutant 2N (lanes 5-8) which can not be phosphorylated by IKK. The kinase reactions were run on 10% SDS-PAGE, dried and exposed to film.

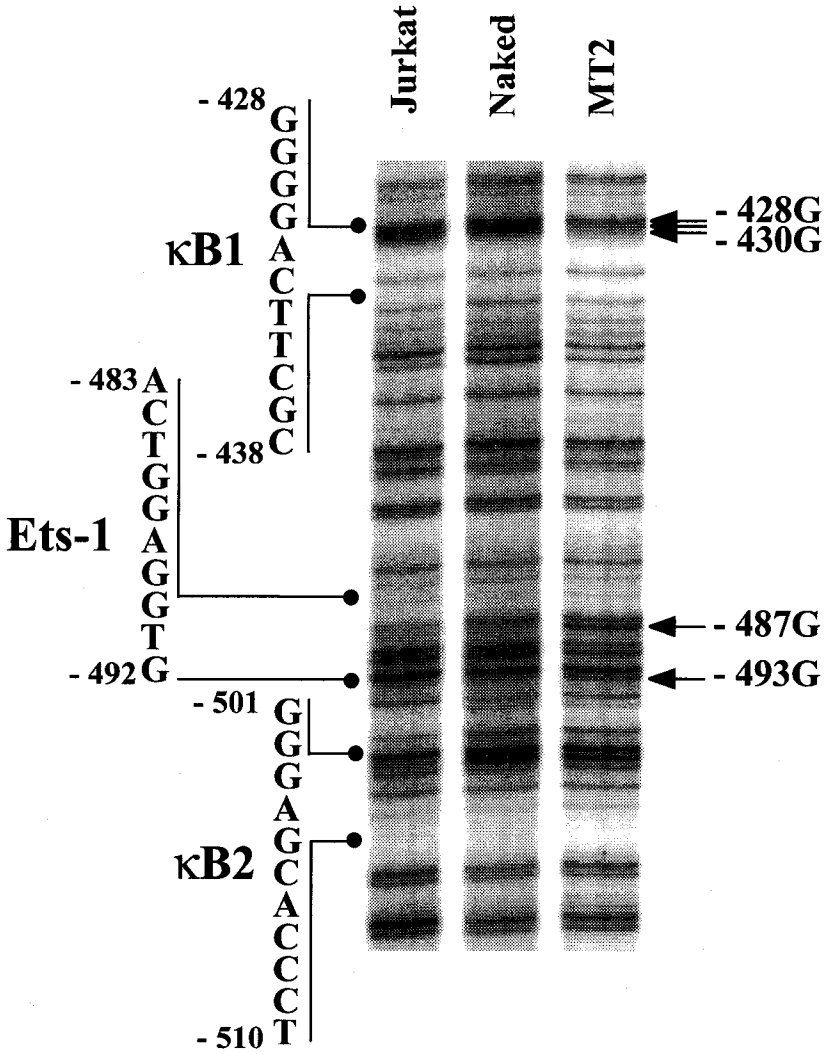

C8166
MT2
T4
Jurkat
C8166
MT2
T4
Jurkat
IκBα
P
1
2
3
4
5
6
7
8
Substrate:
GST-IκBα (aa1-55)
GST-IκBα2N (aa1-55)
IP:
IKKα
IKKα

Genomic footprinting primers were designed to analyze the *in vivo* protein-DNA interactions in the –600 to –400 region of the IRF4 promoter containing the κB1 and κB2 binding sites, two potential NF-κB sites (primers 1, 2 and 3, Figure 37). Dr. Nathalie Grandvaux performed the footprinting analyses. Analyses were performed on the noncoding strand as the κB1 and κB2 sites are significantly G rich on this strand. Protections were observed at the κB1site in MT2 cells, whereas no modification was observed in control Jurkat cells (Figure 40, lanes 1 and 3). Comparison of the in vivo and naked DNA pattern obtained from MT-2 cells revealed decreased methylation of -428G, -429G and –430 G residues and therefore occupation of this site by protein binding (Figure 40, lanes 2 and 3). These results as well as EMSA experiments (SS, data not shown) demonstrated that NF-κB was involved in IRF4 regulation by binding to the κB sites of the IRF-4 promoter. No changes were detected in the κB2 site (NG, data not shown).

A second set of genomic footprinting primers was designed to analyze the noncoding strand of the –460 to –260 region of the IRF-4 promoter that contains the NF-1 and NF-2 sites, two potential NF-AT sites (primers 4, 5 and 6, Figure 37). Analyses of this strand allowed us to characterize protein binding on the NF-2 site as it is particularly G rich on this strand. Experiments performed using primers 4, 5 and 6 showed strong methylation protection of several G residues in and beside the NF-2 site (-385G to –413G) in MT-2 cells but not in Jurkat cells (Figure 41, lanes 1 and 3). Taken together this data demonstrates that in MT2 cells, NF-AT transcription factors are bound to the IRF4 promoter, whereas experiments performed in Jurkat cells did

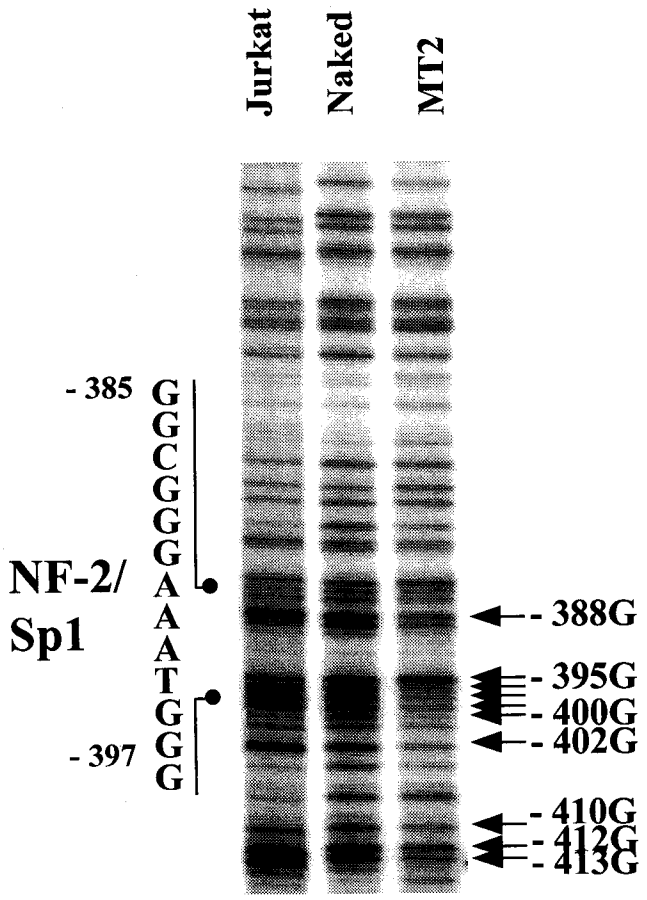
not reveal NF-AT binding to these sites. These results as well as EMSA experiments (SS, data not shown) suggest that NF-AT is also involved in the regulation of IRF4 expression. Both NF-κB and NF-AT are therefore involved in IRF-4 expression in HTLV-I infected cells possibly through the viral transactivator Tax.

Figure 40: In vivo binding to the κB1 site of the IRF4 promoter in Jurkat and MT2 cells. In vivo genomic footprinting of the noncoding strand of the -600 to -400 region of the IRF4 promoter. Genomic DNA isolated from Jurkat (control cells) and MT2 cells was submitted to DMS treatment either before (in vivo) or after extraction (naked DNA). DNA was then cleaved with piperidine and amplified by LM-PCR using primers 1, 2 and 3 (**Figure IRF-4 promoter**). Samples were analyzed on a Long range sequencing gel. Putative NF-kB binding site, kB1 and kB2, are indicated. Ets-1 transcription binding site is also indicated. Arrows indicate G residues that present reproducible (n > 3) protection or hypermethylation.



κB1 site is protected in MT2 cells

Figure 41: In vivo binding to the NF-2 site of the IRF4 promoter in MT2 cells. In vivo genomic footprinting of the noncoding strand of the –460 to –260 region of the IRF4 promoter. Genomic DNA from Jurkat (control cells) and MT2 cells was submitted to DMS treatment either before (in vivo) or after extraction (naked DNA). DNA was then cleaved with piperidine and amplified by LM-PCR using primers 4, 5 and 6 (**Figure IRF-4 promoter**). Samples were analyzed on a Long range sequencing gel. Putative NF-AT binding site, NF-1, NF-2 and NF-3 and CD28 response element (CD28RE) are indicated. Arrows indicate G residues that present reproducible (n > 3) protection or hypermethylation.



NF2 site is protected in vivo in MT2 cells

CHAPTER IV

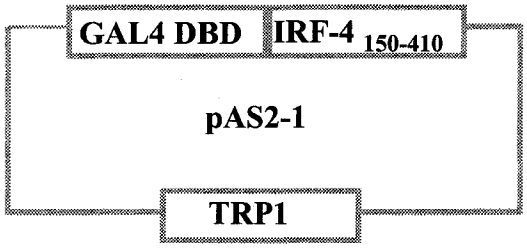
POSTTRANSLATIONAL REGULATION OF IRF-4 BY THE IMMUNOPHILIN FKBP52

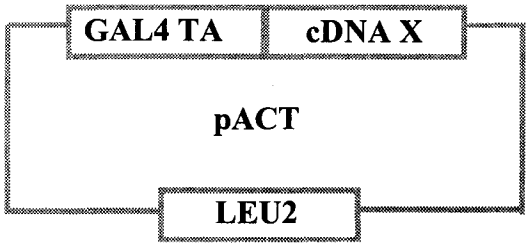
***In vivo* interaction of IRF-4 and FKBP52.** To identify potential partners of IRF-4, a yeast two-hybrid analysis was performed; since full length IRF-4 possessed intrinsic transcriptional activity, the bait utilized was IRF-4 aa150-410 comprising a proline rich domain and the C-terminal IRF association domain (IAD). Among the 10^6 clones screened, 10 clones were positive for mating and were subsequently sequenced for identification (Figure 42A). Two of these clones corresponded to full-length cDNA of the human immunophilin FK506 Binding Protein 52 (FKBP52), a member of the immunophilin family of proteins with peptidyl-prolyl isomerase (PPIase) activity and chaperone-like functions (27, 166, 173) (Figure 42B). FKBP52 contains 3 "FKBP-like" domains: domain 1 possesses features required for PPIase activity and FK506 binding, domain 2 contains GTP and ATP binding sites and domain 3 the tetratricopeptide repeats (TPR) which are responsible for the association with HSP90 (Figure 28) (96, 173).

To determine which domain of FKBP52 interacted with IRF-4, FKBP52 was truncated to an N-terminal fragment [N-FKBP52 (aa1-243)] containing domains 1 and 2 and a C-terminal fragment [ΔN-FKBP52 (aa233-459)] containing the TPR domains and both forms were subcloned into a Myc-tagged vector.

Figure 42: First two-hybrid screening. (A) To identify potential partners of IRF-4, a yeast two-hybrid analysis was performed; since full length IRF-4 possessed intrinsic transcriptional activity, the bait utilized was IRF-4 aa150-410 comprising a proline rich domain and the C-terminal IRF association domain (IAD). The fragment containing the proline rich and the IRF Association domains (aa150-410) of IRF-4 was PCR amplified and subcloned into the pAS2-1 vector (GAL4 $_{1-147}$ DNA binding domain, TRP1) in frame with the DNA binding domain of GAL4 and sequenced. The yeast strain Y190 was cotransformed by the lithium acetate permeabilization method with the pAS2-1-IRF-4 (150-410) and with an EBV-transformed B-cell cDNA library cloned into the pACT vector. Positive yeast clones were selected for prototrophy for leucine, tryptophan and histidine and screened for β–galactosidase expression. Among the $1X10^6$ clones screened, 22 clones were positive for histidine and for βGal. Subsequent two-hybrid assays were carried out by a mating assay between the Y187 and Y190. Diploid cells from the mating assay were selected for Trp- Leu- His- selective medium and screened by replica plating for their ability to produce for βgalactosidase. Of the 22 clones, 10 were positive for mating and sequenced for identification. **(B)** Six out of the ten clones coded for known proteins such as FKBP52, βadrenergic receptor kinase, modulatory recognition factor-1, protein phosphatase 1 (catalytic) and PEBP2alphaC.

A





1 X 10^6 clones screened

56 HIS^+

22 HIS^+ βgal^+

16 HIS^+ βgal^+ Leu^+ $Cyclo^R$

10 $mating^+$ HIS^+ βgal^+ → **Isolation of FKBP52 as a GAL4 TA hybrid**

EBV-transformed B lymphocyte cDNA library

B

CLONES	NAME	FUNCTIONS
1 and 2	Human immunophilin FKBP52	Peptidyl-prolyl isomerase Chaperone-like molecule
3	Human β adrenergic receptor kinase	Phosphorylates the agonist-occupied forms of the β-adrenergic receptor and related G protein-coupled receptors.
4	Human modulatory recognition factor 1	
5	Phosphatase 1 catalytic subunit	
6	Human PEBP2αC Runt	Transcription factor-implicated in tissue specific transcriptional regulation.

In order to confirm the interaction between FKBP52 and IRF-4, Myc-tagged forms of FKBP52 and FLAG-tagged IRF-4 (aa150-410) were cotransfected into COS-7 cells. After immunoprecipitation of Myc-FKBP52 from cell extracts with anti-Myc antibody, immunoblot analysis using α-FLAG antibody revealed that IRF-4 (aa150-410) co-precipitated with full length FKBP52 (Figure 43A, lane 2), as well as the C-terminal fragment (Figure 43A, lane 4) but did not co-immunoprecipitate with the N-terminal 1-243aa fragment (Figure 43A, lane 6), indicating that the TPR mediated protein-protein interactions between FKBP52 and IRF-4 (Figure 44). The lower panel of Fig. 43B revealed that Myc-tagged forms of FKBP52 were expressed and immunoprecipitated with anti-Myc antibody.

Mapping the IRF-4 interaction domains. To localize the region of IRF-4 interacting with the immunophilin, deletion mutants of IRF4 and full length FKBP52 were co-transfected into COS-7 cells and analyzed by co-immunoprecipitation. FKBP52 coprecipitated with IRF-4 (aa1-237), IRF-4 (aa1-340), IRF-4 (aa1-410), IRF-4 (aa150-340) and IRF-4 (aa150-410) (Figure 43B, lanes 2, 4, 6, 9 and 11), indicating that the interaction domain of IRF-4 was located within the N-terminal 237aa. FKBP52 did not co-precipitate with IRF-4 (aa1-150) (Figure 43C, lane 8) or with IRF-4 containing the internal deletion aaΔ150-340 (Figure 43C, lane 6).

Figure 43: Mapping the IRF-4 and FKBP52 interaction domains through immunoprecipitations. The proline-rich region of IRF-4 (aa150-237) interacts with the tetratricopeptide repeats of FKBP52 (aa233-459). COS-7 cells were transfected with Myc-tagged FKBP52 and FLAG or HA-tagged IRF-4 constructs (15 μg each) and whole cell extracts (WCE) (300 μg) were immunoprecipitated (IP) with anti-Myc antibody 9E10 (αmyc), immunoprecipitated complexes were run on a 15% SDS-PAGE and immunoblotted (IB) with anti-FLAG antibody to detect FLAG-IRF-4 expression (in upper panels A and B), anti-HA antibody for IRF-4 (upper panel C). The membranes were reprobed with αmyc to verify protein expression and successful IP (lower panels in A, B and C). WCE (30 μg) were used as input and extract from untransfected COS-7 cells (300 μg) was immunoprecipitated with αmyc as control IP.

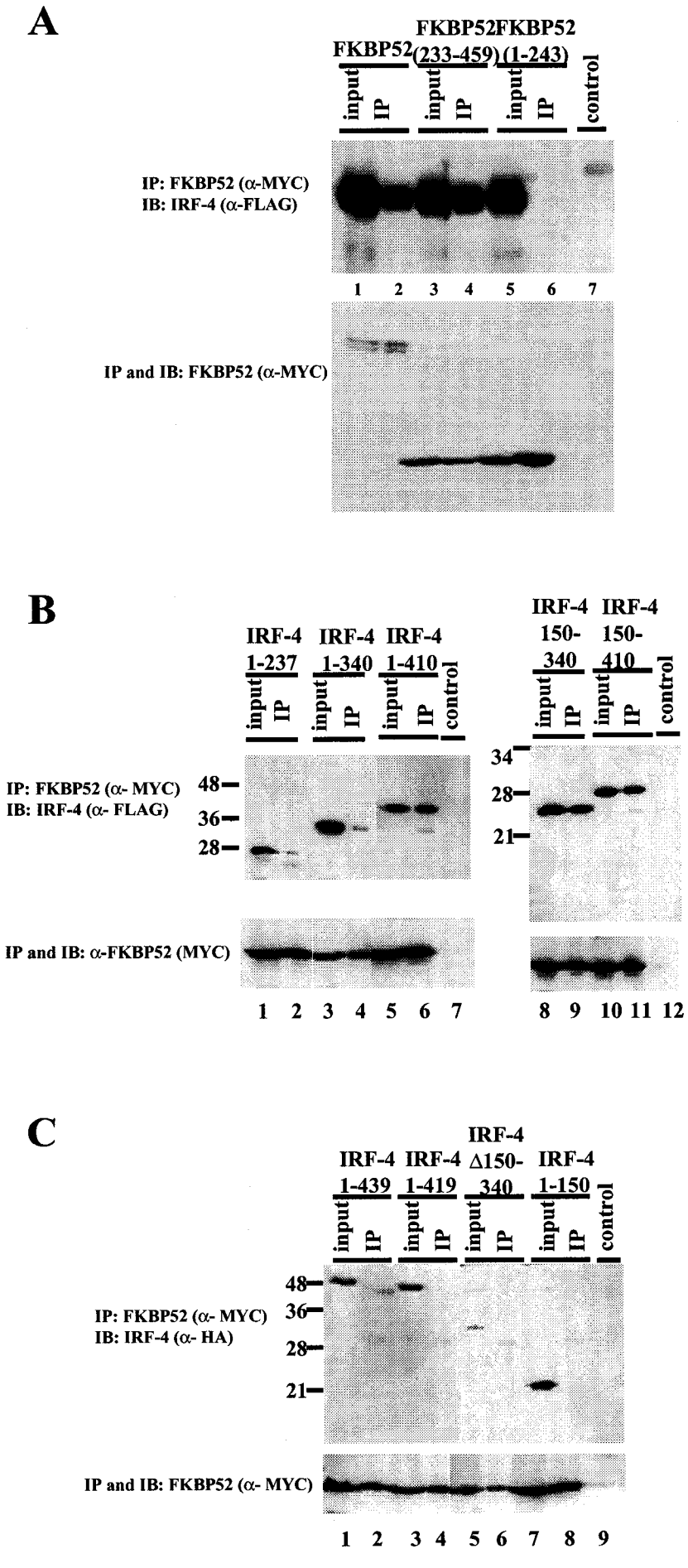
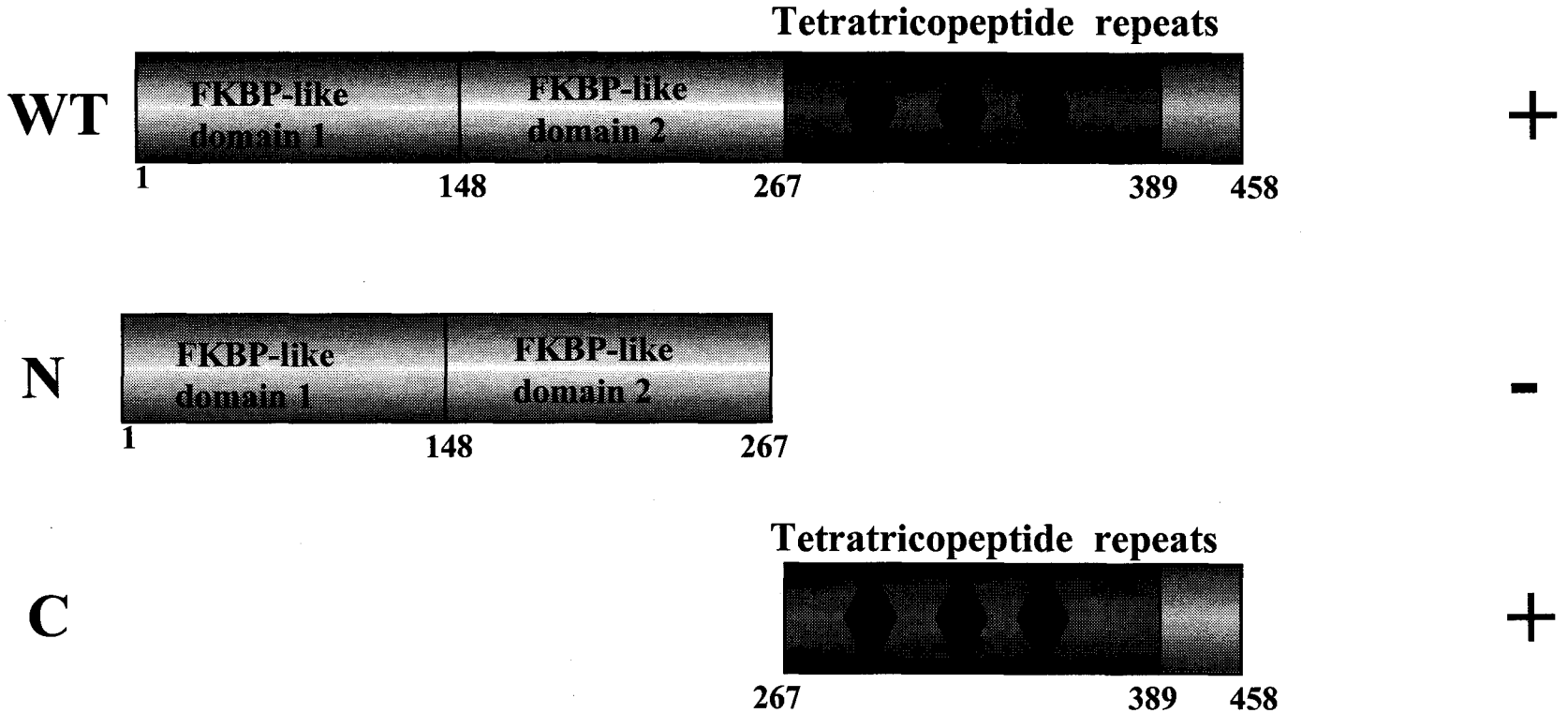

A
FKBP52
FKBP52 (233-459)
FKBP52 (1-243)
input
IP
control
IP: FKBP52 (α-MYC)
IB: IRF-4 (α-FLAG)
1 2 3 4 5 6 7
IP and IB: FKBP52 (α-MYC)
B
IRF-4 1-237
IRF-4 1-340
IRF-4 1-410
IRF-4 150-340
IRF-4 150-410
IP: FKBP52 (α- MYC)
IB: IRF-4 (α- FLAG)
48
36
28
34
28
21
IP and IB: α-FKBP52 (MYC)
1 2 3 4 5 6 7 8 9 10 11 12
C
IRF-4 1-439
IRF-4 1-419
IRF-4 Δ150-340
IRF-4 1-150
IP: FKBP52 (α- MYC)
IB: IRF-4 (α- HA)
48
36
28
21
IP and IB: FKBP52 (α- MYC)
1 2 3 4 5 6 7 8 9

Figure 44: **The minimal FKBP52 interaction domain**. FKBP52 possesses three functional domains (brackets): FKBP-like domain 1 with the PPIase activity (aa1-148), FKBP-like domain 2 with an ATP/GTP binding site (aa148-267) and 3 tetratricopeptide repeats (aa267-389) which mediate protein-protein interactions. Immunoprecipitation results indicated that the tetratricopeptide repeats region (aa267-389) is the minimal domain required for IRF-4 interaction in cells.

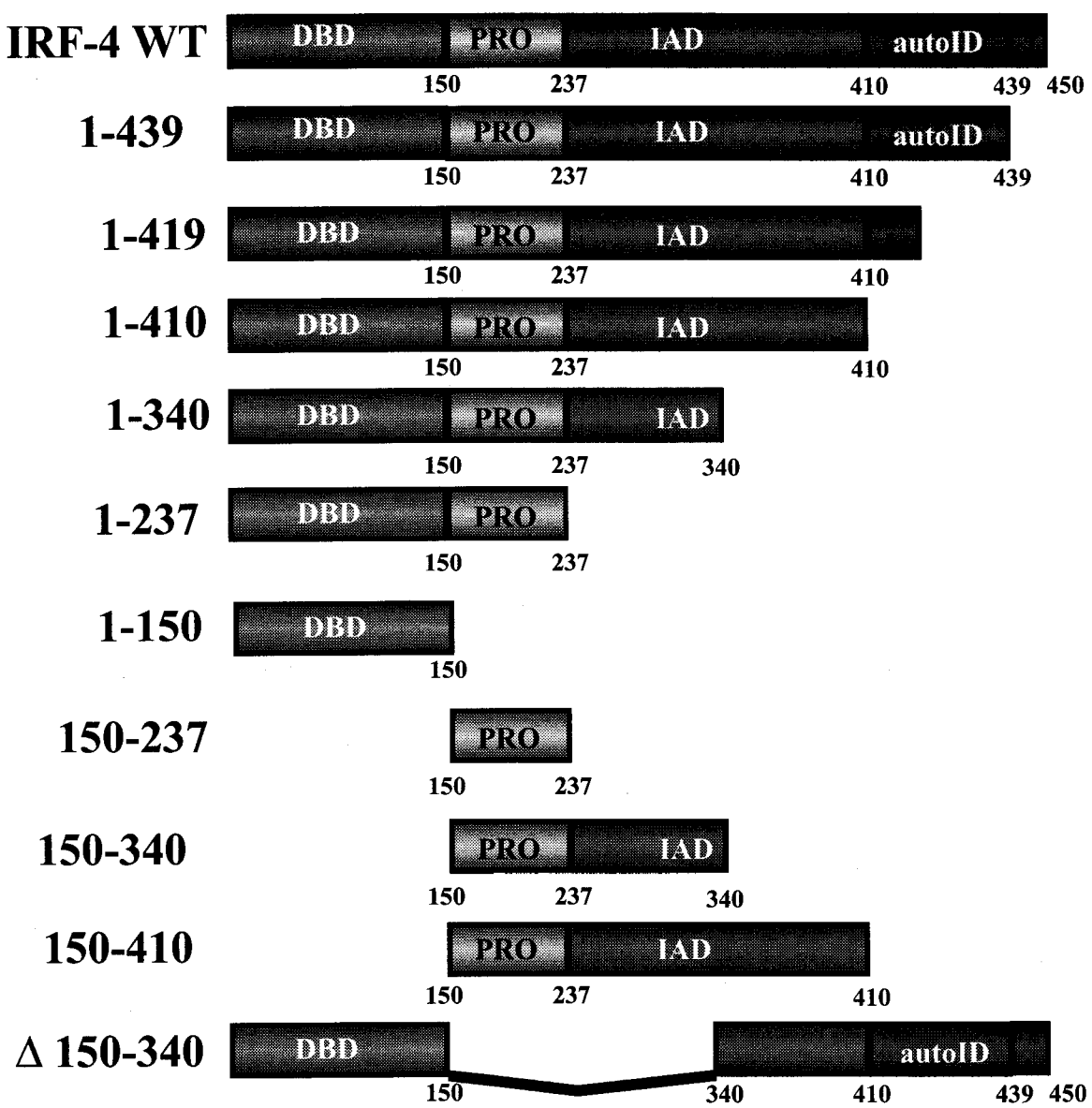
Interaction with IRF-4 150-410



The results of the domain interaction studies are summarized schematically in Figure 45. Based on these results, the proline-rich region of IRF-4 (aa150-237) appears to be the minimal region required for FKBP52 interaction. However, despite repeated efforts, this domain alone was not stably expressed as a tagged peptide and therefore was not tested directly, hence the parentheses in Figure 45 (YM, data not shown).

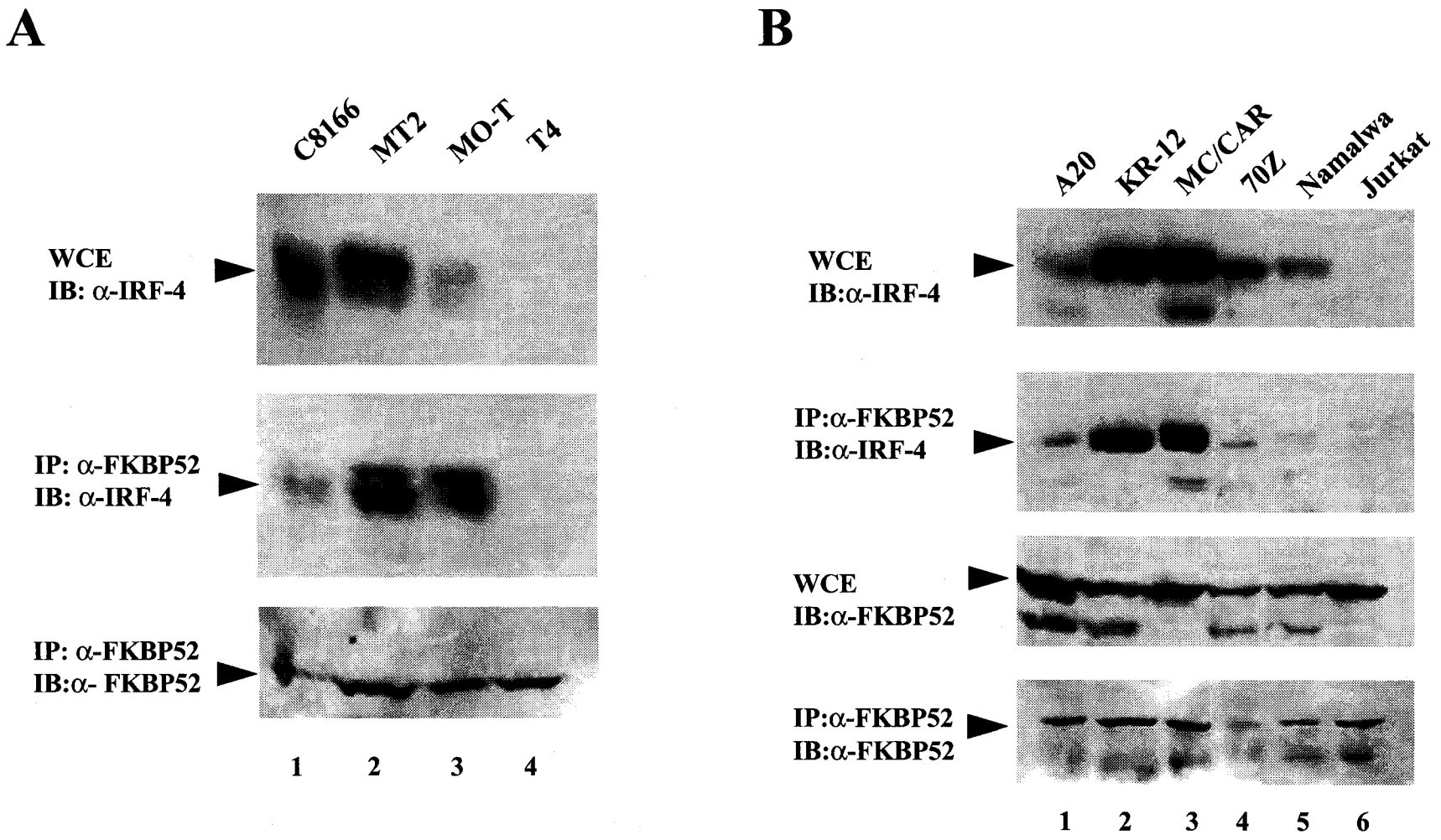
Interestingly, with the inclusion of additional C-terminal amino acids beyond aa410, FKBP52 did not associate with IRF-4 (aa1-439), IRF-4 (aa1-419), (Figure 43C, lanes 2 and 4) or with full length IRF-4 (YM, data not shown); similarly IRF-4 did not interact with the N or C-terminal parts of FKBP52 (YM, data not shown). The conformation of IRF-4 may be inaccessible to FKBP52 because of structural constraints imposed by the C-terminal autoinhibition element which folds back on the N-terminal DNA binding domain (15, 16, 39).

Figure 45: The minimal IRF-4 interaction domain. Schematic summary of the IRF-4 interaction domains with full length FKBP52. The different domains of IRF-4 - DNA binding domain with the tryptophan repeats seen in all IRF member (DBD; aa1-150), the proline-rich region (Pro; aa150-237), the IRF association domain (IAD; aa237-410) and the C-terminal autoinhibition element (AIE; aa410-450) - are illustrated. The 150-237aa construct could not be stably expressed as a tagged peptide and therefore was not tested directly, hence the parentheses.


FKBP52
Interaction
IRF-4 WT
DBD
PRO
IAD
autoID
150
237
410
439
450
-
1-439
DBD
PRO
IAD
autoID
150
237
410
439
-
1-419
DBD
PRO
IAD
150
237
410
-
1-410
DBD
PRO
IAD
150
237
410
+
1-340
DBD
PRO
IAD
150
237
340
+
1-237
DBD
PRO
150
237
+
1-150
DBD
150
-
150-237
PRO
150
237
(+)
150-340
PRO
IAD
150
237
340
+
150-410
PRO
IAD
150
237
410
+
Δ 150-340
DBD
autoID
150
340
410
439
450
-

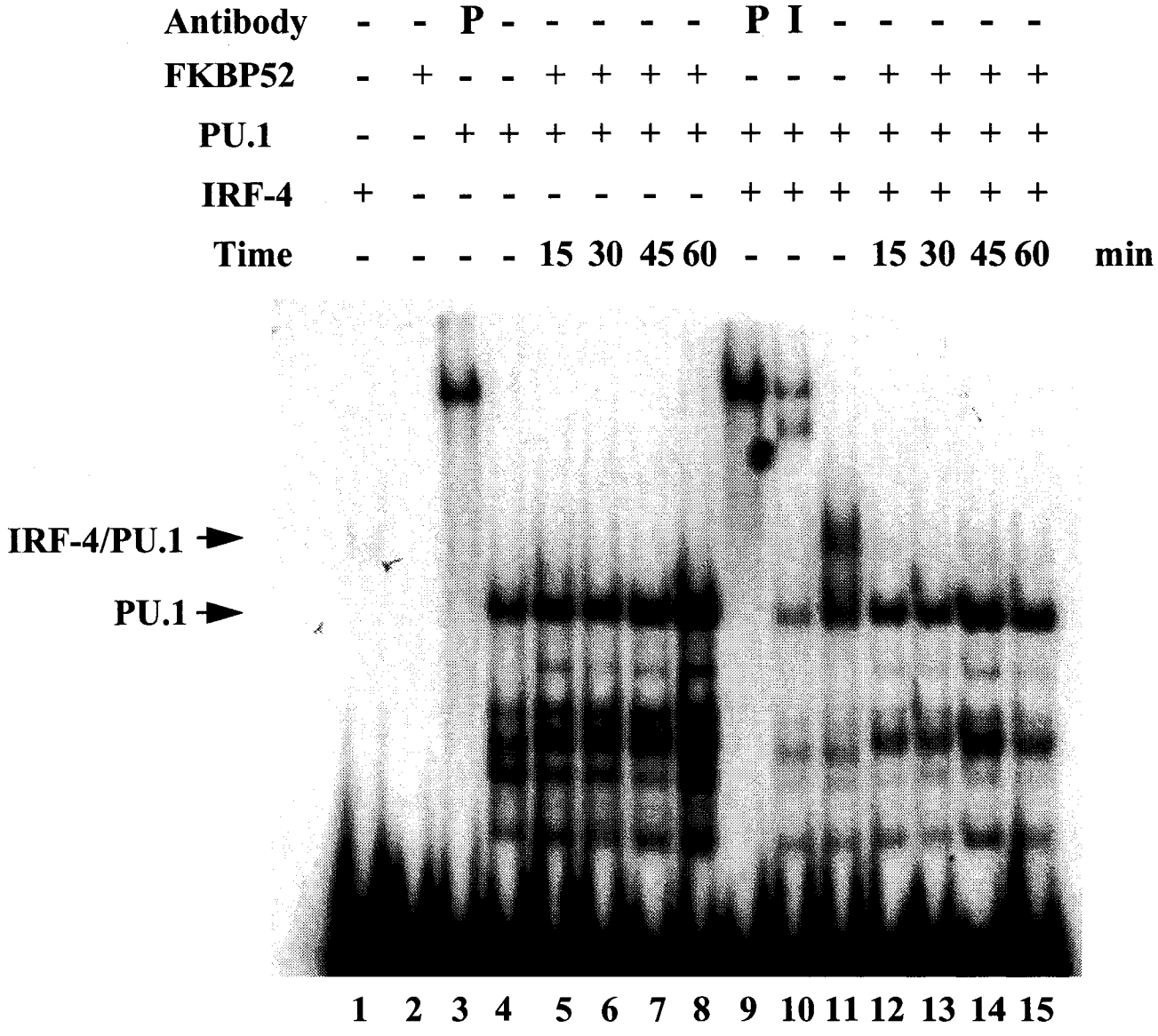
In vivo interaction between IRF-4 and FKBP52 in HTLV-1 transformed cells and in B cells. IRF-4 has been previously shown to be expressed in all Tax-expressing HTLV-I transformed cells as well as in B cells (15, 131, 239). To investigate IRF-4-FKBP52 interactions, two HTLV-I transformed T cell lines C8166 and MT-2, one HTLV-II transformed T cell line MO-T and five B lymphoid cell lines were examined. C8166, MT-2 and MO-T expressed high amounts of IRF-4 whereas T4 which does not express Tax, contained no IRF-4 (Figure 46A, upper panel, lanes 1-4). The levels of FKBP52 were approximately equal in all the T cell lines (Figure 46A, lower panel, lanes 1-4). Co-precipitation assays using an α-FKBP52 antibody revealed that endogenous IRF-4 interacted with endogenous FKBP52 in C8166, MT-2 and MO-T but not in the T4 cell line (Figure 46A, middle panel, lanes 1-3). This interaction was also examined in B cells: two murine cell lines A20 and 70Z/3, and three human cell lines KR-12, MC/CAR and Namalwa cells. Variable amounts of IRF-4 and PU.1 were detected in all B cell lines (Figure 46B, upper panel, lanes 1-5, Figure 31) but not in Jurkat T cells (Figure 46B, upper panel, lane 6). IRF-4-FKBP52 interaction was also detected in all B cells examined but not in Jurkat T cells (Figure 46B, middle panel, lanes 1-6). The amount of IRF-4 interacting with FKBP52 did vary amongst the cell lines, possibly due to differences in IRF-4 expression level in the different cell types (Figure 46B, upper panel, lanes 1-5).

Figure 46: Co-immunoprecipitation demonstrates interaction between endogenous FKBP52 and IRF-4 in HTLV-I transformed T cells and B-cells. (A) C8166, MT2, MO-T and T4 WCE (500 μg) were immunoprecipitated with an αFKBP52 antibody, complexes were run on 10% SDS-PAGE and immunoblotted using αIRF-4 antibody (middle panel) and αFKBP52 antibody (lower panel). Input extracts consisted of 50 μg of WCE (upper panel). The control IP was performed by incubating WCE (500 μg) with normal precleared mouse serum (data not shown). **(B)** A20, KR-12, MC/CAR, 70Z/3, Namalwa and Jurkat WCE (500 μg) were immunoprecipitated with an αFKBP52 antibody, complexes were run on 10% SDS-PAGE and immunoblotted using αIRF-4 antibody (second panel) and αFKBP52 antibody (fourth panel). Input extracts consisted of 50 μg of WCE (first and third panels). The control IP was performed by incubating WCE (500 μg) with normal precleared mouse serum (data not shown).


A
C8166
MT2
MO-T
T4
WCE
IB: α-IRF-4
IP: α-FKBP52
IB: α-IRF-4
IP: α-FKBP52
IB:α- FKBP52
1
2
3
4
B
A20
KR-12
MC/CAR
70Z
Namalwa
Jurkat
WCE
IB:α-IRF-4
IP:α-FKBP52
IB:α-IRF-4
WCE
IB:α-FKBP52
IP:α-FKBP52
IB:α-FKBP52
1
2
3
4
5
6

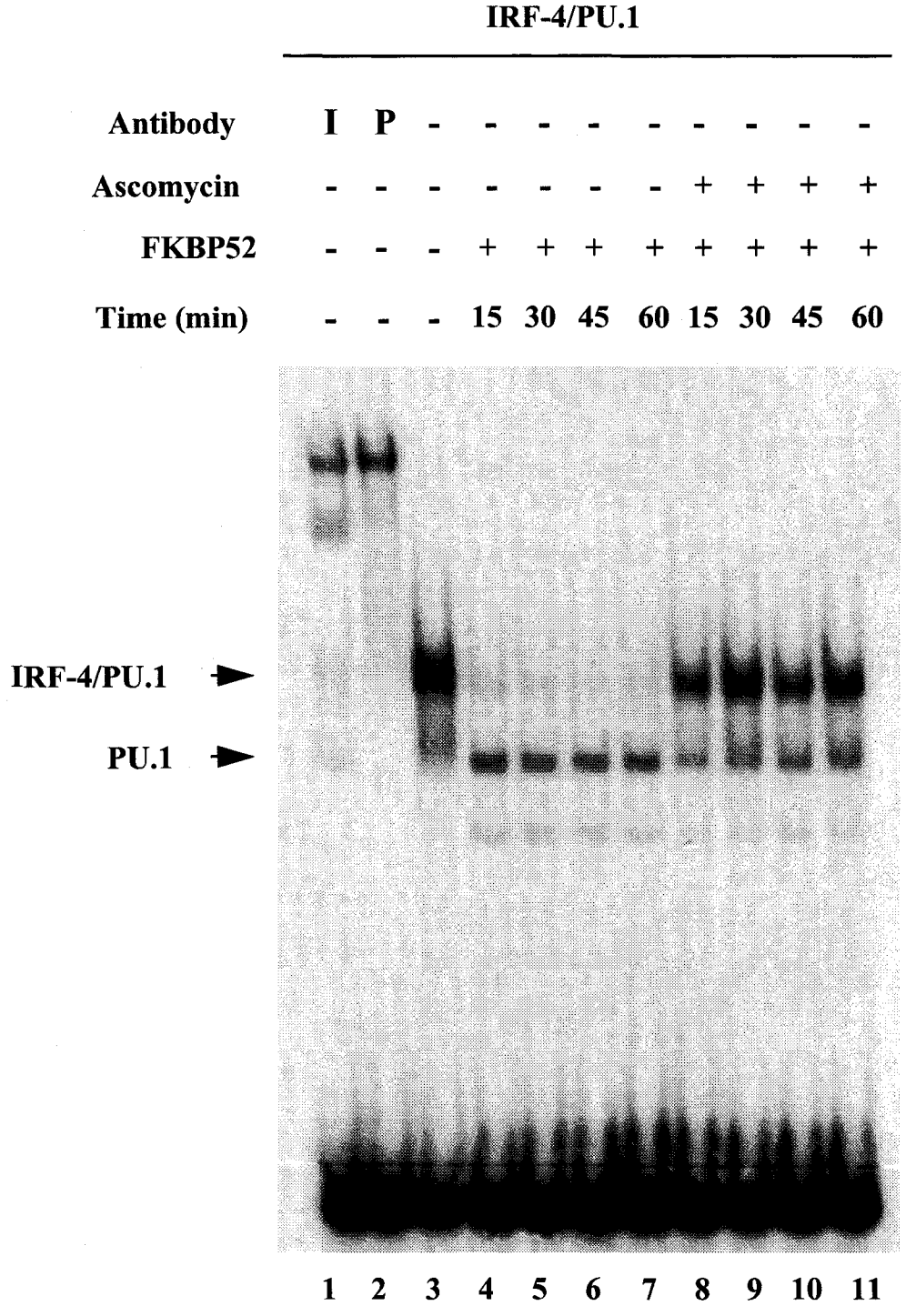
FKBP52 inhibits IRF-4 interaction with PU.1 and subsequent binding to the λB element. IRF-4 was originally cloned as a murine transcription factor recruited to composite elements within immunoglobulin light-chain gene enhancer Eλ2-4 through specific interaction with PU.1, an Ets family member (38). We therefore investigated the effect of FKBP52 on the formation of the IRF-4-PU.1 complex. Nuclear extracts from COS-7 cells transfected with IRF-4 and PU.1 were analyzed by EMSA using the λB element which includes partially overlapping binding sites for IRF-4 and PU.1 (15, 16, 39). As previously shown, IRF-4 alone did not bind to the λB element (Figure 47, lane 1); FKBP52 likewise did not have intrinsic DNA binding activity (Figure 47, lane 2). In contrast, the PU.1 DNA complex was detected in the absence of IRF-4 (Figure 47, lane 4) and the complex was shifted with anti-PU.1 antibody (Figure 47, lane 3). Addition of FKBP52 to the PU.1 containing extracts for different times did not affect the PU.1-DNA complex (Figure 47, lanes 5-8). Nuclear extracts from cells transfected with both IRF-4 and PU.1 expression plasmids were also incubated with the λB element and two complexes were identified - the PU.1 specific complex as well as a slower migrating complex containing both PU.1 and IRF-4 (Figure 47, lane 11 and Figure 48, lane 3) as determined by supershift analysis with anti-PU.1 and anti-IRF-4 antibody (Figure 47, lanes 9 and 10, respectively). FKBP52 had no effect on PU.1 DNA binding (Figure 47, lanes 5-8) but blocked IRF-4 binding to the λB element (Figure 47, lanes 12-15 and Figure 48, lanes 4-7).

Figure 47: **FKBP52 inhibits IRF-4 DNA binding activity to the λB probe**. Nuclear extracts were collected from COS-7 cells transfected with IRF-4, PU.1 or both IRF-4 and PU.1 expression plasmids (10 μg each). EMSA was performed using 3μg of nuclear extracts incubated with the 32P-labeled probe corresponding to the λB element (Eisenbeis, 1995; Brass,1996). PU.1 nuclear extracts were incubated with GST-FKBP52 for increasing times at 25°C (lanes 5-8). The complex (lower arrowhead) contained PU.1 as determined by supershift analysis (P, lane 3). IRF4/ PU.1 nuclear extracts were also incubated with GST-FKBP52 (lanes 12-15). The two complexes (arrowheads) contained IRF-4 and PU.1 as seen by supershifting with αPU.1 (P; lane 9) and αIRF-4 (I; lane 10). As controls, IRF-4 nuclear extracts and GST-FKBP52 were incubated with the λB probe (lanes 1 and 2 respectively).


Antibody - - P - - - - - P I - - - - -
FKBP52 - + - - + + + + - - - + + + +
PU.1 - - + + + + + + + + + + + + +
IRF-4 + - - - - - - - + + + + + + +
Time - - - - 15 30 45 60 - - - 15 30 45 60 min
IRF-4/PU.1
PU.1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

To determine if functional PPIase activity was required for FKBP52 inhibition of IRF-4/PU.1 DNA binding, ascomycin - an ethyl analogue of FK506, an immunosuppressant that inhibits PPIase activity - was incubated with cell extracts for different times. The presence of as little as 10μM ascomycin in the DNA binding assay restored IRF-4/PU.1 binding to the λB probe (Figure 48, lanes 8-11), whereas DMSO (used to dilute the drug) had no effect on IRF-4 binding (YM, data not shown). This concentration of immunosuppressant was previously shown to abolish all PPIase activity (113, 159, 164), but had no effect on IRF-4 or PU.1 binding in the absence of FKBP52 (YM, data not shown). This result indicated that formation of the ternary complex (IRF-4/PU.1/DNA) was inhibited due to the sequestration of IRF-4 by FKBP52. Likewise, ascomycin had no effect on IRF-4/FKBP52 immunoprecipitations when incubated with cell extracts from the cell lines described in Figure 46 (YM, data not shown).

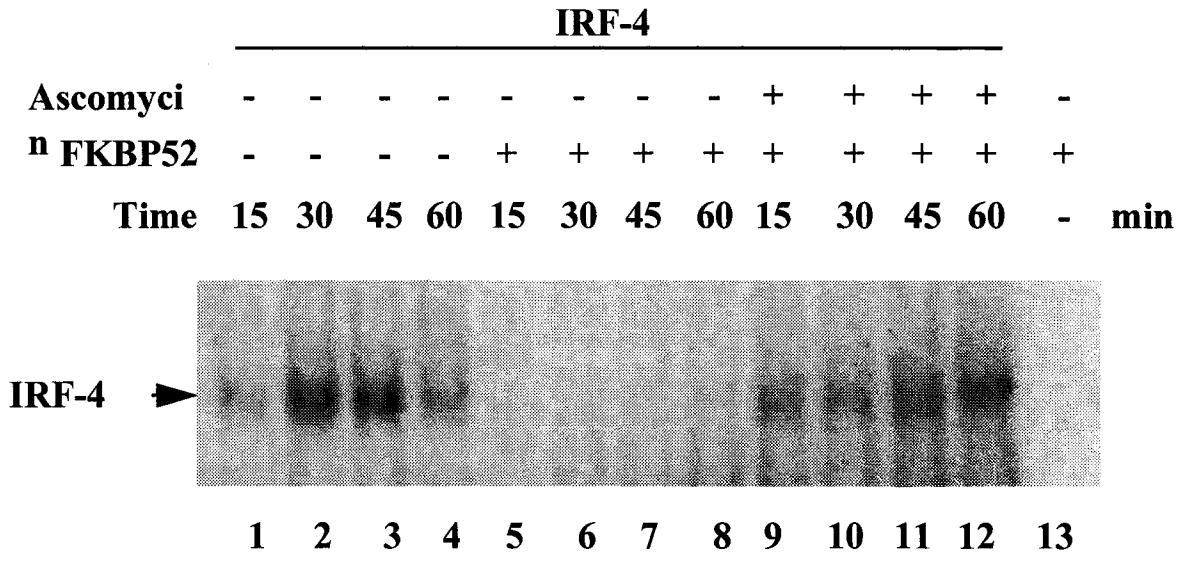
Figure 48: FKBP52 inhibition IRF-4 DNA binding requires functional PPIase activity. Nuclear extracts were collected from COS-7 cells transfected with IRF-4 and PU.1 (10 μg each). EMSA was performed as described in the previous figure using the λB probe. The two complexes (arrowheads) contained IRF-4 and PU.1 as seen by the supershift with αIRF-4 (I; lane 1) and αPU.1 (P; lane 2). IRF4/ PU.1 nuclear extracts were incubated with GST-FKBP52 (lanes 4-11) and in the presence of 10μM of ascomycin (lanes 8-11).


IRF-4/PU.1
Antibody I P - - - - - - - - -
Ascomycin - - - - - - - + + + +
FKBP52 - - - + + + + + + + +
Time (min) - - - 15 30 45 60 15 30 45 60
IRF-4/PU.1
PU.1
1 2 3 4 5 6 7 8 9 10 11

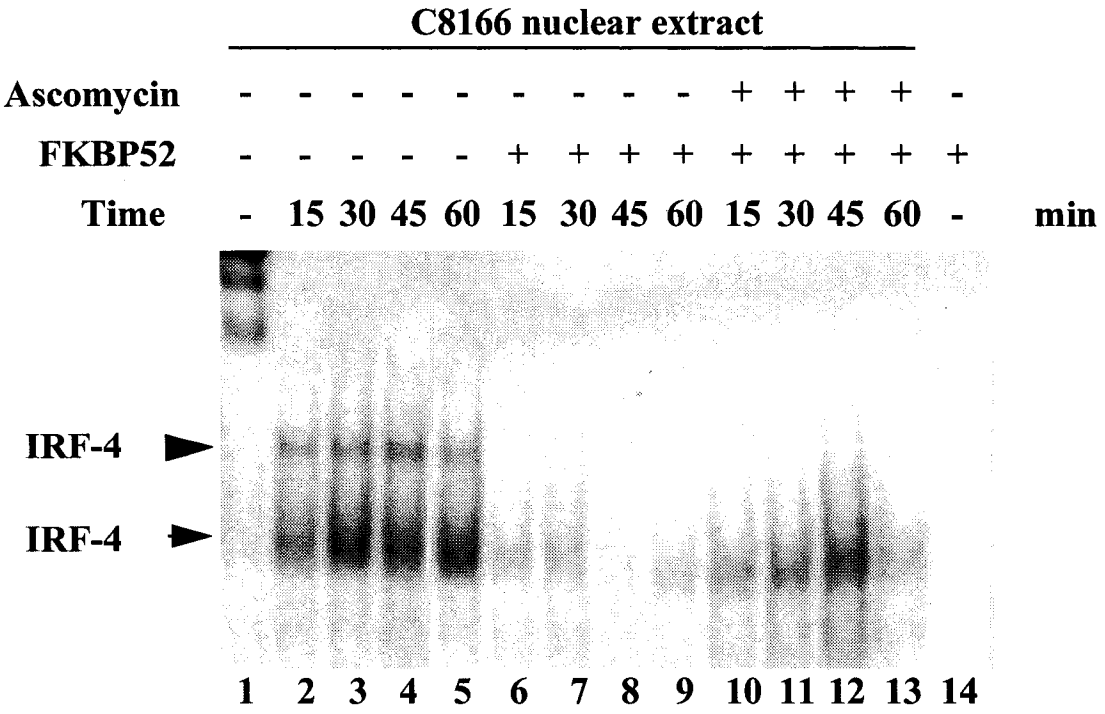
FKBP52 peptidyl-prolyl isomerase activity is required for inhibition of IRF-4 DNA binding. To examine the effect of FKBP52 on IRF-4 DNA binding, recombinant IRF-4 and extracts from C8166 cells were analyzed by EMSA using the ISRE element of ISG-15. Complexes were detected using the ISRE probe with recombinant IRF-4 or C8166 nuclear extracts incubated for different times (Figure 49A, lanes 1-4 and Figure 49B, lanes 2-5 respectively). Although one complex was seen with recombinant IRF-4 (Figure 49A, lanes 1-4), two complexes were detected with C8166 nuclear extracts (Figure 49B, lanes 2-5). The presence of IRF-4 in both complexes was confirmed by supershift analysis with anti-IRF-4 antibody (Figure 49B, lane 1 and data not shown). Recombinant IRF-4 or C8166 extracts were also incubated with the ISRE probe in the presence of GST-FKBP52 (Figure 49A, lanes 5-8; Figure 49B, lanes 6-9); although FKBP52 itself did not form a complex (Figure 49A, lane 13; Figure 49B, lane 14), it prevented IRF-4 from binding to DNA (Figure 49A, lanes 5-8 and Figure 49B, lanes 6-9, respectively). To determine if functional PPIase activity was required for FKBP52 inhibition of IRF-4 binding, recombinant IRF-4 or C8166 nuclear extracts were incubated with recombinant FKBP52 and ascomycin (10μM) for different times (Figure 49A, lanes 9-12 and Figure 49B, lanes 10-13); again the presence of ascomycin in the DNA binding assay mix restored IRF-4 binding to the ISRE probe. The nature of the upper IRF-4 complex in C8166 nuclear extracts, which is not restored by ascomycin is not clear (Figure 49B, lanes 2-5). This complex may represent an IRF-4 dimer, IRF-4 and an unidentified host cellular protein, or a complex that is irreversibly inhibited by FKBP52.

Figure 49: FKBP52 inhibits IRF-4 DNA binding to an ISRE probe in a PPIase dependent manner. (A) EMSA was performed using His-tagged IRF-4 (100 ng) and GST-FKBP52 (200 ng). The 32P-labeled probe corresponded to the ISRE of the ISG15 gene. Recombinant IRF-4 was incubated in the absence (lanes 1-4) or presence of FKBP52 (lanes 5-12) for increasing times at 25°C. Recombinant IRF-4 and FKBP52 were also incubated in the presence of 10mM of ascomycin (lanes 9-12). The control consisted of FKBP52 incubated with the ISG15 probe (lane 13). The arrowhead indicates the IRF-4/ISG15 complex. **(B)** Using C8166 nuclear extracts (3 μg) with the ISRE probe, two complexes were detected (arrowheads). Supershift analysis using an αIRF-4 antibody demonstrated the presence of IRF-4 in both complexes (lane 1). C8166 extracts were incubated in the absence (lanes 2-5) and in the presence of FKBP52 (lanes 6-13) for increasing times at 25°C. Ascomycin (10μM) was added to lanes 10-13 to inhibit the PPIase activity of FKBP52.

A

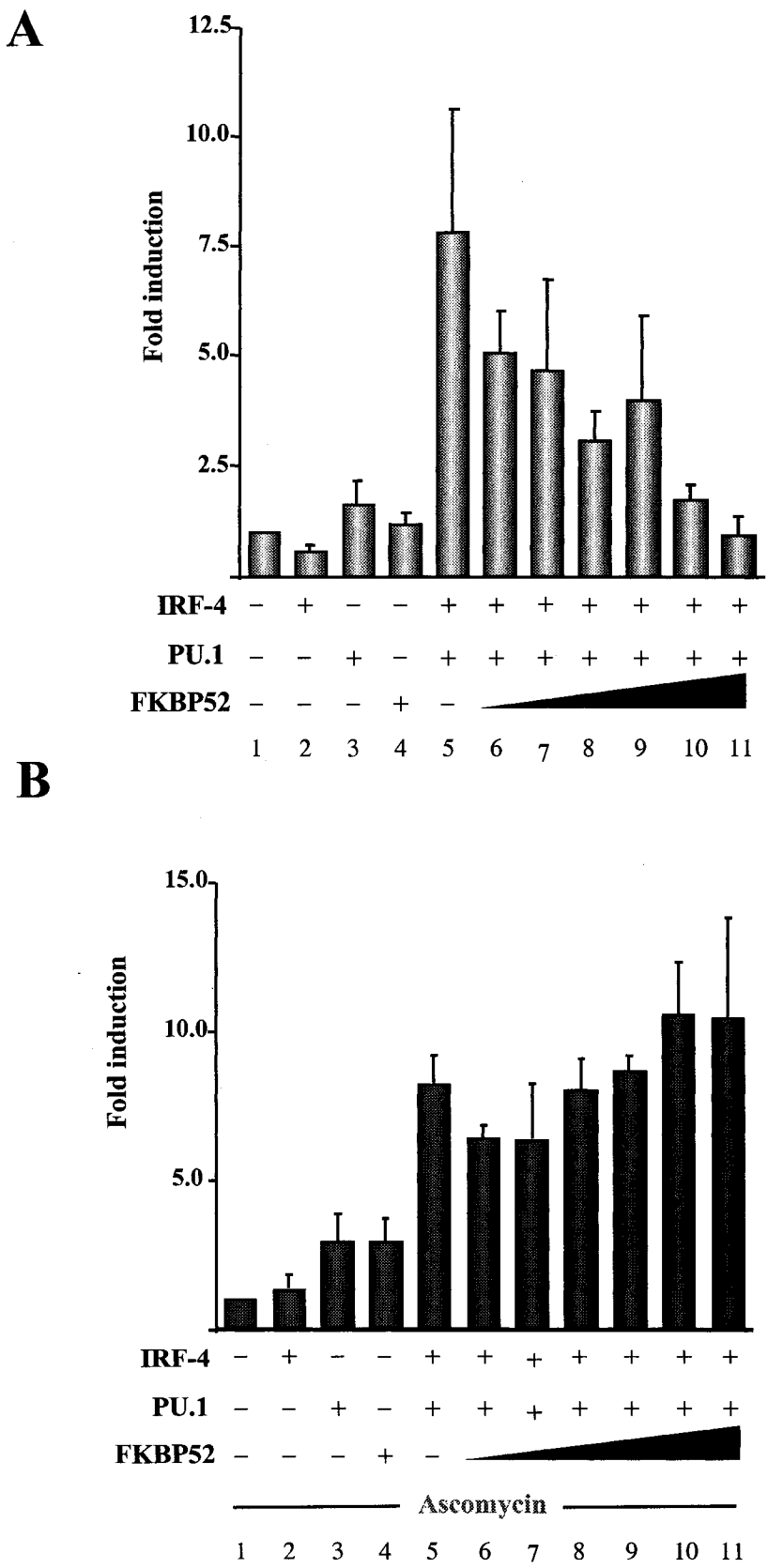

IRF-4
Ascomycin - - - - - - - - + + + + -
FKBP52 - - - - + + + + + + + + +
Time 15 30 45 60 15 30 45 60 15 30 45 60 - min
IRF-4
1 2 3 4 5 6 7 8 9 10 11 12 13

B

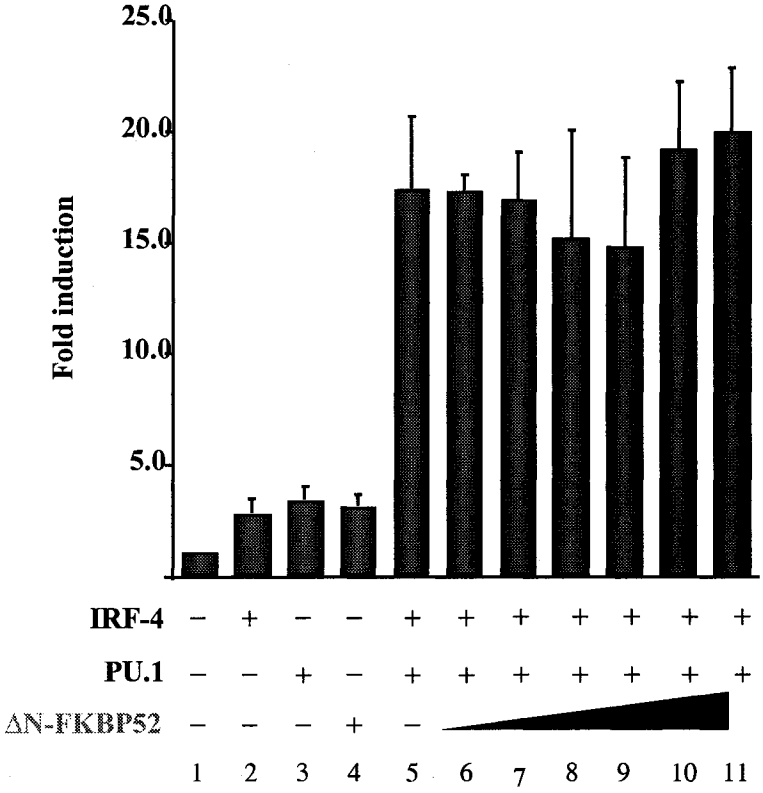

C8166 nuclear extract
Ascomycin - - - - - - - - - + + + + -
FKBP52 - - - - - + + + + + + + + +
Time - 15 30 45 60 15 30 45 60 15 30 45 60 - min
IRF-4
IRF-4
1 2 3 4 5 6 7 8 9 10 11 12 13 14

FKBP52 inhibits IRF-4 transactivation. Previously, the IRF-4-PU.1 dimer was shown to stimulate the transcriptional activity of the $E\lambda_{2\text{-}4}$ λB element in a synergistic manner (15, 39). The ability of FKBP52 to interfere with IRF-4 and IRF-4/PU.1 transactivation was next investigated using COS-7 cells transfected with IRF-4, PU.1 and increasing amounts of FKBP52 expression plasmid. Using the B4TK-CAT construct with a tetramer of the $E\lambda_{2\text{-}4}$ λB element driving the CAT reporter, a greater than 7-8 fold transactivation was produced by the synergistic combination of IRF-4 and PU.1 (Figure 50A, lane 5), while IRF-4 or PU.1 alone did not possess a strong transactivation potential (Figure 50A, lanes 2 and 3 respectively). FKBP52 itself had no effect on the B4TK-CAT (Figure 50A, lane 4), whereas increasing amounts of FKBP52 in the presence of IRF-4 and PU.1 resulted in a dramatic decrease in IRF-4-PU.1 transactivation capacity (Figure 50A, lanes 6-11). Next, transfected cells were incubated with ascomycin (2 μM) for 16 hours; ascomycin completely blocked the inhibition of IRF-4/PU.1 transactivation by FKBP52 (Figure 50B, lanes 6-11), whereas the drug had no effect on IRF-4/PU.1 transactivation in the absence of FKBP52 (Figure 50B, lane 5). Similar results were obtained when a truncated FKBP52 lacking the PPIase domain (ΔN-FKBP52) was co-transfected in increasing amounts; no decrease in transactivation potential was observed (Figure 50C, lanes 6-11). Inhibition of IRF-4 was also IRF-4-specific, based on the observation that increasing FKBP52 did not affect NF-κB p65 mediated transactivation of an NF-κB containing CAT reporter plasmid (Figure 50 D, lanes 4-9).

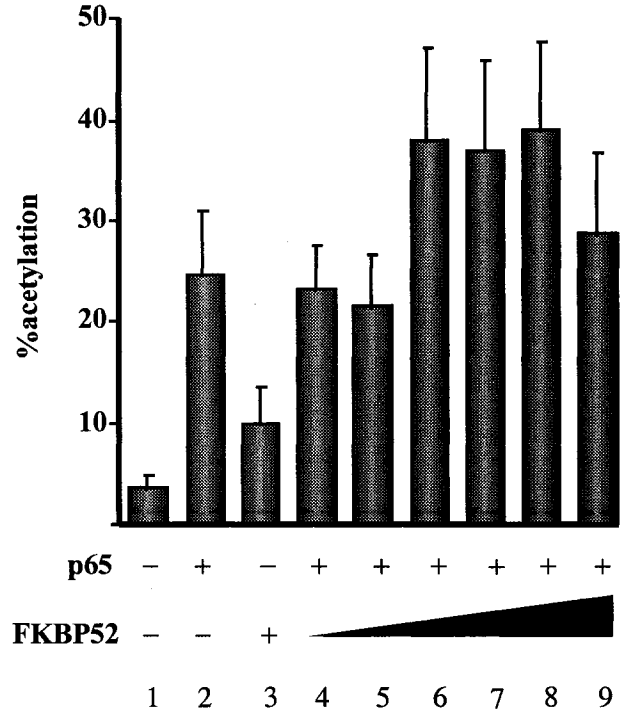
Figure 50: Analysis of IRF-4 transactivation potential in the presence of FKBP52 on a λB-responsive reporter. (A) COS-7 cells were transfected with 2 μg of the B4TK-CAT reporter (Eisenbeis,1995; Brass,1996), IRF-4 and/or PU.1 expressing plasmids, as well as increasing amounts of FKBP52 expression plasmid (2, 5, 10, 15, 20 and 25 μg). **(B)** COS-7 cells were transfected as described in (A) and were treated with 2 μM of ascomycin for 16h. **(C)** COS-7 cells were transfected with 2 μg of the B4TK-CAT reporter, IRF-4 or PU.1 expressing plasmids, as well as increasing amounts of ΔN-FKBP52 (aa233-459) expressing plasmid (2, 5, 10, 15, 20 and 25 μg). **(D)** COS-7 cells were transfected with 2 μg of the PRDII-CAT reporter and the NF-κB p65 expression plasmid as well as increasing amounts of FKBP52 expressing plasmid (2, 5, 10, 15, 20 and 25 μg). CAT activity was analyzed 48h post-transfection using 10 μg of WCE for 4h at 37°C. CAT activity values correspond to an average of 3-6 independent experiments and were normalized to βGal expression.


A
12.5
10.0
7.5
5.0
2.5
Fold induction
IRF-4 – + – – + + + + + + +
PU.1 – – + – + + + + + + +
FKBP52 – – – + –
1 2 3 4 5 6 7 8 9 10 11
B
15.0
10.0
5.0
Fold induction
IRF-4 – + – – + + + + + + +
PU.1 – – + – + + + + + + +
FKBP52 – – – + –
Ascomycin
1 2 3 4 5 6 7 8 9 10 11

C


25.0
20.0
15.0
10.0
5.0
Fold induction
IRF-4 – + – – + + + + + + +
PU.1 – – + – + + + + + + +
ΔN-FKBP52 – – – + –
1 2 3 4 5 6 7 8 9 10 11

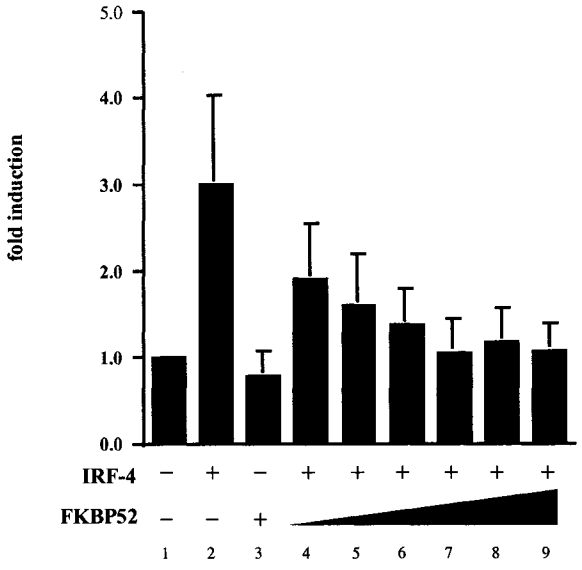
D


50
40
30
20
10
%acetylation
p65 – + – + + + + + +
FKBP52 – – +
1 2 3 4 5 6 7 8 9

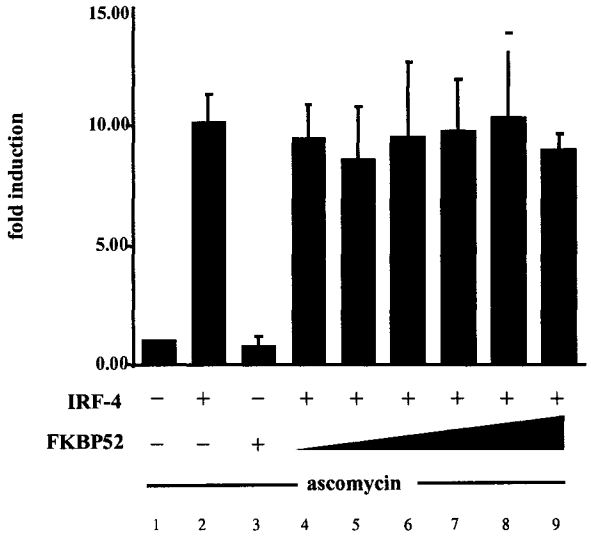
Using a different reporter construct, IRF-4 produced a moderate increase in ISG-15-CAT activity (2-3 fold) (Figure 51A, lane 2). Although FKBP52 itself had no effect on reporter gene activity (figure 51A, lane 3) increasing amounts of FKBP52 also produced a concentration dependent inhibition of IRF-4 transactivation (figure 51A, lanes 4-9). FKBP52-mediated inhibition also required functional PPIase activity since the use of ascomycin and ΔN-FKBP52 co-transfection restored IRF-4 activity (Figure 51B, lanes 4-9, figure 51C, lanes 4-9).

Figure 51: Analysis of IRF-4 transactivation potential in the presence of FKBP52 on an ISRE-responsive reporter. (A) COS-7 cells were transfected with 2 μg of the ISG15-ISRE-CAT reporter (Lin, 1999), IRF-4 expressing plasmid, as well as increasing amounts of FKBP52 expression plasmid (2, 5, 10, 15, 20 and 25 μg). **(B)** COS-7 cells were transfected as described in (A) and were treated with 2 μM of ascomycin for 16h. **(C)** COS-7 cells were transfected with 2 μg of the ISG15-ISRE-CAT reporter, IRF-4 expressing plasmid, as well as increasing amounts of ΔN-FKBP52 (aa233-459) expressing plasmid (2, 5, 10, 15, 20 and 25 μg). CAT activity was analyzed 48h post-transfection using 10μg of WCE for 4h at 37°C. CAT activity values correspond to an average of 3-6 independent experiments and were normalized to βGal expression.

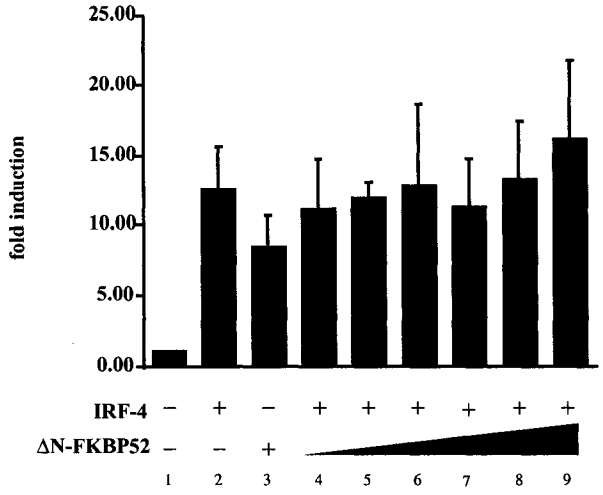
A


5.0
4.0
3.0
2.0
1.0
0.0
fold induction
IRF-4 – + – + + + + + +
FKBP52 – – +
1 2 3 4 5 6 7 8 9

B

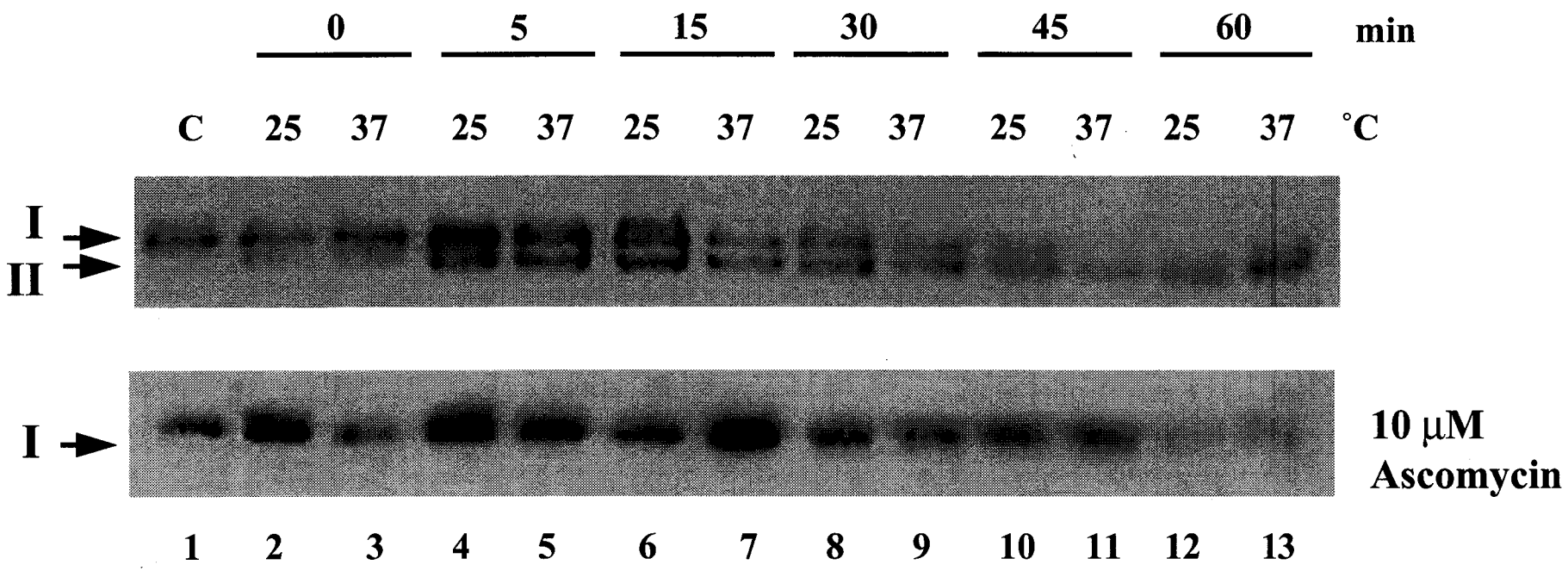

15.00
10.00
5.00
0.00
fold induction
IRF-4 – + – + + + + + +
FKBP52 – – +
ascomycin
1 2 3 4 5 6 7 8 9

C


25.00
20.00
15.00
10.00
5.00
0.00
fold induction
IRF-4 – + – + + + + + +
ΔN-FKBP52 – – +
1 2 3 4 5 6 7 8 9

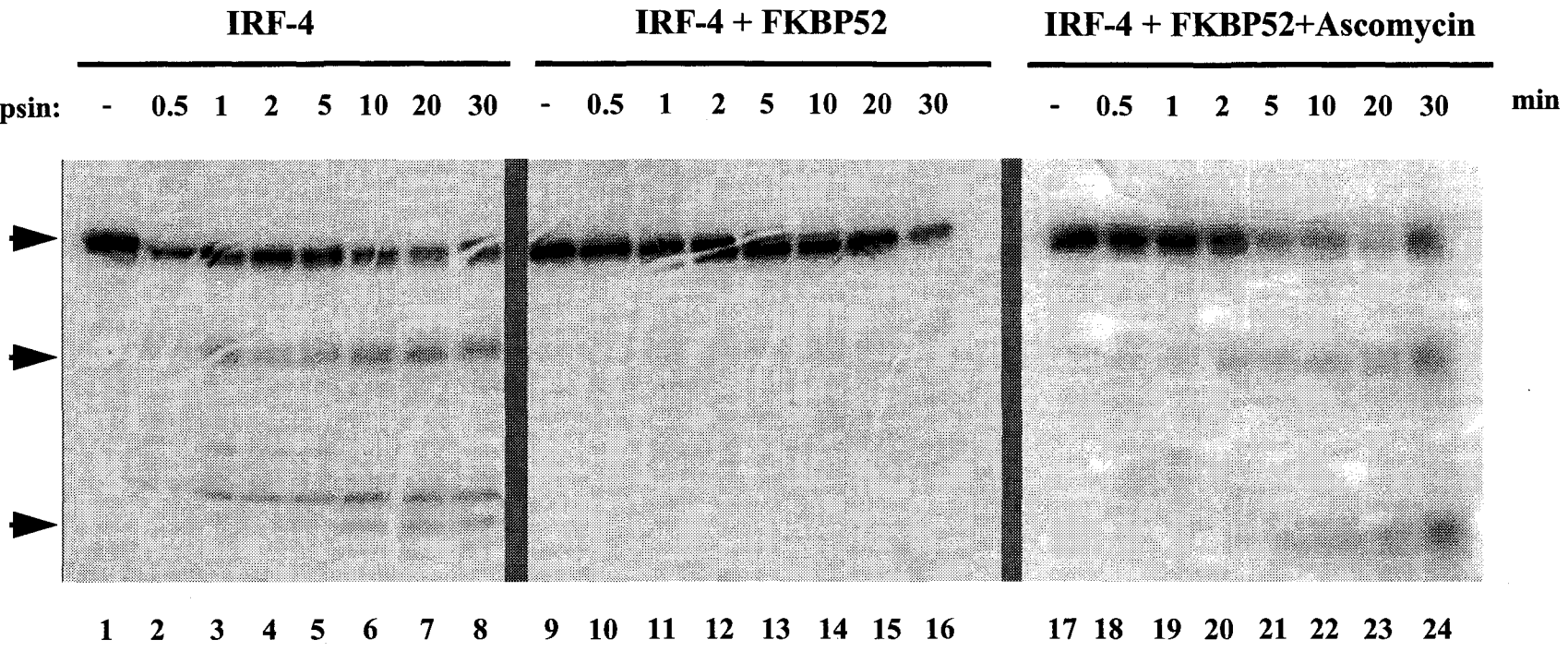
FKBP52 induces a conformational change in IRF-4. Analysis of the expression pattern of IRF-4 in extracts from HTLV-1 infected cells revealed 2 closely migrating bands corresponding to IRF-4. The slower migrating form of IRF-4 did not appear to correspond to phosphorylated IRF-4, since calf intestinal phosphatase treatment did not affect IRF-4 migration (YM, data not shown). To examine the relationship between the PPIase activity of FKBP52 and the distinct IRF-4 forms observed on SDS-PAGE, recombinant IRF-4 and FKBP52 were incubated at 25°C and 37°C for different times; recombinant IRF-4 alone was detected as a unique form by immunoblot (I) (Figure 52, upper panel, lane 1). When incubated with FKBP52, a second band (II) with a slightly faster migration accumulated with time (Figure 52, upper panel, lanes 2-9). The appearance of the faster migrating form was observed as early as 5 min (Figure 52, upper panel, lanes 2 and 3) and by 45 minutes, IRF-4 was completely converted into the faster migrating form (Figure 52, upper panel, lanes 10 and 11). This effect was specific to FKBP52 since IRF-4 alone remained as a single form (Figure 52, upper panel, lane 1 and data not shown). Furthermore, the appearance of the faster migrating form was completely inhibited in the presence of the PPIase inhibitor ascomycin (Figure 52, lower panel, lanes 2-11). Thus the appearance of the faster migrating form is dependent on functional PPIase activity of FKBP52.

Figure 52: FKBP52 induces a conformational change in IRF-4. Recombinant His-tagged IRF-4 (100 ng) was incubated with GST-FKBP52 (200 ng) at 25° and 37 °C for different times. IRF-4 alone (lane 1, upper panel) was incubated at 37°C for 1h as control. The same experiment was repeated in the presence of 10μM of ascomycin (lower panel). The control consisted of IRF-4 and 10μM of ascomycin incubated at 37°C for 1h (lane 1, lower panel). Samples were run on 8% SDS-PAGE, transferred to a membrane and immunoblotted with anti-IRF-4 antibody.


0
5
15
30
45
60
min
C
25
37
25
37
25
37
25
37
25
37
25
37
°C
I
II
I
10 μM
Ascomycin
1
2
3
4
5
6
7
8
9
10
11
12
13

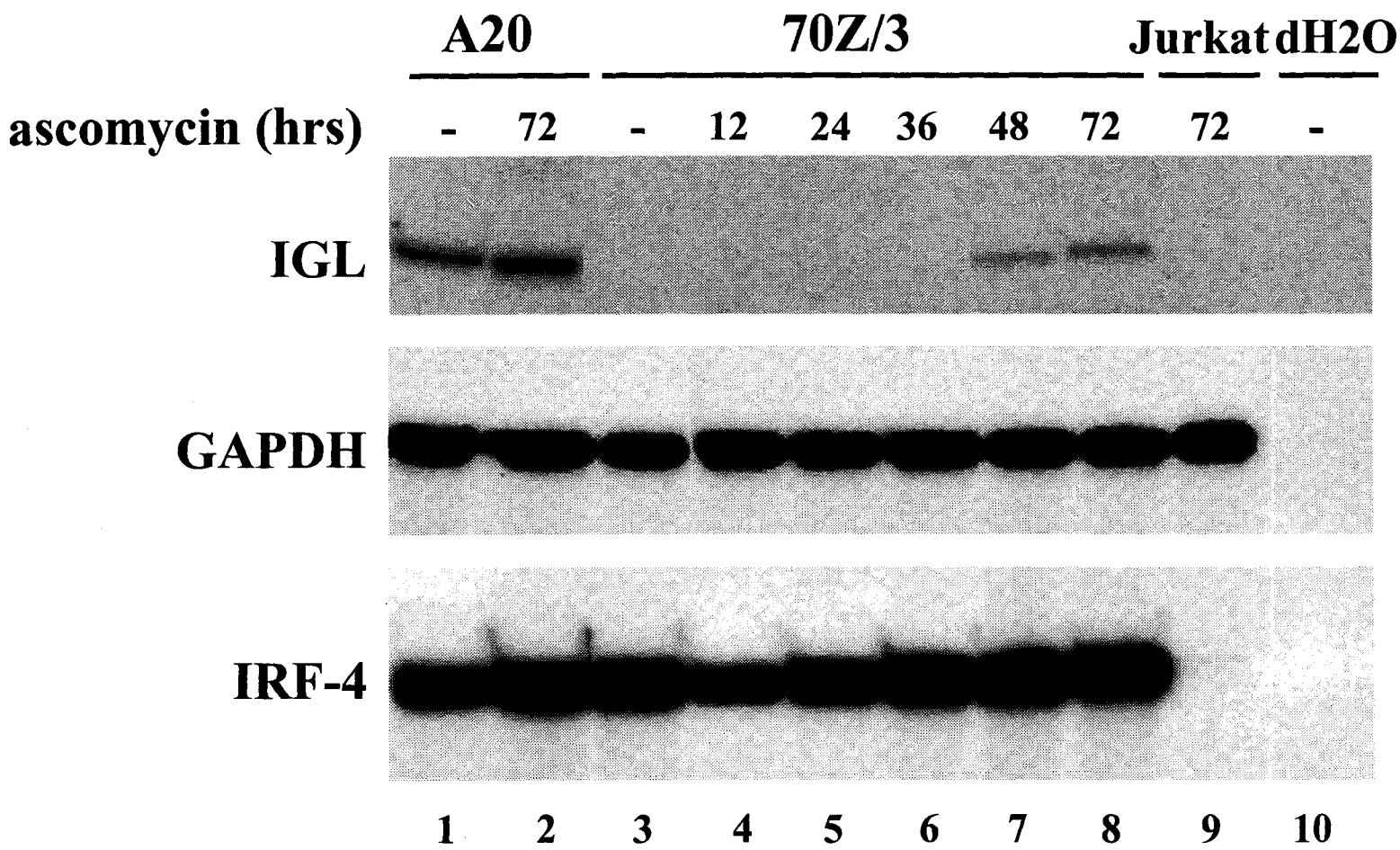
To further confirm the conformational alteration in IRF-4, partial proteolytic digestion of recombinant IRF-4 was performed in the presence or absence of recombinant FKBP52 and ascomycin (Figure 53, lanes 2-8, lanes 10-16 and lanes 18-24). Partial proteolytic cleavage of ^{35}S-labeled IRF-4 by chymotrypsin was blocked by preincubation of IRF-4 with FKBP52 for 1h (Figure 53, lanes 10-16). Strikingly, the presence of 10μM ascomycin restored the partial proteolytic digest pattern and confirmed the importance of the PPIase activity of FKBP52 in mediating proteolysis-resistant conformation of IRF-4 (Figure 53, lanes 18-24). Ascomycin had no effect on the proteolytic digest of IRF-4 alone and similar results were obtained with trypsin proteolysis (YM, data not shown).

Figure 53: FKBP52 induces a conformational change in IRF-4 and protects from proteolytic degradation. *In vitro* translated ^{35}S-labeled His-IRF-4 (1µl) was preincubated with BSA (200ng, lanes 1-8), GST-FKBP52 (200ng, lanes 9-24) or GST-FKBP52 and ascomycin (1µM, lanes 17-24) for 1h at 25°C, IRF-4, IRF-4/FKBP52 and IRF-4/FKBP52/ascomycin reactions were subjected to chymotrypsin digestion (2ng per reaction) for different times at 25°C. Digestion reactions were terminated by the addition of 1X SDS sample loading buffer, loaded on 15% SDS-PAGE and visualized by autoradiograghy. Controls (lanes 1, 9 and 17) were incubated for 30 min at 25°C in the absence of chymotrypsin.


IRF-4
IRF-4 + FKBP52
IRF-4 + FKBP52+Ascomycin
chymotrypsin: - 0.5 1 2 5 10 20 30 - 0.5 1 2 5 10 20 30 - 0.5 1 2 5 10 20 30 min
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

Ascomycin induces the expression of IgL λ in the murine pre-B cell line 70Z/3. 70Z/3, a mouse pre-B lymphoma cell line has been used extensively as a model to study the transcriptional regulation of immunoglobulin genes (102, 161). 70Z/3 expresses both IRF-4 and PU.1 at levels similar to those in differentiated B cells (Figures 31 and 46) but does not express surface immunoglobulins (ATCC references). Furthermore, the PU.1/IRF-4 complex does not stimulate IgL λ mRNA production in these cells. To determine if the PPIase activity of FKBP52 was responsible for the absence of IRF-4/PU.1 activity, the pre-B cell line 70Z/3 was treated with ascomycin (2μM) for 0-72h. Using a RT-PCR based assay, IgLλ expression was detected at 48 and 72h after ascomycin treatment (Figure 54, upper panel, lanes 7 and 8); IgLλ mRNA expression was also detected at 36h (YM, data not shown). The differentiated B cell line A20 was used as a positive control that already expressed high amounts of IgLλ (Figure 54, upper panel, lane 1). Inhibition of PPIase activity also increased IgLλ expression in these cells by 4-fold (Figure 54, upper panel, lane 2), whereas no such increase was detected in Jurkat T cells treated with the drug (Figure 54, upper panel, lane 9). Finally, ascomycin had no effect on GAPDH or IRF-4 mRNA expression levels (Figure 54, middle and bottom panels).

Figure 54: Ascomycin induces IgLλ mRNA expression in the pre-B cell line 70Z/3. Three cell lines, A20, 70Z/3 and Jurkat T cells (1 x 10^7 cells each) were treated with ascomycin (2 μM) for different times. Total RNA was extracted from these cells and RT-PCR was performed on 2 μg of RNA using labeled IgLλ (top panel), GAPDH (middle panel) and IRF-4 (bottom panel) specific primers. Due to high IgLλ expression in A20, the RT reaction was diluted 100 fold for the PCR reaction. The RT reaction was also diluted 100-fold and 10-fold for GAPDH and IRF-4 amplification, respectively. Cells not treated with ascomycin, were incubated with DMSO (used to dilute the drug) for 72h.

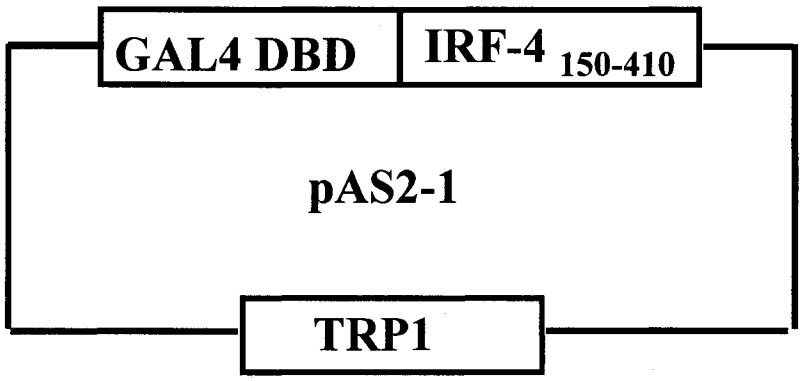

A20
70Z/3
Jurkat
dH2O
ascomycin (hrs)
- 72 - 12 24 36 48 72 72 -
IGL
GAPDH
IRF-4
1 2 3 4 5 6 7 8 9 10

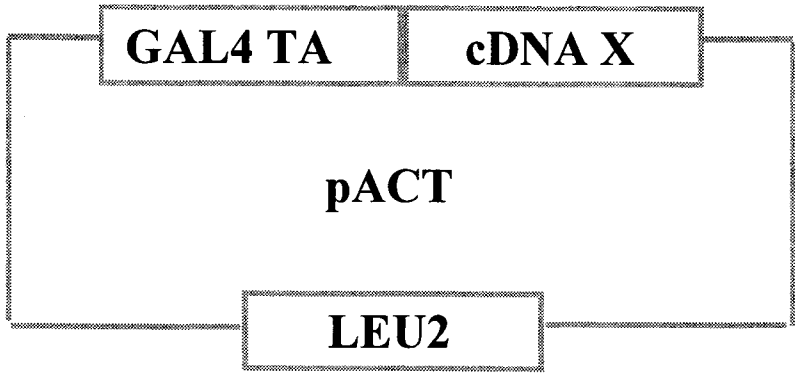
CHAPTER V

IRF-4 INTERACTING PARTNERS IN HTLV-I INFECTED T CELLS

Yeast two-hybrid assay. To identify new partners of IRF-4 in HTLV-I infected T cells, a yeast two-hybrid analysis was performed; since full length IRF-4 possessed intrinsic transcriptional activity, the bait utilized was IRF-4 aa150-410 comprising a proline rich domain and the C-terminal IRF association domain (IAD) (Figure 55). The fragment containing the proline rich and the IRF Association domains (aa150-410) of IRF-4 was PCR amplified with primer A (5'-ATCAGAATTC CCGGGAGTGTACAGGATTGTTCC-3') and primer B (5'-ATCAGTCGACGGATCCT GAGGGTCTGGAAACTCC-3') and subcloned into the pAS2-1 vector (GAL4 $_{1-147}$ DNA binding domain, TRP1; Clontech) in frame with the DNA binding domain of GAL4 and sequenced. The yeast strain Y190 was cotransformed by the lithium acetate permeabilization method with the pAS2-1-IRF-4 (150-410) and with an HTLV-I-transformed T-cell cDNA library cloned into the pACT2 vector (Clontech). Positive yeast clones were selected for prototrophy for leucine, tryptophan and histidine and screened for β–galactosidase expression. Among the $2X10^6$ clones screened, 239 clones were positive for histidine and for βGal. Subsequent two-hybrid assays were carried out by a mating assay between the Y187 and Y190. Diploid cells from the mating assay were selected for Trp- Leu- His- selective medium and screened by replica plating for their ability to produce for β–galactosidase. Of the 239 clones, 91 were positive for mating (Figure 55). Plasmid DNA was isolated from the 91 positive yeast clones and transformed in *E.coli* DH5α by electroporation. The constructs were sequenced using 5'PACT primer (5'-TACCACTACAATGGATG-3'). A list of the known clones interacting with IRF-4 is seen on Table 10.

Figure 55: **Second two-hybrid screening**. To identify potential partners of IRF-4, a yeast two-hybrid analysis was performed; since full length IRF-4 possessed intrinsic transcriptional activity, the bait utilized was IRF-4 aa150-410 comprising a proline rich domain and the C-terminal IRF association domain (IAD). The fragment containing the proline rich and the IRF Association domains (aa150-410) of IRF-4 was PCR amplified and subcloned into the pAS2-1 vector (GAL4 $_{1-147}$ DNA binding domain, TRP1) in frame with the DNA binding domain of GAL4 and sequenced. The yeast strain Y190 was cotransformed by the lithium acetate permeabilization method with the pAS2-1-IRF-4 (150-410) and with an HTLV-I-transformed T-cell cDNA library cloned into the pACT2 vector. Positive yeast clones were selected for prototrophy for leucine, tryptophan and histidine and screened for βgalactosidase expression. Among the $2X10^6$ clones screened, 239 clones were positive for histidine and for βGal. Subsequent two-hybrid assays were carried out by a mating assay between the Y187 and Y190. Diploid cells from the mating assay were selected for Trp- Leu- His- selective medium and screened by replica plating for their ability to produce for βgalactosidase. Of the 239 clones, 91 were positive for mating and sequenced for identification.





2 X 10^6 clones screened

↓

269 HIS^+

↓

236 HIS^+ βgal^+

↓

220 HIS^+ βgal^+ Leu^+ $Cyclo^R$

↓

91 $mating^+$ HIS^+ βgal^+ ⟶ **Isolation of many interesting clones**

HTLV-I-transformed T lymphocyte cDNA library

Table 10: List of clones from the second two-hybrid screen. Thirty-five out of the 91 clones coded for known proteins and are listed with some of their cellular functions.

Clones	Functions
1) MHC Class II	- Antigen Presentation
2) Protein Phosphatase 2α	-Involved in kinase/phophatase activation or inactivation
3) PML	-Tumor suppressor -Nuclear body formation
4) Replication Factor C	-DNA pol accessory molecule
5) Gi3 protein alpha subunit	-Involved in activation of Gi-coupled receptor
6) Lamin A/C	-Nuclear env. protein -Mutated in Emery-Dreifuss muscular dystrophy
7) EIF4 gamma1	-Recognition of mRNA cap
8) Rho GDP dissociation inhibitorα	-Modulates activity of Rho proteins
9) NY-REN-24	-Antigen recognized by autologous antibodies
10) RhoA-specific nucleotide exchange factor	-Modulates activity of Rho proteins -Involved in cytoskeletal organisation
11) TNF-receptor like-2	-Patent sequence
12) Ras-related GTP binding protein 4B	-
13) Selectin P ligand	-Counter-receptor for P-selectin on myeloid cells and activated T cells
14) Rad51c	-Strand transfer protein
15) Oct-6	-Neuronal cell development and apoptosis
16) Prolyl 4-hydroxylase	-Hydroxylation of Prolines in peptide linkages
17) Ubiquitin-conjugating enzyme E21	-Ubiquitination of Lys on proteins

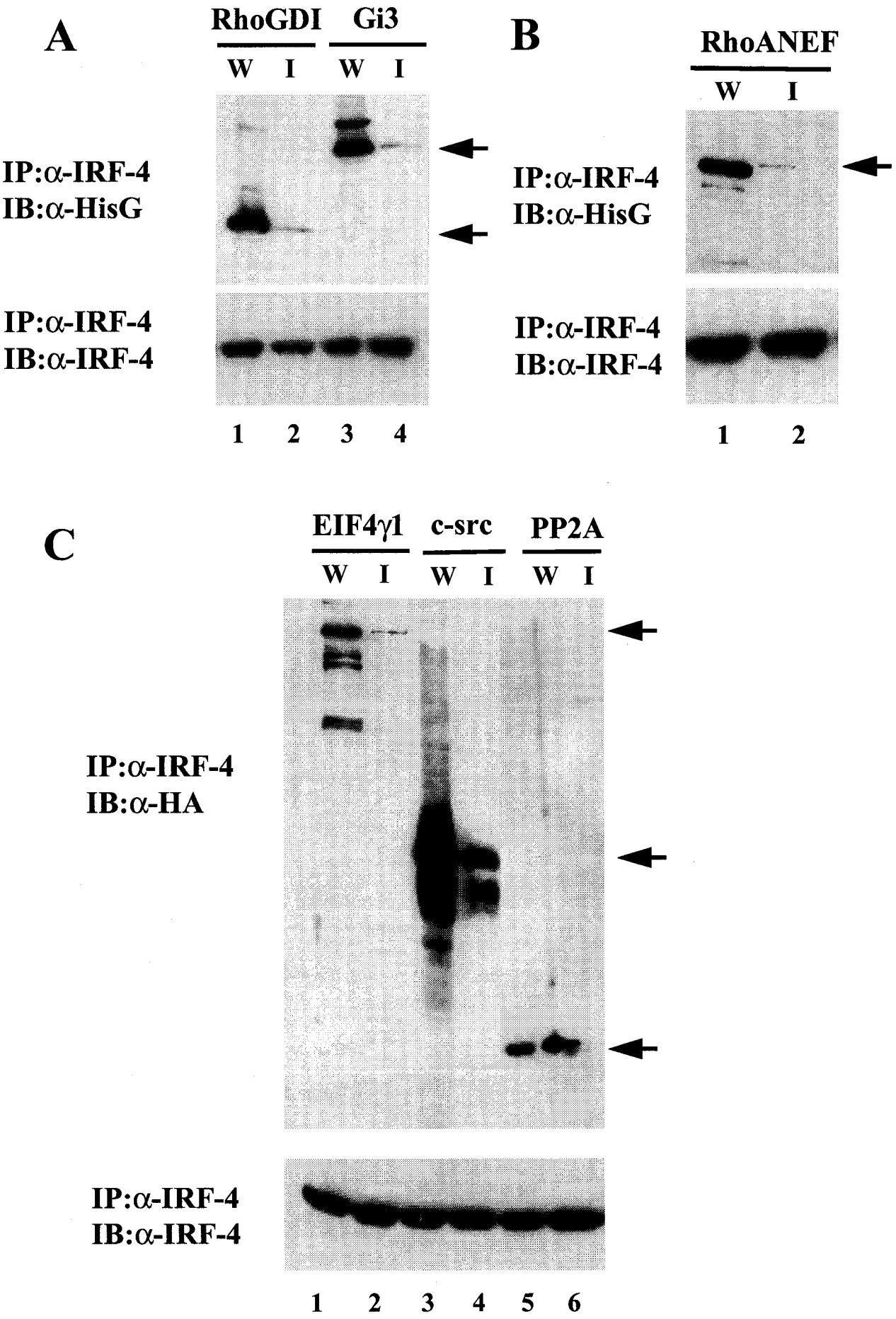
Clones	Functions
18) Polycystic kidney disease 1	-Gene disrupted in autosomal dominant polycystic kidney disease
19) SMIF	-SMAD-4 interacting partner
20) AML2	-SMAD-TGFβ signaling
21) LAT	-Associates with Phospholipase Cγ1 and Grb2
22) Calreticulin	-Implicated in autoimmune processes
23) Sodium-dep. mutli-vitamin transporter	-Uptake of pantothenate and biotin
24) tetracycline transporter-like protein	-
25) TBP-associated factor 170 (TFII170)	-RNA Pol II initiation factor
26) Gli-1	-Kruppel zinc finger protein
27) PP1α	-Involved in kinase/phophatase activation or inactivation
28) βagrenergic receptor kinase 1 (GRK2)	-Homologous receptor densensitization
29) Protaglandin E receptor 4	-
30) Slit homolog 1	- CNS development in Drosophila
31) Endothelial monocyte-activation cytokine	-Monocyte and granulocyte activating cytokine
32) REV1	-Involved in UV mutagenesis repair
33) NFKBIL1	-
34) RAN	-Nuclear env. structure -Nucleocytoplamic transport
35) c-SRC	-Signal transduction of cytokines and growth factors

***In vivo* interaction with IRF-4.** The cDNA of the different clones were obtained from several researchers and transfected with IRF-4 full length into Cos7 cells. Coimmunoprecipitation assays with an α-IRF-4 antibody was used to confirm interaction. Preliminary results are shown in Figure 56. IRF-4 positively interacted with Rho Guanine Dissociation Inhibitor (Figure 56A, lane 2), Gi3 (Figure 56A, lane 4), RhoA Nucleotide Exchange Factor (Figure 56B, lane 2), eIF4γ1 (Figure 56C, lane 2), c-src (Figure 56C, lane 4) and Protein Phosphatase 2A catalytic (Figure 56C, lane 6). Whole cell extracts were loaded as controls of plasmid expression (Figure 56A, lanes 1 and 3, Figure 56B, lane1 and Figure 56C, lanes 1, 3 and 5). The immunoprecipitations were verified by blotting with an α-IRF-4 antibody (Figures 56A, B, C, bottom panels).

We further investigated the functions of these novel interacting partners. Rho Guanine Dissociation Inhibitor (RhoGDI) keeps unstimulated Rho proteins in a GDP-bound state and therefore inactive. Rho GTPases switch between inactive GDP-bound and active GTP-bound state; they are mediator of G protein-coupled receptor signaling and are involved in cell adhesion, motility, transcription, cell cycle progression, cytokinesis and cell fate determination. RhoA Nucleotide Exchange Factor (RhoANEF) also controls Rho GTPase by stimulating the exchange of GDP for GTP (194). eIF4γ1 is part of the eIF4 translation initiation factors responsible for recruiting the mRNA to the ribosome. eIF4γ1 is a large polypetide which acts as a scaffolding protein, bringing together other eIFs to the mRNA and the translational machinery (77). c-src is the normal homologue of v-src, the transforming gene in

Rous Sarcoma Virus. It is a non-receptor tyrosine kinase associated with the plasma membrane involved in growth factor, integrin adhesion receptor, stress and G protein-coupled receptor signaling pathways (8). Gi3 is a member of the heterotrimeric GTP-binding proteins. It is involved in vesicle trafficking, platelet activation, opioid receptor and G protein-coupled receptor signaling pathways (194). Protein Phosphatase 2A (catalytic) is a serine-threonine phosphatase involved in the regulation of many signal transduction pathways (193).

Figure 56: Co-immunoprecipitation analyses of potential IRF-4 interacting partners. Cos7 cells were transfected with 10 μg of IRF-4, RhoGDI, Gi3, RhoANEF, EIF4γ1, c-src and PP2A expression plasmids. Whole cell extracts were collected 48 hours post-transfection and used in co-immunoprecipitation assay. IRF-4 was coimmunoprecipitated from the Cos7 WCE (500 μg) using G protein coated sepharose beads crosslinked to an anti-IRF-4 antibody. **(A)** Immunoprecipitated complexes were run on 15% SDS-PAGE and immunoblotted using αHisG antibody (upper panel) and αIRF-4 antibody (lower panel). Input extracts consisted of 50 μg of WCE (upper and lower panels, lanes 1 and 3). **(B)** Immunoprecipitated complexes were run on 10% SDS-PAGE and immunoblotted using αHisG antibody (upper panel) and αIRF-4 antibody (lower panel). Input extracts consisted of 50 μg of WCE (upper and lower panels, lane 1). **(C)** Immunoprecipitated complexes were run on 10% SDS-PAGE and immunoblotted using αHA antibody (upper panel) and αIRF-4 antibody (lower panel). Input extracts consisted of 50 μg of WCE (upper and lower panels, lanes 1, 3 and 5). The control IPs were performed by incubating WCE (500 μg) with normal precleared mouse serum (data not shown).


A
RhoGDI
Gi3
W
I
W
I
IP:α-IRF-4
IB:α-HisG
IP:α-IRF-4
IB:α-IRF-4
1
2
3
4
B
RhoANEF
W
I
IP:α-IRF-4
IB:α-HisG
IP:α-IRF-4
IB:α-IRF-4
1
2
C
EIF4γ1
c-src
PP2A
W
I
W
I
W
I
IP:α-IRF-4
IB:α-HA
IP:α-IRF-4
IB:α-IRF-4
1
2
3
4
5
6

CHAPTER VI

THE IDENTIFICATION OF NOVEL IRF-4 REGULATED GENES

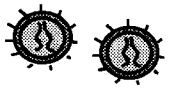
IRF-4 stably expressing Jurkat cell line

To date, very little is known about IRF-4 gene regulation in lymphoid cells. Besides the immunoglobulin light chain gene in B cells, which IRF-4 regulates with PU.1, other IRF-4 regulated genes in B cells and in activated T cells are to date, still unknown. We therefore investigated the role of IRF-4 gene regulation in T cells. An IRF-4 stably expression Jurkat cell line was produced using the pMSCV neo system (Clontech) with an amphotropic packaging cell line (ATCC; Dr. Gary Nolan) (Figure 57). IRF-4 was subcloned into pMSCV neo plasmid and transiently transfected into the amphotropic packaging cell line. As a control, pMSCV wt neo plasmid was also transiently transfected into the packaging cell line. 48 hours post-transfection, virus-containing media was collected and used to infect Jurkat T cells. 48 hours post-infection, aliquots from the IRF-4 infected Jurkat cells as well as wt infected cells were analyzed for IRF-4 protein expression. IRF-4 protein, although faint was only seen in Jurkats infected with the IRF-4 virus and not the wt virus (Figure 57, lanes 1-2). The rest of the cells were put under antibiotic selection for two weeks using neomycin. After selection, increasing amount of whole cell extracts from the pMSCV IRF-4 Jurkat cells, the pMSCV wt Jurkat cells as well as regular Jurkats were analyzed by Western blot using an α-IRF-4 antibody. Greater amounts of IRF-4 protein were detected only in the pMSCV IRF-4 Jurkat cells and not in the pMSCV wt Jurkat cells or in non-transduced Jurkats (Figure 57, lanes 3-5, lanes 6-8 and lane 9, respectively).

Figure 57: Generation of an IRF-4 stably expressing Jurkat cell line. An IRF-4 stably expression Jurkat cell line was produced using the pMSCV neo system with an amphotropic packaging cell line. IRF-4 was subcloned into pMSCV neo plasmid and transiently transfected into the amphotropic packaging cell line. As a control, pMSCV wt neo plasmid was also transiently transfected in the packaging cell line. 48 hours post-transfection, virus-containing media was collected and used to infect Jurkat T cells. 48 hours post-infection, aliquots from the IRF-4 infected Jurkat cells as well as wt infected cells were analyzed for IRF-4 expression by Western blot. WCE (100 μg) were run on 10% SDS-PAGE and blotted with an anti-IRF-4 antibody. The rest of the cells were put under antibiotic selection using neomycin for two weeks. After selection, increasing amount of whole cell extracts (25, 50 and 100 μg) from the pMSCV IRF-4 Jurkat cells, the pMSCV wt Jurkat cells as well as regular Jurkats were analyzed by Western blot using an α-IRF-4 antibody.

pMSCV-IRF4
pMSCV-WT

→ Amphotropic Packaging cell line



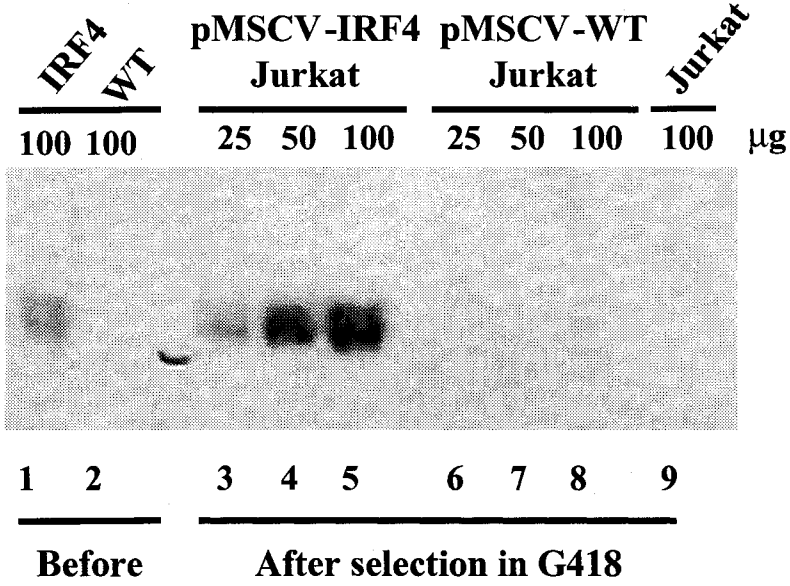
→ Collect virus (48hrs after)

↓

Infect Target Cells (Jurkat)

← G418

Stably Infected Cell lines



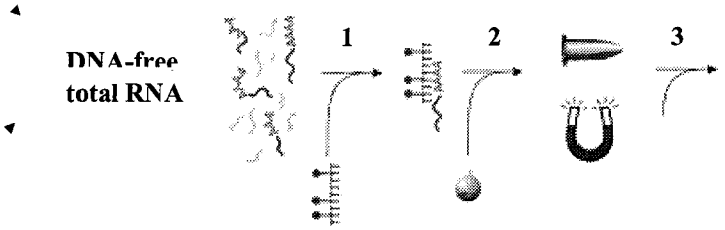
cDNA array analysis of IRF-4 regulated genes.

Cells (50 million) from the stably expressing cell line Jurkat-MSCVneo-IRF-4 as well as the control cell line Jurkat MSCVneo-wt were collected and lysed using the denaturation solution supplied by the Clontech kit. Total RNA isolation was performed as described in the "Atlas Pure Total RNA Labeling System" (Clontech Inc.). RNA purity and integrity was assessed by spectrophotometer analyses and agarose gel as described by the manual provided.

cDNA probe synthesis was performed in three steps: 1) Poly A mRNA from the two cell lines were annealed to biotinylated oligo dT 2) Streptavidin coated magnetic beads were incubated with the RNA which bound to the biotynilated oligo dT and 3) After washing of the beads using a magnet, the mRNAs were eluted of the beads and the cDNA probes were synthesized using gene-specific primers, reverse transcriptase and α-32P-dATP (Figure 58). The newly synthesized probes were used for hybridization and detection on 2 separate Atlas Human 1.2 Arrays (1,176 characterized cDNA spotted on each; Clontech Inc.). Analysis of the membranes was possible through the use of the software "AtlasImage 1.5" (Clontech Inc.) (Figure 58). A series of three separate experiments (RNA extraction, probe synthesis and hybridization) were performed in order to determine the genes regulated by IRF-4 (Table 11).

Figure 58: cDNA array protocol for the determination of IRF-4 regulated genes. RNA was isolated from the stably expressing cell line, Jurkat-MSCVneo-IRF-4 as well as the control cell line Jurkat MSCVneo-wt. cDNA probe synthesis was performed in three steps: 1) Poly A mRNA from the two cell lines were annealed to biotynilated oligo dT 2) Streptavidin coated magnetic beads were incubated with the RNA which bound to the biotynilated oligo dT and 3) After washing of the beads using a magnet and eluting the bound mRNAs, cDNA probe was synthesized using gene-specific primers, reverse transcriptase and α-32P-dATP. The newly synthesized probes were used for hybridization and detection on 2 separate Atlas Human 1.2 Arrays (1,176 characterized cDNA spotted on each). Analysis of the membranes was possible through the use of the software "AtlasImage 1.5". A series of three separate experiments (RNA extraction, probe synthesis and hybridization) were performed in order to determine the genes regulated by IRF-4.

Jurkat- MSCVwt



Hybridization with
Atlas human 1.2 Array -
1,176 characterized cDNA spotted
On a nylon membrane

Jurkat-MSCV/IRF4

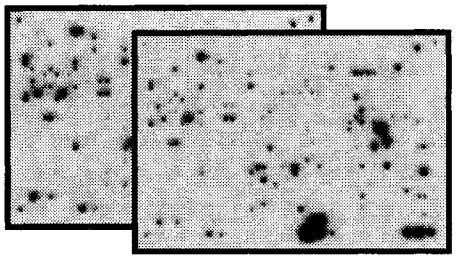
1-Poly-A$^+$anneal with Biotynilated Oligo (dT)

2-Biotin binds to streptavidin Magnetic beads

3- cDNA probe synthesis (gene-specific primer mix) - [α^{32}P] dATPlabeling directly on beads

Jurkat -MSCV/wt

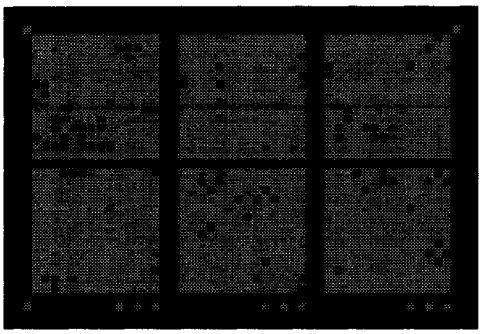
Jurkat-MSCV/IRF4



cDNA probe synthesis (gene-specific primer mix) - [α^{32}P] dATP labeling directly on beads

Hybridization with Atlas human 1.2 Array - 1,176 characterized cDNA spotted on a nylon membrane (Clontech)

Atlas Image software Analysis



Identification of up-& down regulated genes → Correlation of results by RT-PCR

Table 11: **List of potential IRF-4 regulated genes**. A series of three separate experiments (RNA extraction, probe synthesis and hybridization) were performed in order to determine the genes regulated by IRF-4 listed in the table. Genes highlighted in yellow were further analyzed by RT-PCR.

Genes up-regulated by IRF-4

Coordinate	Gene/Protein name	Fold induction 1st exp	2nd exp	3rd exp
Facilitated diffusion proteins				
A09m	E16 amino acid transporter	3.6	no data	1.3
Intracellular protein kinases				
B03l	S6KII-alpha1	no data	> 1.7	1.2
B06h	MAPKAP kinase (3pK)	1.9	1.9	1.1
B10f	c-src kinase	2.1	1	1.1
B11h	MAPKK2	1.6	2	1.2
B12h	MAPKK3	2.1	2.1	no data
G proteins				
B10n	protein G-I/G-s/G-t beta subunit 2	2	1	1.1
DNA polymerases, replication factors & recombination proteins				
C01m	MCM2 DNA replication licensing factor	1.5	1.5	1.3
C03m	MCM5 DNA replication licensing factor	2.1	no data	
DNA synthesis, repair & recombination proteins				
C01n	ERCC1 DNA excision repair protein	2	> 1.5	1
C02i	calpain	1.6	1.8	
GTPase inhibitors				
C09d	Rho-GDI	1.8	1	no data
Tyrosine phosphatases				
C12a	PP-1A	1.6	1.7	1
Proteinases				
C13h	calpain 1 large catalytic subunit	no data	1.7	1
Transcription factors				
D10k	ADA3-like protein	no data	1.9	1.1
E11a	ETR-101	no data	> 10	1.1
Cell surface antigen and adhesion protein				
E03g	cadherin 5	2.4	1.4	1
E05h	complex I-B18	no data	2.1	1
Hormones				
F04h	ribonuclease/angiogenin inhibitor	1.7	1.7	1.5
Protein turnover				
F13m	cathepsin D precursor	1.7	> 1.6	>1.6
F14l	bikunin	no data	1.8	1.9
F12j	parathymosin			2.1
B04k	MLK-3			2.2
E01h	leukosialin precursor			1.9
D08k	PCAF associated factor 65			2.1
C04e	hint protein, PKC inhibitor	1.9		1.5

Genes down-regulated by IRF-4

Coordinate	Gene/Protein name	Fold decrease 1st exp	2nd exp	3rd exp
Facilitated diffusion proteins				
A06m	erythrocyte glucose transporter1	1.7	no data	1.1
Intracellular protein kinases				
B03i	PKC-alpha	2.4	3.1	1.1
B05j	c-jun N-terminal kinase 2	3.8	no data	1.2
B14h	MAPKK5	1.9	2	1.7
Receptors				
A12e	ERBB-3	2.1	2.5	> 1.6
A01f	VEGFR1	2.5	1.7	2.1
B01e	U-PAR	1.6	1.8	1
B13d	SDF-1 receptor CXCR4	2.7	1.9	1.7
Receptors-associated proteins				
B14f	GRB2	3.4	1.6	> 2.1
G proteins				
A01g	RhoA	2.6	1.8	> 1.7
B08m	RhoB	no data	2	1.2
B10m	RAB-7	1.6	1.8	1
B13m	p21-rac	2	1.7	no expression
Tumors suppressors & related proteins				
A03b	EB1 protein	> 10	2.2	1
A04b	ezrin	2.5	1	1.3
Cell cycle regulators				
A02h	G2/mitotic -specific cyclin B1	> 8	> 10	3
A04k	CDK 4 inhibitor p16-INK4	> 3	no data	2.6
A05k	CDK 4 inhibitor D p19-INK4D	>2.1	1	1.6
A09h	fte-1	6.3	no data	1.1
A11j	CDC-like kinase 3	1.8	no data	1.1
A13j	CDK regulatory subunit 1	2.3	no data	up 1.6
A14j	CDK regulatory subunit 2	3	no data	1.2
A03l	prothymosin alpha	3	> 1.6	no data
A01m	transmenbrane 4 superfamily prot. SAS	1.9	no data	2.6
A09k	ubiquitin conjugating enzyme E2H10	1.9	1	1.4
DNA polymerases, replication factors & recombination proteins				
C06l	PCNA	7	7.5	> 3
C08l	replication protein A	2	no data	> 2.3
Apoptosis related proteins				
C11k	ALG-2 calcium binding protein	5	no variation	
C12j	Cell-arrest and DNA-damage inducible protein 153	2.6	1.7	
C14i	NIP3	5	> 1.8	1.7
Kinases substrates and inhibitors				
C14d	14-3-3n protein eta	> 3.4	3.2	2.7
DNA synthesis, repair & recombination proteins				
D01a	DNA-repair protein XRCC1	1.6	no variation	
D07a	recA like protein	3.7	no variation	1.8
D14a	deoxyribonuclease II	1.7	1.4	

Genes down-regulated by IRF-4...

Coordinate	Gene/Protein name	Fold decrease 1st exp	2nd exp	3rd exp
Transcription factors				
D02n	60S ribosomal protein L 6	>2.3	1	up 2.3
D05n	hSNF2b	1.3	1.7	1.2
D06j	EGF response factor	2.2	2.4	2.3
D14j	activated RNA transcriptional coactivator p15	6	no data	no expression
E02e	ATF4	>1.6	no data	1
E04b	nuclease -sensitive element DNA-binding protein	> 2.5	2	no data
E08c	trans-acting T cell specific transcription factor GATA 3	2.5	1.9	1.1
Neuromediators				
D09f	acyl-Coa-binding protein	2.1	1	1.5
Cell surface antigen and adhesion protein				
E03i	LFA-1 beta subunit	2.3	1	1.6
E14i	LFA-1 alpha subunit	1.7	1.9	2.3
Protein turnover				
F01l	proteasome component C2	4.5	1.7	no data
F02l	proteasome component C3	3.1	no data	1
Homeostasis				
F02a	heat shock cognate 71kDA protein	5	1.6	no data
Extracellular communication proteins				
F13j	thymosin beta 4	6.3	1	up> 4.9
A01l	CDC25B			> 1.9
A09 b	c-myc purine-binding transcription factor puf			> 2
C05k	cytoplasmic dynein light chain (HDLC1)		up	2.4
A02j	ser/thr protein kinase PLK1			> 2.9
C12m	DNA ligase I			>2.6
A02f	tyr protein kinase receptor tyro 3 (rse)	1.4		2.6
C10l	RFC40			>1.6
C14n	uracyl DNA glycosylase precursor			3.5
D14l	PRB-binding protein E2F1			1.9
E08n	soluble epoxide hydrolase			2.5
A02k				
F07b	cytosolic superoxide dismutase	2.4		

Results from the list of potential IRF-4 regulated genes seem to suggest that IRF-4 functions more as a repressor of gene expression than an activator, in T cell context. A greater number of gene categories were decreased in the IRF-4 expressing Jurkat cell line; intracellular protein kinases, receptors, receptor associated proteins, G proteins, tumor suppressors, DNA polymerases, replication factors, apoptosis related proteins, transcription factors, cell surface antigens and proteins involved in turnover (Table 11). Some genes were also increased but the fold induction was rarely over two and therefore not very significant according to the Clontech manual.

RT-PCR of selected genes

A series of genes were selected for further analysis by RT-PCR. Selection was based on the reproducibility of the results in the 3 separate cDNA array experiments as well as the potential role of these genes in HTLV-I-induced T cell leukemogensis: HSC70 is a molecular chaperone part of the Heat Shock Protein family (155). RhoA is a small G protein which controls several cellular events such as cellular growth, movement, proliferation and differentiation by rearranging actin and cytoskeleton proteins (194). RP-A is a single-stranded DNA binding protein involved in DNA replication, repair and recombination (11). erbB3 is part of the erbB glycoprotein receptors with tyrosine kinase activity which have been implicated in the development of several human cancers (112). GRB2 is an adaptor protein found linked to many growth factor receptors. It is involved in signal transduction of various pathways such as the Ras signaling pathway (204). 14-3-3 family members play a key regulatory role in signal transduction, checkpoint control, apoptotic and

nutrient-sensing pathways (47). CXCR4 is the receptor for Stromal Derived Factor-1 (SDF-1) and one of the co-receptors for HIV-I entry (148). Cyclin B1 is involved in the G2 to M checkpoint in cell cycle progression (214). EB1 localizes to the mitotic spindle and cytoplasmic microtubules. It regulates microtubule stability and is important for the positioning of the mitotic spindle (215). PCNA is required for eukaryotic cell DNA synthesis and nucleotide excision repair (220). MAPKK3 is involved in the MAP kinase signal transduction and activates p38 proteins (24). NIP3 is a pro-apoptotic member of the Bcl2 family of cell death factors which is strongly induced during hypoxia (18). Finally, p19 is a cell cycle inhibitor part of the INK4 family (197).

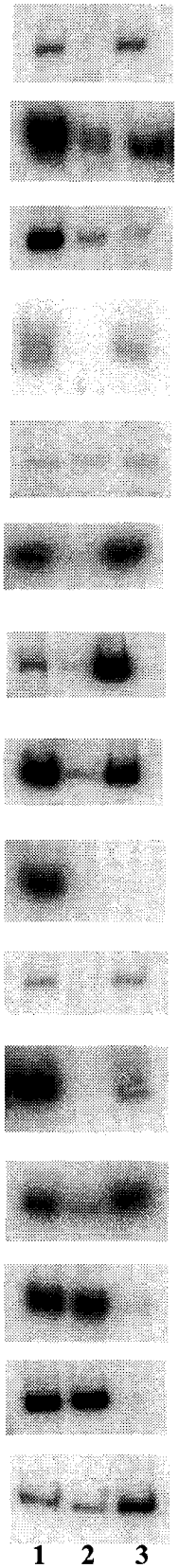
A set of gene specific primers was purchased from Clontech for all the genes mentioned above. The RNA (1μg of each) used in cDNA array analyses (Jurkat-MSCVneo-IRF-4 and Jurkat MSCVneo-wt) as well from the HTLV-I infected T cell line, MT2, were reverse transcribed into cDNA and 5μl of cDNA was used for PCR. At first, the proper PCR conditions were assessed for each gene. "Real- time"-PCR was performed on each gene from the three sample RNAs (Jurkat-MSCVneo-IRF-4, Jurkat MSCVneo-wt and MT2) by taking 5ul aliquots from each PCR reaction at different cycles: 18, 23, 28, 33 and 38 (Clontech Inc.). These aliquots were loaded on 2% agarose gel and visualized by ethidium bromide staining and UV shadowing (YM, data not shown). These initial PCRs permitted the determination of the appropriate number of PCR cycles needed for the amplification of each gene within a linear and non-saturated range. Listed below are the number of PCR cycles used for

each gene amplification: HSC70 = 28, RhoA = 23, RP-A = 28, ERBB3 = 33, GRB2 = 28, 14-3-3η = 28, CXCR4 = 28, cyclin B1 = 28, EB1 = 28, PCNA = 28, MAPKK3 = 28, NIP3 = 28, p19 = 28, LFA-I = 28 and GAPDH = 23.

The above mentioned conditions were then used for RT-PCR analyses in the presence of α-32P-dCTP. RT-PCR samples (5 μl) were loaded on 4% polyacrylamide gels, dried and exposed to film (Figure 59). Results from at least two separate RT-PCRs were quantified using the NIH program and gel plotting macros. The results were also quantified relative to the amount of GAPDH amplified. As seen on Figure 59, the levels of GAPDH were set as 1 for Jurkat MSCVneo-wt, 0.81 for Jurkat MSCVneo-IRF-4 and 2.83 for MT2 cells depending on their intensities. The intensity of the RT-PCR bands from each gene was also analyzed using the same NIH program. Each activity (band intensity) was normalized to the amount of GAPDH in each cell line. In Figure 59, the first three columns represent percentage values of expression compared to Jurkat MSCVneo-wt, which was set at 100%. The next two columns are the fold increase or decrease of each mRNA in Jurkat MSCVneo-IRF-4 and MT2 cells when compared to Jurkat MSCVneo-wt. Interestingly, most of the mRNAs downregulated in Jurkat MSCVneo-IRF-4 cell line were also decreased in the MT2 HTLV-I infected T cell (Figure 59). HSC70 was decreased by 2.8 and 2.6-fold in the Jurkat MSCVneo-IRF-4 cell line and the MT2 cells, respectively. RhoA was decreased by 5 X and 7.7 X, respectively. RP-A was decreased by 8.3 X and 50 X. erbB3 decreased 8.3 X and 5.3 X. GRB2 decreased 1.5 X and 3 X. 14-3-3η decreased 2.2 X and 2.6 X. Cyclin B1 was decreased by 5.3- and 4-fold, respectively. EB1 was

decreased by 25-fold in both cell lines. PCNA decreased 4 X and 2.7 X. NIP3 decreased 1.5 X and 16.7 X. CXCR4 was decreased by 1.3-fold in the Jurkat MSCVneo-IRF-4 cell line but it was increased by 2.3-fold in MT2 cells. p19 was slightly increased by 1.1 X in the Jurkat MSCVneo-IRF-4 cell line but was dramatically decreased in MT2 cells by 50-fold. Finally, MAPKK3 which seemed upregulated by 2-fold in the cDNA array results (Table 11), was decreased by 9-fold in the Jurkat MSCVneo-IRF-4 cell line and by 16.7-fold in MT2 cells.

Figure 59: **RT-PCR analyses of IRF-4 regulated genes**. The RNA (1μg of each) used in cDNA array analyses (Jurkat-MSCVneo-IRF-4 and Jurkat MSCVneo-wt) as well as from the HTLV-I infected T cell line, MT2, were reverse transcribed into cDNA array and 5μl was used in PCR in the presence of α-32P-dCTP. Samples (5μl) were loaded on 4% polyacrylamide gels, dried and exposed to film. Results from two separate RT-PCRs were quantified using the NIH program and gel plotting macros. They were also quantified relative to the amount of GAPDH amplified. The level of GAPDH was set as 1 for Jurkat MSCVneo-wt, 0.81 for Jurkat MSCVneo-IRF-4 and 2.83 for MT2 cells depending on the intensities. The intensity of the RT-PCR bands from each gene was analyzed using the NIH program and each activity was normalized to the amount of GAPDH in each cell line. The first three columns are percentage values of expression compared to Jurkat MSCVneo-wt, which was set at 100%. The next two columns are the fold increase (green) or decrease (red) of each gene in Jurkat MSCVneo-IRF-4 and MT2 cells when compared to Jurkat MSCVneo-wt.



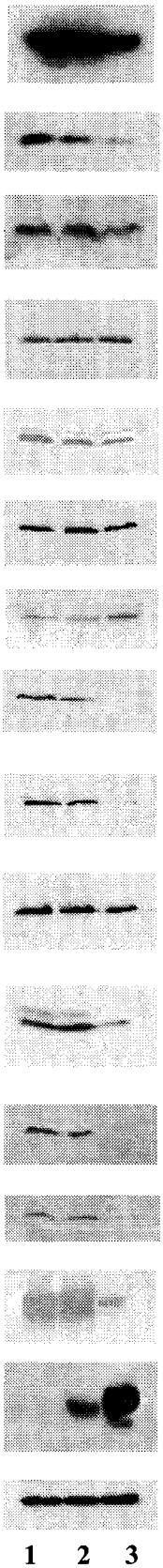
	Induction relative to GAPDH levels			Fold induction	
	Jurkat wt	IRF-4	MT2	IRF-4	MT2
HSC70	100	35	38	2.8X	2.6X
RhoA	100	20	13	5X	7.7X
RP-A	100	12	2	8.3X	50X
ERBB3	100	12	19	8.3X	5.3X
GRB2	100	70	34	1.5X	3X
14-3-3η	100	45	39	2.2X	2.6X
CXCR4	100	75	232	1.3X	2.3X
Cyclin B1	100	19	25	5.3X	4X
EB1	100	4	1	25X	100X
PCNA	100	25	37	4X	2.7X
MAPKK3	100	11	6	9X	16.7X
NIP3	100	65	6	1.5X	16.7X
p19	100	100	2	-	50X
LFA-1	100	100	2	-	50X
GAPDH	1.00	0.81	2.83		

Western blot analyses of IRF-4 regulated genes

Protein extracts were obtained from the cell lines used in the cDNA array and RT-PCR analyses. 50 μg of whole cell extracts were loaded on 10 or 15% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blotted with the various antibodies. Results from at least two separate Western blots were quantified using the NIH program and gel plotting macros. They were also quantified relative to the amount of actin present in the cells. As seen on Figure 60, the levels of actin were set to 1 for Jurkat MSCVneo-wt, 1 for Jurkat MSCVneo-IRF-4 and 1.2 for MT2 cells, depending on their intensities. The intensity of each protein band was analyzed using the same program and each activity was normalized to the amount of actin in each cell line. In Figure 60, the first three columns represent percentage values of expression compared to Jurkat MSCVneo-wt, which was set at 100%. The next two columns are the fold increase or decrease of each protein in Jurkat MSCVneo-IRF-4 and MT2 cells when compared to Jurkat MSCVneo-wt. Interestingly, the results found by RT-PCR, in general, agreed with the protein analyses (Figures 59 and 60). HSC70 was decreased by 1.3 X and 5 X in the Jurkat MSCVneo-IRF-4 cell line and the MT2 cells, respectively. RhoA was decreased by 3 X and 16.7 X, respectively. RP-A was not decreased in the Jurkat MSCVneo-IRF-4 but was decreased by 2 X in MT2 cells. erbB3 protein levels seemed constant in the three cell lines examined. GRB2 was decreased by 1.4 X and 2.3 X, respectively. 14-3-3η protein only decreased by 1.4 X in MT2 cells. CXCR4 protein was decreased by 1.4-fold in Jurkat MSCVneo-IRF-4 but was increased by 1.4-fold in MT2 cell. Cyclin B1 was decreased by 2.3 X and 50 X, respectively. EB1 decreased by 2 X and 50 X. PCNA

decreased by 1.2 X and 3 X. MAPKK3 was only decreased by 6.3 X in MT2 cells. NIP3, decreased by 1.7 X and 5.6 X. p19 protein expression was only decreased in MT2 cells by 11-fold compared to Jurkat MSCVneo-wt cells. IRF-4 expression was also quantified in these cell lines. As expected, no IRF-4 protein was detected in Jurkat MSCVneo-wt cells. Jurkat MSCVneo-IRF-4 cells had a 7-fold increase in IRF-4 expression when compared to Jurkat MSCVneo-wt cells. The MT2 cells had a 16-fold increase in IRF-4 protein expression when compared to Jurkat MSCVneo-wt cells. It should be noted that the HTLV-I infected cell line, MT2, had a 2.5-fold increase in IRF-4 protein expression compared to the Jurkat MSCVneo-IRF-4 cell line.

Figure 60: Western blot analyses of IRF-4 regulated genes. Protein extracts (50 μg) were obtained from the three cell lines used in RT-PCR analyses, Jurkat MSCVneo-wt, Jurkat MSCVneo-IRF-4 and MT2. Samples were loaded on 10 or 15% SDS-PAGE, transferred to nitrocellulose and blotted with the different antibodies. Results from two separate Western Blots were quantified using the NIH program and gel plotting macros. They were also quantified relative to the amount of actin amplified. The level of actin was set as 1 for Jurkat MSCVneo-wt and for Jurkat MSCVneo-IRF-4 and 1.2 for MT2 cells depending on the intensities. The intensity of the protein bands was analyzed using the NIH program and each activity was normalized to the amount of actin in each cell line. The first three columns are percentage values of expression compared to Jurkat MSCVneo-wt, which was set at 100%. The next two columns are the fold increase (green) or decrease (red) of each gene in Jurkat MSCVneo-IRF-4 and MT2 cells when compared to Jurkat MSCVneo-wt.



	Induction relative to actin levels			Fold induction	
	Jurkat wt	IRF-4	MT2	IRF-4	MT2
HSC70	100	75	20	1.3X	5X
RhoA	100	33	6	3X	16.7X
RP-A	100	100	54	-	2X
ERBB3	100	110	105	-	-
GRB2	100	71	43	1.4X	2.3X
14-3-3η	100	107	74	-	1.4X
CXCR4	100	75	139	1.3X	1.4X
Cyclin B1	100	44	2	2.3X	50X
EB1	100	49	2	2X	50X
PCNA	100	84	34	1.2X	3X
MAPKK3	100	100	16	-	6.3X
NIP3	100	60	18	1.7X	5.6X
p19	100	106	9	-	11X
LFA-1	100	106	33	-	3X
IRF-4	100	715	1668	7X	16X
actin	1.00	1.00	1.20		

1 2 3

CHAPTER VII

DISCUSSION

IRF-4 expression in lymphoid cells and HTLV-I infected T cells.

One of the initial cloning sources of IRF-4 was from an Adult T cell Leukemia cell line (239). The specific detection of high levels of IRF-4 expression in HTLV-1 infected cells was unexpected, since IRF-4 expression in activated T cells was often difficult to detect (239). Interestingly, HTLV-1 Tax expression appears to correlate with IRF-4 expression and may be acting in an indirect manner by stimulating gene expression through an interaction with host transcription factors such as NF-κB and NF-AT which are constitutively activated in these virally infected cells (Figure 39) (63, 79, 205).

Grumont et al. as well as our laboratory have demonstrated that NF-κB, more specifically c-Rel, is involved in IRF-4 expression. In the murine promoter, two κB sites (-1763 and -686) were shown to bind c-Rel and involved in IRF-4 transcription. The human IRF-4 promoter also contains two κB sites (-510 and -438), one of which seems involved in IRF-4 expression (Figures 37 and 40). IRF-4 regulation by cRel has important implications for the immune response and cellular division. While NF-κB is an important activator of virally induced IFNβ expression, cRel appears to modulate IFN-regulated gene expression in lymphocytes by inducing IRF-4 which has been characterized as a repressor of ISRE-regulated transcription (239). IRF-4 may block IFN-induced transcription by direct competition with activators for binding to ISREs or through protein-protein interactions with such activators as IRF-1 or IRF-9/ISGF3γ (15, 239). It has also been proposed that the IRF-4 binding

partner, IRF-8/ICSBP, is also involved with IRF-4 in repression of IFN-induced transcription.

In contrast to most IRFs, the expression of IRF-4 is not regulated by type I or type II IFNs, but is instead induced by cRel in response to mitogenic stimulation. Since IRF-4 is required for lymphocyte proliferation (137) and inhibits IFN-induced transcription (239), it has been proposed that this novel mode of IRF regulation may permit lymphocyte activation under conditions normally refractory to cellular proliferation. The growth inhibitory activities of IFNs would be detrimental to lymphocyte function during an immune response. Therefore, by attenuating the effects of IFNs, IRF-4 could maximize the antiviral immune response of B and T cells in a microenvironment with high local concentrations of IFNs. This dual role of IRF-4 in regulating lymphocyte proliferation and IFN gene expression is compatible with the finding that IRF-4 is a cellular gene induced by HTLV-I infection. Since retroviral replication depends on the division of target cells, induction of IRF-4 in infected T cells would contribute in maintaining an activated cellular state, while helping HTLV-I infected T cells evade the anti-viral response by repressing IFN-regulated gene expression.

The role of IRF-4 in HTLV-1-induced leukemogenesis remains unclear. However, the relationship between IRF-4 expression and oncogenicity has been highlighted by the observation that in some patients with multiple myeloma, a chromosomal translocation - t(6;14)(p25;q32) - juxtaposes the immunoglobulin heavy-chain (IgH)

locus to MUM1 (multiple myeloma 1); the MUM1 locus at 6p25 is identical to IRF-4. This chromosomal translocation involving IRF-4 may contribute to leukemogenesis since MUM1/IRF-4 has oncogenic activity *in vitro* (84). ATL patients also show several genetic alterations (loss or gain) at chromosome 6p where the IRF-4 gene is found (219).

The finding that IRF-4 is a transcriptional target for cRel also has important implications for v-Rel-mediated transformation. Because v-Rel lacks part of the Rel transactivation domain, the transcriptional activity of this viral oncoprotein differs from that of cellular cRel (57, 58). Consequently, a v-Rel-induced change in the normal pattern of IRF-4 expression could disturb cellular growth and may help to explain the basis for lymphoid-specific transformation by v-Rel. Furthermore, chicken IRF-4 was recently cloned and shown to increase v-Rel-induced transformation in fibroblasts (Pose, unpublished data, 2000).

Although Tax was shown to be involved IRF-4 expression (239), it is not sufficient on its own to induce IRF-4 protein expression in non-HTLV-I infected T cells. A Tax-inducible Jurkat cell line, JPX9, was used to analyze IRF-4 expression. Tax expression in these cells was induced over a 3-day period by using zinc chloride. Over this same period, no IRF-4 protein expression was detected (Figure 34). Other groups have directly implicated Tax in the upregulation of IRF-4 transcription. No protein data to date has ever demonstrated an increase in IRF-4 protein by Tax expression in non-infected T cells. We believe that Tax is involved in IRF-4

expression but only in the context of HTLV-I- infected, ATL cells. Through the constitutive activation of NF-κB and NF-AT family members, as well as in the presence of Tax, these infected T cells can express IRF-4 protein.

NF-AT family members also represent interesting regulators of IRF-4 gene expression. NF-ATs are a family of enhancer binding proteins that participate in the regulation of a large number of cytokine genes. This family consists of four classical members NFAT1 (p, c2), NFAT2 (c, c1), NFAT3 (c4) and NFAT4 (x, c3). A fifth member has been recently added, NFAT5/TonEBP (98). In normal T cells, the NF-AT proteins are sequestered in the cytoplasm as hyperphosphorylated inactive precursors. Mitogenic T cell stimuli results in rapid dephosphorylation of NF-ATs by the phosphatase calcineurin. Dephosphorylation of NF-ATs leads to nuclear translocation and activation of their DNA binding activity. The activation of NF-ATs could easily lead to an upregulation of the mitogenically-induced IRF-4. This idea is supported by the use of cyclosporin A, an inhibitor of calcineurin and NF-AT signal transduction, which downregulates IRF-4 mRNA expression in Concavalin A-treated T cells (131). A personal communication with Dr. L. Glimcher (Harvard Medical School, Boston, USA) has provided insight into the regulation of IRF-4 expression. Double knockouts of NF-AT1 (p, c2) and NF-AT4 (X, c3) T and B cells have decreased IRF-4 expression. IRF-4 expression in these knockout T cells can no longer be upregulated by stimuli.

The role of NF-ATs in HTLV-I infection has not been studied as extensively as the involvement of the NF-κB family members. HTLV-I Tax has been shown to transactivate the IL-2 gene through an enhancer termed the CD28 responsive element (CD28RE). This activation by Tax is partially mediated through NFAT1. HTLV-I infected T cells were also shown to possess constitutively dephosphorylated and activated NFAT1 (63). This factor is also involved in Tax-mediated Fas ligand promoter transactivation in HTLV-I infected T cells which may serve as a mechanism of viral pathogenesis (178).

IRF-4 is also constitutively expressed in B cells, macrophages and surprisingly in lens cells. The regulation of IRF-4 expression in these cells remains to be understood. B cells possess constitutive NF-κB activation (140) and this may be involved in constitutive IRF-4 expression. Further promoter analysis in the different cell types will provide insight on this matter. In this study, we provided an analysis of the dysregulation of the IRF-4 gene in HTLV-I transformed cells and the specific cellular components involved in driving IRF-4 overexpression during the course of HTLV-I infection and transformation. Our studies have revealed several DNA binding domains within the human IRF-4 promoter that are important in transcriptional activation of the IRF-4 gene and implicate members of the NF-κB and NF-AT transcription factors in HTLV-I induced upregulation of the IRF-4 promoter.

Posttranslational regulation of IRF-4 by FKBP52.

In the present study, a novel interaction between the interferon regulatory factor IRF-4 and the immunophilin FK506 binding protein 52 (FKBP52) was characterized: 1) The proline rich region of IRF-4 (aa150-237) and the C-terminal tetratricopeptide repeats of FKBP52 were the minimal domains required for interaction. 2) Association of FKBP52 with IRF-4 inhibited IRF-4 -PU.1 binding to the IgLλ enhancer Eλ2-4, as well as IRF-4 interaction with the ISG-15 ISRE element. Furthermore, inhibition of IRF-4 DNA binding required functional peptidyl-prolyl isomerase (PPIase) activity of FKBP52, since the PPIase inhibitor ascomycin restored IRF-4 binding. 3) Transactivation by IRF-4-PU.1 and IRF-4 was also abrogated by FKBP52 in a concentration-dependent manner, an effect that also required functional PPIase activity since both ascomycin and a mutant of FKBP52 (ΔN-FKBP52) lacking PPIase activity blocked the inhibitory effect. 4) Incubation with FKBP52 led to the appearance of a faster migrating IRF-4 band as visualized by immunoblot analysis and by partial proteolysis of IRF-4. 5) Finally, incubation of the pre-B cell line 70Z/3 with ascomycin resulted in the expression of IgL λ, an IRF-4/PU.1 regulated gene in B cells. Based on these observations, we concluded that FKBP52 alters the conformation of IRF-4 by cis-trans isomerization of proline residues; such a modification appears to convert IRF-4 from a configuration capable of interaction with PU.1, DNA binding and activation of gene expression (IgL λ), to a conformation that no longer interacts with PU.1 nor binds DNA. Ascomycin relieves the inhibition catalyzed by FKBP52 by abrogating the PPIase activity of the immunophilin.

The inhibition of IRF-4-PU.1 DNA binding and transactivation potential by FKBP52 has important implications in B cell development. The IRF-4/ PU.1 complex is essential for the expression of the IgLλ and the J chain genes. Both genes are required for the expression of a productively rearranged immunoglobulin lambda gene in plasma cells (16). Therefore, FKBP52 may function as a negative regulator of immunoglobulin gene expression during B cell differentiation, or alternatively may act to prevent expression of certain genes prior to the plasma cell stage. Inhibition of the transactivating complex IRF-4-PU.1 by FKBP52 suggests a new role for immunophilins in the regulation of B cell development and maturation.

Previously, the IRF-4-PU.1 dimer was shown to stimulate the transcriptional activity of the $E\lambda_{2\text{-}4}$ λB element in a synergistic manner (15, 39). A model of IRF-4-PU.1 complex activation proposed that in the absence of PU.1, IRF-4 exists in a closed conformation unable to bind to the $E\lambda_{2\text{-}4}$ λB element. Following interaction of IRF-4 with PU.1 and DNA, IRF-4 undergoes a conformational change that swivels the regulatory domain (aa170-450) away from its own DBD (aa1-150), and into direct contact with the PEST region of PU.1. Furthermore, deletion of the C-terminal segment generated a form of IRF-4 with PU.1-independent binding activity (15, 16). Ortiz et al. further delimited the IRF-4 interaction domain to residues 245-422 (158). Site-directed mutagenesis of conserved amino acids within two predicted α-helices confirmed the importance of these residues for IRF-4-PU.1 DNA binding and transactivation. These two α-helices are also highly conserved amongst the IRF family members and may be involved in heterodimerization with other transcription

factors (158). To analyze the function of the IRF-4/PU.1 dimer *in vivo*, a chimeric repressor was engineered by fusing PU.1 and IRF-4 DNA binding domains through a flexible POU domain. This fused dimer strongly repressed expression of a rearranged immunoglobulin gene (IgLλ) (16).

Deletion analysis of IRF-4 also revealed the presence of a C-terminal segment (aa410-450) important for autoinhibition of DNA binding and ternary complex formation (15). Consistent with these observations, FKBP52 did not immunoprecipitate with full length IRF-4; only after removal of the C-terminal autoinhibition domain (aa411-450) did IRF-4 interact with FKBP52. IRF-4 appears to be modified by FKBP52 through proline isomerization to maintain a closed non-DNA binding conformation, which is relieved through its intermolecular interaction with PU.1. As described previously (15, 16, 39), IRF-4 may be inaccessible to FKBP52 because of structural constraints imposed by the C-terminal autoinhibition element.

Several physiological roles have been ascribed to immunophilins: 1) binding and sequestration of calcineurin; 2) protein folding and assembly; 3) protein trafficking; 4) direct regulation of protein activity; and 5) chaperone-like activity (96). Immunophilins are peptidyl-prolyl isomerases (PPIases) involved in catalyzing cis-trans isomerization of proline residues within proteins. Cis prolines are important to protein structural integrity by introducing bends within proteins. During protein synthesis, the peptide bonds on the amino side of proline residues are found in the

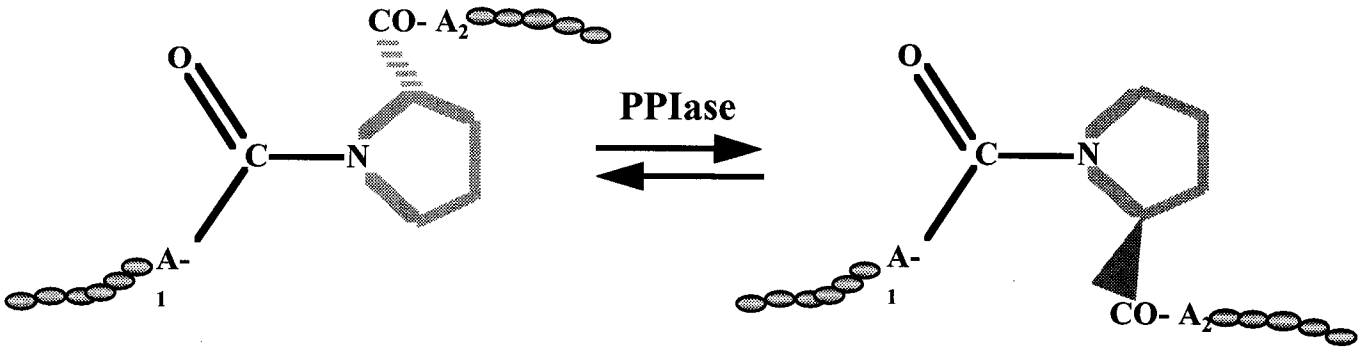
open trans-conformation (Figure 61A). When the three-dimensional structures were determined, 15% of these bonds were in the alternate cis-conformation (96, 129). Enzymes with peptidyl-prolyl isomerase activity such as FKBP52 catalyze the cis-trans interconversion, a modification that would otherwise occur slowly. Immunophilins target specific proline residues preceded by a bulky hydrophobic residue for cis-trans isomerization (83, 96, 129). Within IRF-4, several clustered prolines preceded by hydrophobic residues are available for peptidyl-prolyl isomerization by FKBP52 (Figure 61B). In the model illustrated in Figure 62, we propose that FKBP52 can act as a chaperone that escorts IRF-4 into the nucleus. During transport or once in the nucleus, FKBP52 would alter IRF-4 conformation through proline isomerization, thereby sequestering IRF-4. The interaction with the proper transcriptional partner such as PU.1 would alleviate this inhibitory effect and permit DNA binding and transactivation of target genes such as IgL λ (Figure 62).

One of the first described members of the immunophilin family - cyclophilin A - was shown to be the primary target of cyclosporin A, a clinically used immunosuppressive drug that blocks the calcineurin dependent activation of NF-AT in T cells (42, 133, 134, 146). FKBP52 (HBI, p59, HSP56) was originally discovered in association with unliganded steroid receptor complexed with heat shock proteins HSP70 and HSP90 and involved in cytoplasmic to nuclear shuttling of nuclear receptors (164, 166). Recently, another member of the immunophilin family, the human cyclophilin Cyp-40 was shown to regulate the transcriptional activity of c-Myb transcription factor (61, 62), by interfering with the capacity of c-Myb to bind

DNA. Interestingly, the oncogenic form of c-Myb, v-Myb, evaded this inhibition of DNA binding (113). Point mutations and deletions in v-Myb that contribute to its oncogenic potential also abrogated the cyclophilin-mediated inhibition of DNA binding of v-Myb (61, 120). By analogy with c-Myb and Cyp-40, IRF-4 interacts with an immunophilin that inhibits its DNA binding activity and transactivation potential in a PPIase-dependent fashion. This unexpected convergence of transcription factors and immunophilins suggests a novel post-translational role for immunophilins in the regulation of gene transcription and cell growth in lymphocytes.

Figure 61: Cis-trans isomerization and proline-rich region of IRF-4. (A) PPIase catalyzes cis-trans isomerization at specific proline residues. Peptide bonds on the amino side of proline residues are found mainly in the trans-conformation. The appearance of the cis-conformation is catalyzed by PPIase. Amino acid preceding (A-1) and following the proline residue (A2) are linked to polypeptide chains. Adapted from Kay, 1996. **(B)** Residues within the proline rich domain of IRF-4 (aa150-237) which may be subject to isomerization by FKBP52 are underlined.

A

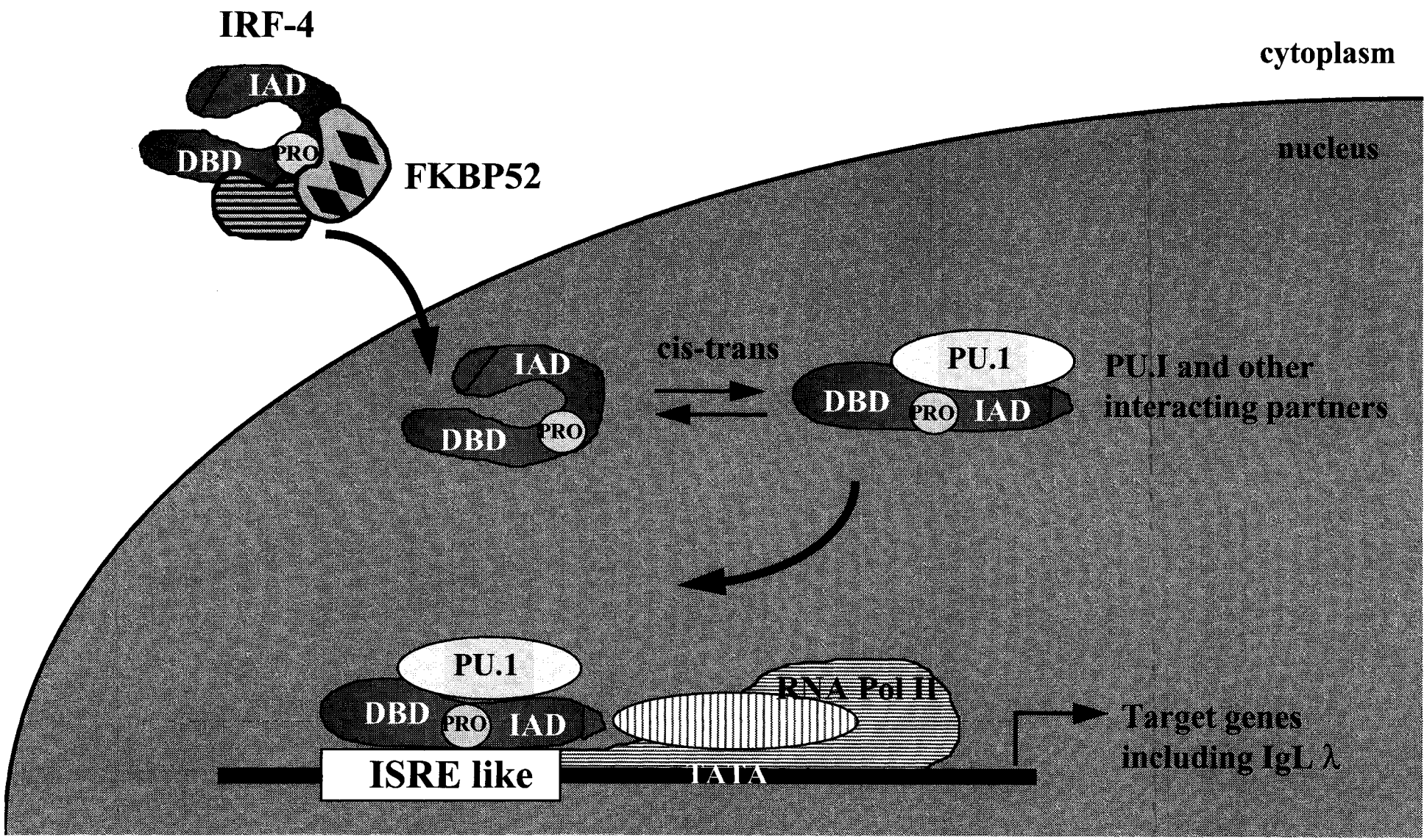


trans proline **cis proline**

B

150 PYPMTAPYGSLPAQQVHNYM
MPPHDRSWRDYAPDQSHPEIP
YQCPVTFGPRGHHWQGPSCEN
GCQVTGTFYACAPPESQAPGIP
IEPSIRS 237

Figure 62: **IRF-4/FKBP52 model**. Schematic model describing the potential functions of FKBP52 in mediating nuclear transport/chaperone function and protein folding by cis-trans isomerization, in regulating IRF-4 functions such as DNA binding and transactivation. DBD: DNA binding domain, IAD: IRF Association Domain, Pro: Proline-rich region, ♦: tetratricopeptide repeats.


IRF-4
IAD
DBD
PRO
FKBP52
cytoplasm
nucleus
IAD
DBD
PRO
cis-trans
PU.1
DBD
PRO
IAD
PU.I and other
interacting partners
PU.1
DBD
PRO
IAD
RNA Pol II
ISRE like
TATA
Target genes
including IgL λ

IRF-4 interacting partners in HTLV-I infected T cells.

The second yeast two-hybrid using IRF-4 as bait and an HTLV-I infected T cell cDNA library provided interesting potential partners for IRF-4. The cDNA of the clones were obtained from different researchers and transfected with IRF-4 full length into Cos7 cells. Coimmunoprecipitation assays using α-IRF-4 was used to confirm interaction. *In vivo*, IRF-4 was shown to interact with Rho Guanine Dissociation Inhibitor, RhoA Nucleotide Exchange Factor, Gi3, c-src, eIF4γ1, and Protein Phosphatase 2A catalytic.

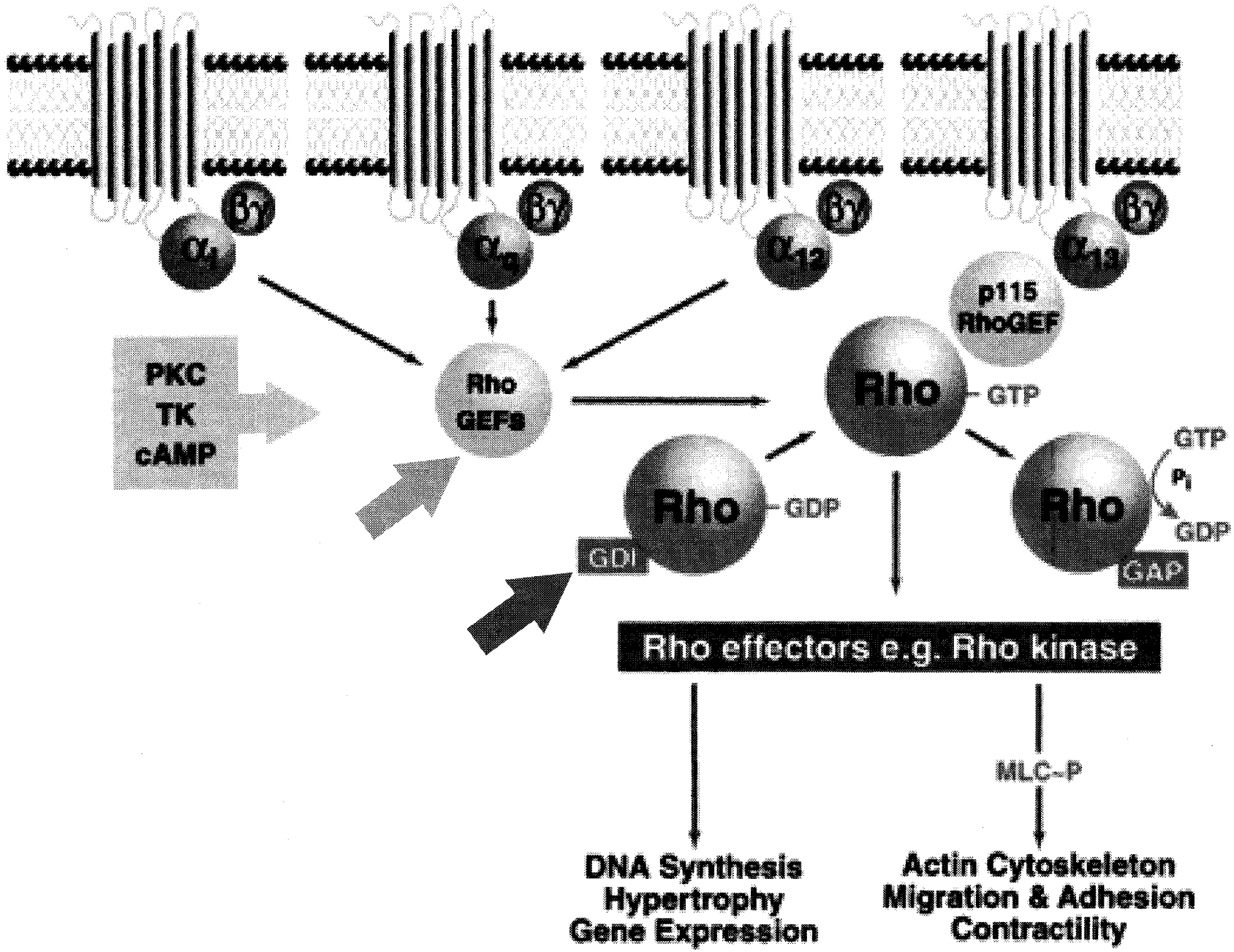
Rho Guanine Dissociation Inhibitor (RhoGDI) maintains unstimulated Rho proteins in a GDP-bound state and thereby rendering them inactive. Rho GTPases act as molecular switches between inactive GDP-bound and active GTP-bound state. They are mediators of G protein-coupled receptor signaling involved in cell adhesion, motility, transcription, cell cycle progression, cytokinesis and cell fate determination. RhoA Nucleotide Exchange Factor (RhoANEF) also controls Rho GTPase activity by stimulating the exchange of GDP for GTP. Gi3 is a member of the heterotrimeric GTP-binding proteins. It is involved in vesicle trafficking, platelet activation, opioid receptor and G protein-coupled receptor signaling pathways (194) (Figure 63). IRF-4 could potentially regulate G protein-coupled receptor signaling and Rho signaling in HTLV-I infected cells though its interaction with RhoGDI, RhoANEF and Gi3. Interestingly, IRF-4 also interacts with c-src, a non-receptor tyrosine kinase associated with the plasma membrane which is also involved in regulates G protein-coupled receptor signaling as well as growth factor, integrin adhesion receptor and

stress signaling pathways. c-src is the cellular homologue of v-src, the transforming gene in Rous Sarcoma Virus (8). c-src is not likely involved in IRF-4 phosphorylation (YM, data not shown) but IRF-4 may potentially regulate c-src activity.

eIF4γ1 is part of the eIF4 translation initiation factor responsible for recruiting the mRNA to the ribosome. eIF4γ1 is a large polypetide which acts as a scaffolding protein, bringing together other eIFs to the mRNA and the translational machinery. IRF-4 has the potential to act on eIF4γ1 and affect translation of viral and cellular proteins. IRF-4 could also interact with DAP5 which is homologous to eIF4γ1 but lacks the region for eIF4E (the cap binding protein) binding. DAP5 has been recently implicated in apoptosis and functions as a negative regulator of translation (77).

Protein Phosphatase 2A (PP2a catalytic) is a serine-threonine phosphatase involved in many cellular pathways. Phosphatases have been viewed as negative regulators of many kinase-activated signaling pathways (193). PP2A has been recently shown to act as a tumor suppressor. Mutations which prevented proper interaction between the three subunits of PP2A were found in lung and colon tumors (182). Interaction of the PP2A catalytic subunit with IRF-4 may prevent interaction with the other regulatory subunits and potentially contribute to HTLV-I induced leukemogenesis.

Figure 63: **Rho signaling pathways**. G protein coupled receptor signal through G proteins which activate Rho guanine nucleotide exchange factors (RhoGEF or RhoNEF). These factors enhance the exchange of GDP to GTP on Rho proteins. Once Rho proteins are bound to GTP, they are activated and are involved in numerous pathways such as DNA synthesis, actin cytoskeleton, migration and adhesion, gene expression and contractility. Other factors such as RhoGDI, negatively regulate Rho proteins by maintaining Rho bound to GDP. RhoGAP also regulate Rho proteins by stimulating the exchange from GTP to GDP. Adapted from Seasholtz, 1999.


βγ
βγ
βγ
βγ
PKC
TK
cAMP
Rho
GEFs
p115
RhoGEF
Rho
GTP
Rho
GDP
GDI
Rho
GTP
Pi
GDP
GAP
Rho effectors e.g. Rho kinase
MLC-P
DNA Synthesis
Hypertrophy
Gene Expression
Actin Cytoskeleton
Migration & Adhesion
Contractility

Novel IRF-4 regulated genes.

Understanding the role of IRF-4 in gene expression is only in its beginning. IRF-4 has been previously shown to be involved with PU.1 in the transcriptional regulation of B cell specific genes such as immunoglobulin κ and λ light chains and CD20, a B cell development and activation marker (15, 39, 78). The IRF-4-PU.1 transactivating complex is also involved in the expression of IL-1β in murine and human macrophages (126-128). IL-1β is a proinflammatory cytokine predominantly secreted by macrophages and monocytes induced by LPS. IRF-4 may therefore be involved immune response activation and inflammation induced by bacterial infections.

IRF-4 can also cooperate with other transcription factors such as E47 and Stat6 to modulate gene expression. IRF-4 interacts with E47, a component of the E2A transcription factor. The ubiquitously expressed E2A is crucial for normal B cell development and is composed of E12 and E47, which are two splice variants of the same gene (149). IRF-4 was shown to bind together with E47 to the immunoglobulin κ 3' light chain enhancer region and to generate a 100-fold transcriptional synergy in a reporter gene assay (149). Stat6 can also interact with IRF-4 and drive the expression of IL-4 -inducible genes such as CD23 (69). This IL-4-inducible gene, CD23, is expressed on activated B cells and macrophages. It is a low affinity receptor for IgE and regulates IgE synthesis. Stat6 is activated primarily by Jak1 and Jak3 bound to the IL-4R upon IL-4 stimulation. IL-13 is functionally similar to IL-4 and is also capable of activating Stat6. Both of these cytokines are important in proliferation, viability, gene expression and differentiation of lymphocytes (236).

IRF-4 and IRF-8/ICSBP were also shown to form specific complexes that bind to the ISG15 gene promoter in B cells and in macrophages. Both IRFs downregulated the expression of the ISG15 gene in macrophages. Synergistic downregulation was also observed in the presence of both IRF-4 and IRF-8 (180). ISG15 has been described as an IFNα/β-stimulated cytokine secreted by macrophages and lymphocytes that increases IFNγ production in these cells. IFNγ is a key regulator of inflammatory responses, and its uncontrolled activity may lead to harmful pathological changes. A possible mechanism for terminating IFNγ response is the IRF-8/ICSBP and the IRF-4-mediated downregulation of ISG15 (180).

Although much work has been done in B cells and macrophages, the role of IRF-4 in T cells more specifically in HTLV-I infected T cells, remains to be elucidated. One candidate gene that may be modulated in HTLV-1 infected cells by IRF-4 is the IL-2 gene. In IRF-4 deficient mice, a normal T- and B-cell distribution was observed at 4 to 5 weeks of age, but with time IRF-4-/- mice gradually exhibited severe lymphadenopathy and defects in both B- and T-cell activation (137). Early T-cell events such as calcium influx and expression of the T-cell activation markers CD25 and CD69 remained normal in IRF-4-/- T-cells, indicating that IRF-4 may function at later stages of T-cell activation, possibly at the level of IL-2 production and/or IL-2 response.

We therefore investigated the role of IRF-4 gene regulation in T cells. An IRF-4 stably expressing Jurkat cell line was produced using the pMSCV neo system (Clontech) and used in cDNA array analysis. Array results suggested that IRF-4 functions more as repressor than an activator in T cells. A great number of gene categories were decreased in the IRF-4 expressing Jurkat cell line: intracellular protein kinases, receptors, receptor associated proteins, G proteins, tumor suppressors, DNA polymerases, replication factors, apoptosis related proteins, transcription factors, cell surface antigens and proteins involved in turnover (Table 11). A series of genes were selected for further analysis by RT-PCR and by Western blot. Selection was based on the reproducibility of the results in the 3 separate cDNA array experiments as well as the potential role of these genes in HTLV-I induced T cell leukemogenesis.

Heat Shock Cognate 70 (HSC70) was found decreased by 3-fold in the cDNA array results. RT-PCR further confirmed that HSC70 mRNA was decreased 2.8 X in Jurkat-MSCVneo-IRF-4 as well as 2.6 X in the HTLV-I infected cell line, MT2, when compared to the Jurkat MSCVneo-wt. HSC70 protein levels were also decreased 1.3 X and 5 X in Jurkat-MSCVneo-IRF-4 and MT2 cells. HSC70 is a constitutive member of the heat shock protein family and is thought to function in multiple cellular processes such as folding of nascent chain polypeptides, the translocation of proteins across membranes, coat disassembly of clathrin-coated vesicles containing cell surface receptors bound to their ligands, and prevention of protein aggregation under stress conditions (155). Interestingly, HSC70 was also

found to be a cell fusion-enhancing factor for HTLV-I. It can promote viral fusion as well as syncytia formation of HTLV-I infected cells. HSC70 is not however a receptor for HTLV-I entry (44). The downregulation of HSC70 by IRF-4 in HTLV-I infected cells is unclear. A possible explanation suggests that by downregulating HSC70 in these infected cells, further infection by HTLV-I would be inhibited. This mechanism has been used by most viruses to prevent superinfection of the same cell. As for syncytia formation in HTLV-I infected cells, the decrease of HSC70 does not seem to perturb syncytia seen in culture (YM, data not shown). Other adhesion molecules in these cells such as ICAM-1, ICAM-3 and VCAM-1 are more likely to be involved in syncytia formation (28).

LFA-1 is another gene which was thought to be regulated by IRF-4. cDNA array data suggested that IRF-4 downregulated LFA-1 by two-fold. RT-PCR and Western blot analyses did not confirm these results. LFA-1 mRNA and protein was not decreased in Jurkat-MSCVneo-IRF-4 when compared to Jurkat-MSCVneo-wt. Moreover, LFA-1 mRNA and protein was greatly decreased in MT2 cells compared to uninfected Jurkats. LFA-1 is a member of the β2 integrin family which is expressed only on leukocytes and plays a major role in leukocyte cell-cell and cell-matrix adhesion during inflammation and other immune responses (34). Downregulation of LFA-1 by HTLV-I could be a mechanism by which the virally-infected T cells avoid contact with other immune cells to prevent harmful immune responses. A decrease in adhesion molecules could also be involved in metastasis and spreading of HTLV-I leukemic cells to other organs.

RhoA was found decreased by 2-fold in the cDNA array results. RT-PCR further confirmed that RhoA mRNA was decreased 5 X in Jurkat-MSCVneo-IRF-4 as well as 7.7 X in the HTLV-I infected cell line, MT2 when compared to the Jurkat MSCVneo-wt. RhoA protein levels were also decreased 3 X and 16.7 X in Jurkat-MSCVneo-IRF-4 and MT2 cells. RhoA is part of the small G protein family that are able to switch between the inactive GDP-bound state to the active GTP-bound state. Small G proteins are mediators of G protein-coupled receptor signaling. RhoA controls several cellular events such as cell growth, movement, proliferation and differentiation by rearranging actin and cytoskeleton proteins (194).

The cytoskeleton can no longer be viewed as simply a structural framework playing a role in cell shape and motile events such as cell movement, intracellular transport, contractile-ring formation and chromosome movement. Recent studies have revealed that the cytoskeleton plays a critical role in the regulation of various cellular processes linked to transformation including proliferation, contact inhibition, anchorage-independent cell growth and apoptosis (163). The proper functioning of RhoA is critical to the cytoskeletal framework. It is involved in actin stress fiber and focal adhesion formation which are essential for intracellular movement, cell motility, adhesion and contact-inhibited cell growth. In cellular transformation, disruption of actin filament leads to a decrease in focal adhesion, changes in microfilament structures, an increase in anchorage-independent growth and in cellular tumorigenecity (163). This suggests fundamental roles for actin filaments in

oncogenic transformation; Overexpression of RhoA produces growth arrest by disrupting actin cytoskeleton and microtubules (202). Therefore, a decrease in RhoA would be advantageous for an oncogenic virus such as HTLV-I. Indeed in ATL cells, spontaneous actin spreading and polymerization has been observed in the absence of stimulation (210). This effect may be mediated by a change in Rho protein expression.

Rho family proteins have also been implicated in the development of human cancers. First, the transforming ability of Ras and other oncoproteins is dependent on Rho family protein function. Second, constitutively active forms of RhoA, RhoB, Rac1 and Cdc42 have been shown to cause tumorigenic transformation in NIH 3T3 cells (233). The exact role of RhoA in cellular transformation is most likely cell-type specific although this remains to be clarified. In HTLV-I infected cells, RhoA levels are decreased. This decrease may be advantageous in certain cases of cancer formation.

Intracellular pathogens, such as viruses, which subvert the host machinery, have also provided great insight into the regulation of actin cytoskeleton and cell motility. Once a virus enters a cell, most often their genome requires transport to the nucleus for transcription. Viral mRNAs must then be transported out of the nucleus and back into the cytoplasm for protein translation. Once viral proteins are made, they assemble at the cell surface to form new virions. All of these events are mediated by subverting the host cytoskeleton as their own viral transport system (170). The actin

cytoskeleton in an uninfected cell is not suited to achieve a perinuclear localization. Therefore viruses need to reorganize the actin cytoskeleton in order to shuttle in and out of the nucleus (170). A decrease in RhoA would lead to an actin cytoskeleton rearrangement and potentially aid in HTLV-I virion production. Interestingly, RhoA activity inhibits HIV-I replication. This activity mediated by RhoA is distinct from its other cellular functions such as GDP/GTP binding and actin stress fiber formation activities (228). A similar mechanism of inhibiting replication may be present in HTLV-I infected cells, therefore, a decrease in RhoA would be beneficial to the virus.

RhoA as well as another small G protein, Cdc42, are involved in the formation of actin protrusions such as filipodia (thin spikes) or lamelopodia (flat sheets). These structures are critical for T cell activation by 1) changing T cell shape and movement and 2) by forming signaling components at the cell surface. Indeed, RhoA is a critical regulator of cell-mediated killing by cytotoxic T lymphocytes (106). By downregulating RhoA, HTLV-I could evade immune recognition and destruction by cytotoxic T cells.

Interestingly, the second yeast two-hybrid using IRF-4 as bait and an HTLV-I-transformed T-cell cDNA library has provided potential insight into the role of IRF-4. IRF-4 was found to interact *in vivo* with RhoANEF and RhoGDI. Both of these factors are critical regulators of Rho activity. Rho Guanine Dissociation Inhibitor (RhoGDI) keeps unstimulated Rho proteins in a GDP-bound state rendering them inactive. RhoA Nucleotide Exchange Factor (RhoANEF) also controls RhoA GTPase

by stimulating the exchange of GDP for GTP. RhoB, another member of the Rho family of small G proteins, was also decreased 2 X in the IRF-4 expressing Jurkat cells (Table 11). RhoB was recently found to mediate apoptosis in neoplastically transformed cells after DNA damage (121). IRF-4 may therefore control Rho signaling at two levels: 1) at the transcriptional level, by downregulating RhoA and RhoB mRNAs and subsequently protein levels and 2) at the posttranslational level, by modulating Rho activity through GTP exchange by interacting with RhoGDI and RhoANEF factors (Figure 63).

Replication Protein-A (RP-A, 14kDa subunit) demonstrated a 2-fold decrease in the cDNA array analysis. RT-PCR further confirmed that RP-A mRNA was decreased 8.3 X in Jurkat-MSCVneo-IRF-4 as well as 50 X in the HTLV-I infected cell line, MT2 when compared to the Jurkat MSCVneo-wt. RP-A protein levels were not decreased in Jurkat-MSCVneo-IRF-4. However, RP-A protein levels were decreased in MT2 cells compared to uninfected Jurkats. RP-A is a single-stranded DNA binding protein and a central player in DNA replication, repair and recombination. It is composed of three subunits : 70kDa, 32kDa and 14kDa (11).

Proliferating Cyclic Nuclear Antigen (PCNA) was also found to be decreased 6-fold by cDNA array analysis. RT-PCR further confirmed that PCNA mRNA was decreased 4 X in Jurkat-MSCVneo-IRF-4 as well as 2.7 X in the HTLV-I infected cell line, MT2 when compared to the Jurkat MSCVneo-wt. PCNA protein levels were also decreased 1.3 X and 3 X in Jurkat-MSCVneo-IRF-4 and MT2 cells, respectively.

PCNA was originally characterized as a DNA polymerase accessory protein and an essential component for eukaryotic chromosomal DNA replication. It is also involved in a number of other processes such as Okazaki fragment joining, DNA mismatch repair, base excision repair and chromatin reorganization (220). Moreover, RP-A stimulates PCNA-dependent base excision repair of apurinic/apyrimidinic sites in DNA (33). IRF-4 downregulation of both RP-A and PCNA could be of important consequence in HTLV-I infected T cells such as a decrease in DNA repair within these cells. The PCNA promoter also possesses 4 potential ISRE site around the -500 bp region to which IRF-4 may bind and mediate gene repression.

Cancer is a multi-step process, which involves many phenomenons, such as an increase in cellular DNA mutations. HTLV-I infected cells and ATL cells are karyotypically abnormal and are frequently found as pleiomorphic multinucleated giant cells. The expression of Tax correlates with progressive accumulation of DNA damage in these cells (138, 167). Tax was also shown to repress DNA polymerase β expression, which is also involved in DNA repair (169, 246, 247). The effects of Tax and of IRF-4 in HTLV-I infected cells may lead to an overall decrease in DNA repair, an increase in the accumulation of DNA mutations and ultimately contribute to cellular transformation.

erbB3 demonstrated a 2-fold decrease by the cDNA array analysis. RT-PCR further confirmed that ErbB3 mRNA was decreased 8.3 X in Jurkat-MSCVneo-IRF-4 as well as 5.3 X in the HTLV-I infected cell line, MT2 when compared to the Jurkat

MSCVneo-wt. erbB3 protein levels seemed to remain constant in both Jurkat-MSCVneo-IRF-4 and MT2 cells when compared to Jurkat-MSCVneo-wt. erbB3 is part of the erbB glycoprotein receptors with tyrosine kinase activity. This protein has been implicated in the development of several human cancers (112). The transcriptional downregulation of erbB3 mRNA by IRF-4 remains unclear.

GRB2 was also found decreased by 2-fold in the cDNA array analysis. RT-PCR further confirmed that GRB2 mRNA was decreased 1.5 X in Jurkat-MSCVneo-IRF-4 as well as 3 X in the HTLV-I infected cell line, MT2 when compared to the Jurkat MSCVneo-wt. GRB2 protein levels were also decreased 1.4 X and 2.3 X in Jurkat-MSCVneo-IRF-4 and MT2 cells. GRB2 is an adaptor protein found linked to many growth factor receptor such as platelet-derived growth factor and epidermal growth factor receptors. It possesses SRC Homology domain 2 (SH2) and SH3 domains which are involved in protein-protein interaction. GRB2 is also involved in signal transduction of various pathways such as the Ras signaling pathway (8). GRB2 has been implicated in the development of human cancers because it facilitates the downregulation of the cyclin-dependent inhibitor p27kip1 (204). A decrease in GRB2 in HTLV-I infected cells seems at first disadvantageous to these leukemic cells (see discussion below). GRB2 is also an important mediator of MAP kinase signaling (8).

MAP kinase kinase3 (MAPKK3 or MKK3) was increased by 2-fold in the cDNA array analysis. RT-PCR did not confirm the expected result, MAPKK3 mRNA was actually decreased 9 X in Jurkat-MSCVneo-IRF-4 as well as 16.7 X in the HTLV-I

infected cell line, MT2 when compared to the Jurkat MSCVneo-wt. MAPKK3 protein was also decreased 6.3 X in MT2 cells. MAPKs are critical regulators of cell-surface receptor signal transduction. They also respond to chemical and physical stresses, thereby controlling cell survival and adaptation (24). MAPKK3 and MAPKK6 are specifically involved in the activation of p38 proteins which have been linked to the induction of apoptosis (24). A reduction in MAPKK3 could potentially lead to a decrease in p38-induced apoptosis. HTLV-I, through the overexpression of IRF-4 may downregulate MAPKK3 and GRB2, decrease p38 activation and thereby avoid apoptosis. Interestingly, the MAPKK3 knockout mice were defective in IL-12 production (123). IL-12 is an important regulator of cell-mediated immune responses and possesses tumoricidal activity. The reduction of IL-12 in HTLV-I infected cells, may promote their survival.

14-3-3η was decreased by 3-fold in the cDNA array analysis. RT-PCR further confirmed that 14-3-3η mRNA was decreased 2.2 X in Jurkat-MSCVneo-IRF-4 as well as 2.6 X in the HTLV-I infected cell line, MT2 when compared to the Jurkat MSCVneo-wt. 14-3-3η protein levels were only decreased in MT2 cells by 1.4 X. 14-3-3 proteins play key regulatory roles in signal transduction, checkpoint control, apoptotic and nutrient-sensing pathways (47). 14-3-3η is involved in glucocorticoid receptor signaling. It interacts with the glucocorticoid receptor and stimulates its transactivation capacity (227). Its exact cellular functions are unclear.

CXCR4 was also decreased by 2-fold in the cDNA array analysis. RT-PCR further confirmed that CXCR4 mRNA was decreased 1.3 X in Jurkat-MSCVneo-IRF-4 but it was increased 2.3 X in the HTLV-I infected cell line, MT2 when compared to the Jurkat MSCVneo-wt. CXCR4 protein levels were also decreased 1.3 X in Jurkat-MSCVneo-IRF-4 but increased 1.4 X in MT2 cells. CXCR4 is the receptor for Stromal Derived Factor-1 (SDF-1) and one of the co-receptors for HIV-I entry (148). Tax has been previously shown to be involved in the CXCR4 promoter activation through Nuclear Respiratory Factor 1 (145). This upregulation of CXCR4 by Tax has potential implications for individuals co-infected with HIV-1 and HTLV-I. HTLV-I infection has been associated with the rapid progression of HIV disease; an increase in CXCR4 could be a possible mechanism for HIV-I increased disease progression in these patients. Tax also induces SDF-1 chemokine (4) which is a good candidate for explaining the infiltration of lymphoid cells into HTLV-I infected tissues and the B cell abnormalities seen in HTLV-I infected individuals.

Cyclin B1 was decreased by 7-fold in the cDNA array analysis. RT-PCR further confirmed that cyclin B1 mRNA was decreased 5.3 X in Jurkat-MSCVneo-IRF-4 and 4 X in the HTLV-I infected cell line, MT2 when compared to the Jurkat MSCVneo-wt. Cyclin B1 protein levels were also decreased 2.3 X and 50 X in Jurkat-MSCVneo-IRF-4 and MT2 cells, respectively. Cyclin B1 is involved in the G2/M (mitotic) checkpoint in the cell cycle (214). The progression through mitosis is monitored by surveillance mechanism such as the mitotic checkpoint, which ensures the proper segregation of chromosomes during cell division as well as the repair of

chromosomal abnormalities (80). The mitotic spindle checkpoint prevents the onset of anaphase and subsequent commitment to cellular division until chromosomes are aligned properly on a bipolar spindle. Disruption of this checkpoint would ultimately lead to chromosomal abnormalities such as breakage, duplication and mutations. Interestingly, a decrease in cyclin B1 by IRF-4 would abrogate the mitotic checkpoint and lead to improper chromosomal segregation. The cyclin B1 promoter does possesses two IRF sites (ISRE: -900 and -800 region) and several IRF-4 binding motifs (128) which could be involved in its downregulation. IRF-4 also seems to downregulate another gene, EB1, which is also involved in chromosome stability.

EB1 was decreased by 6-fold in the cDNA array analysis. RT-PCR further confirmed that EB1 mRNA was decreased 25 X in Jurkat-MSCVneo-IRF-4 and in the HTLV-I infected cell line, MT2 when compared to the Jurkat MSCVneo-wt. EB1 protein levels were also decreased 2 X and 50 X in Jurkat-MSCVneo-IRF-4 and MT2 cells, respectively. EB1 localizes to the mitotic spindle and cytoplasmic microtubules. It regulates microtubule stability and is important for the positioning of the mitotic spindle. EB1 is also involved in the regulation of the adenomatous polyposis coli (APC) tumour suppressor (215). Disruption of EB1 and cyclin B1 by IRF-4 could lead to the chromosomal abnormalities seen in HTLV-I infected cells and ATL cells.

The failure to maintain proper cell cycle control is recognized as a contributing factor to genetic instability and a hallmark of human and experimental cancers. Experiments in yeast have shown that the disruption of genes required for spindle checkpoint

during mitosis dramatically increased the rate of chromosome loss. Several proteins in yeast and in humans have been identified as factors required for preanaphase arrest or delay in response to microtubule depolymerization. These factors are called mitotic arrest-defective (MAD) proteins. Recent studies have shown that Tax interacts with human MAD1 (HsMAD1) protein and compromises the functions of HsMAD1 by preventing homodimerization and heterodimerization with HsMAD2, thus leading to the loss of the M checkpoint (90). ATL cells are karyotypically abnormal and are frequently present as pleiomorphic multinucleated giant cells. The expression of Tax correlates with progressive accumulation of DNA damage in cells (138, 167). The Tax-HsMAD1 interaction and the IRF-4 mediated downregulation of cyclin B1 and EB1 provides a molecular explanation for HTLV-I-induced karyotypic abnormalities and potential for transformation seen in ATL cells.

NIP3 was decreased by 3-fold in the cDNA array analysis. RT-PCR further confirmed that NIP3 mRNA was decreased 1.5 X in Jurkat-MSCVneo-IRF-4 and 16.7 X in the HTLV-I infected cell line, MT2 when compared to the Jurkat MSCVneo-wt. NIP3 protein levels were also decreased 1.7 X and 5.6 X in Jurkat-MSCVneo-IRF-4 and MT2 cells, respectively. NIP3 is a mitochondrial proapoptotic member of the Bcl-2 family of cell death factors and it is strongly induced in response to hypoxia (18). NIP3 was initially reported to physically interact with adenovirus E1B 19K and Bcl-2. Bcl-2 can suppress NIP3-induced apoptosis although resistance is rapidly overcome (25). Downregulation of pro-apoptotic genes such as

Bax and p53 have been previously documented in HTLV-I infected T cells. The decrease in NIP3 by IRF-4 could also prevent apoptosis of leukemic cells.

p19INK4D was decreased by 2-fold in the cDNA array analysis. RT-PCR only confirmed that p19 mRNA was decreased 50 X in the HTLV-I infected cell line, MT2 when compared to the Jurkat MSCVneo-wt. p19 protein levels were also decreased in MT2 cells by 11-fold. p19 is part of the INK4 cyclin dependent kinase inhibitors. The INK4 family, p15, p16, p18 and p19, specifically interact with cdk4 and cdk6 and prevent their association with D-type cyclins. The cdk4-cyclin D and cdk6-cyclin D complexes are crucial in G1 to S phase transition (93). Inhibition of these cyclin dependent kinases inhibitors are frequently seem in many human cancers. HTLV-I is also involved in cell cycle deregulation. The binding of Tax to $p16^{INK4A}$ induces a reduction in the $p16^{INK4A}$- CDK4 complex, with subsequent activation of CDK4 kinase (206). The activated CDK4 kinase phosphorylates the tumor suppressor Rb protein which in turn releases E2F and induces transcription of genes involved in cellular progression from G1 to S. By inhibiting $p16^{INK4A}$, Tax causes deregulated cell cycle progression and cellular proliferation.

Tax can also bind and sequester $p15^{INK4B}$ in a similar fashion to $p16^{INK4A}$. $p15^{INK4B}$ is also a cell cycle inhibitor of CDK4 and is important in the regulation of cell growth and transformation, especially in T lymphocytes. Tax binding to $p15^{INK4B}$ inactivated its function and made cells resistant to growth arrest induced by TGFβ (207). Inactivation of $p15^{INK4B}$ gene has been found in acute lymphoblastic

leukemia and murine primary T-cell lymphoma independently of p16 ^{INK4A} alteration. p15^{INK4B}, like p16^{INK4A} is a tumor suppressor.

In contrast to p15^{INK4B} and p16^{INK4A}, Tax was also shown to suppress the expression of p18^{INK4C} in HTLV-I-infected cells through the E-box element of the p18 promoter (207). Tax has been previously implicated in repression of gene expression through the modulation of the basic helix-loop-helix family of transcription factors which bind to E-box motifs (223). Genetic alterations in p18 ^{INK4C} have only been found in a few cases of human tumors. However, p18^{INK4C} deficient mice provided suggestive data for its role in T cells; as T and B cells of these mice exhibited a higher proliferative rate upon mitogenic stimulation, suggesting an important role of p18^{INK4C} in the regulation of lymphocytes (207).

Another cell cycle inhibitor, p19^{INK4D} expression was also decreased in HTLV-I-infected T cells when compared to uninfected T cells (207). Tax was not involved in this downregulation and the mechanism of p19^{INK4D} repression still remains unclear. It is possible that IRF-4 is involved in the downregulation of p19^{INK4D} expression in HTLV-I infected cells.

Other possible IRF-4-regulated genes from the cDNA array, which were not directly studied, have the potential to be involved in cell cycle deregulation, cellular proliferation, DNA repair and apoptosis. Further studies of the IRF-4 regulated genes will elucidate its role in HTLV-I induced leukemogenesis and potentially lead to new

avenues for treatment. IRF-4 gene regulation also needs to be studied in a cell-specific manner. Its roles in B cells, macrophages and lens cells remain to be studied.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

The present studies investigating the activation and regulation of IRF-4 have contributed to a better understanding of its function in lymphoid cells, HTLV-I-infected T cells and in HTLV-I induced leukemogenesis. The candidate's major contributions to original knowledge are listed below:

1. Analysis of the IRF-4 promoter revealed the importance of the NF-κB and NF-AT family members in regulating its expression. Both transcription factor families were shown to be constitutively activated in HTLV-I infected cells and ATL cells. IRF-4 expression was also dramatically upregulated by antigen-mimetic stimuli which makes it a good marker of T cell activation.

2. Investigation of new IRF-4 interacting partners lead to the discovery of a novel posttranslational mechanism for transcription factor regulation. The immunophilin FKBP52 through its interaction with IRF-4, decreased its DNA binding and transactivation potential. This FKBP52-mediated repression was dependent on its functional PPIase activity. Through cis-trans isomerization of proline peptide bonds, FKBP52 altered IRF-4 conformation and subsequently its activity.

3. Investigation of new IRF-4 interacting partners in an HTLV-I infected T cell context also revealed interesting IRF-4 interacting partners such as eIF4γ, c-

src, RhoGDI, RhoANEF, Gi3 and PP2A which could potentially be involved in leukemogenesis.

4. Using cDNA array technology and an IRF-4 stably expressing Jurkat cell line, novel genes regulated by IRF-4 were identified. These IRF-4 regulated genes such as RhoA, cyclin B1, EB1, PCNA, RP-A and many others are involved in cellular processes which are often deregulated in transformation such as DNA repair, cellular proliferation and apoptosis. IRF-4, through transcriptional regulation and protein-protein interactions, seems to play a significant role in HTLV-I induced leukemogenesis.

REFERENCES

1. **Agadjanyan, M. G., B. Wang, S. B. Nyland, D. B. Weiner, and K. E. Ugen.** 1998. DNA plasmid based vaccination against the oncogenic human T cell leukemia virus type 1. Curr Top Microbiol Immunol **226:**175-92.

2. **Algarte, M., H. Kwon, P. Genin, and J. Hiscott.** 1999. Identification by in vivo genomic footprinting of a transcriptional switch containing NF-kappaB and Sp1 that regulates the IkappaBalpha promoter. Mol Cell Biol **19:**6140-53.

3. **Andrada-Serpa, M. J., D. Schor, A. Q. Araujo, and V. M. Rumjanek.** 1996. Immunological features of HTLV-I myelopathy in Rio de Janeiro, Brazil, and in vitro effects of cyclosporin A. J Neurol Sci **139:**7-14.

4. **Arai, M., T. Ohashi, T. Tsukahara, T. Murakami, T. Hori, T. Uchiyama, N. Yamamoto, M. Kannagi, and M. Fujii.** 1998. Human T-cell leukemia virus type 1 Tax protein induces the expression of lymphocyte chemoattractant SDF-1/PBSF. Virology **241:**298-303.

5. **Baughman, G., G. J. Wiederrecht, N. F. Campbell, M. M. Martin, and S. Bourgeois.** 1995. FKBP51, a novel T-cell-specific immunophilin capable of calcineurin inhibition. Mol Cell Biol **15:**4395-402.

6. **Baughman, G., G. J. Wiederrecht, F. Chang, M. M. Martin, and S. Bourgeois.** 1997. Tissue distribution and abundance of human FKBP51, and FK506-binding protein that can mediate calcineurin inhibition. Biochem Biophys Res Commun **232:**437-43.

7. **Bazarbachi, A., and O. Hermine.** 1996. Treatment with a combination of zidovudine and alpha-interferon in naive and pretreated adult T-cell

leukemia/lymphoma patients. J Acquir Immune Defic Syndr Hum Retrovirol **13:**186-90.

8. **Belsches, A. P., M. D. Haskell, and S. J. Parsons.** 1997. Role of c-Src tyrosine kinase in EGF-induced mitogenesis. Front Biosci **2:**d501-18.

9. **Bex, F., and R. B. Gaynor.** 1998. Regulation of gene expression by HTLV-I Tax protein. Methods **16:**83-94.

10. **Bluyssen, H. A. R., J. E. Durbin, and D. E. Levy.** 1996. ISGF3g p48, a specificity switch for interferon activated transcription factors. Cytokine and Growth Factor Reviews **7:**11-17.

11. **Bochkareva, E., S. Korolev, and A. Bochkarev.** 2000. The role for zinc in replication protein A. J Biol Chem **275:**27332-8.

12. **Borel, J. F.** 1976. Comparative study of in vitro and in vivo drug effects on cell-mediated cytotoxicity. Immunology **31:**631-41.

13. **Boshoff, C., and Y. Chang.** 2001. Kaposi's sarcoma-associated herpesvirus: a new DNA tumor virus. Annu Rev Med **52:**453-70.

14. **Braganca, J., and A. Civas.** 1998. Type I interferon gene expression: Differential expression of IFN-A genes induced by viruses and double-stranded hRNA. Biochimie **80:**673-687.

15. **Brass, A., E. Kehrli, C. Eisenbeis, U. Storb, and H. Singh.** 1996. Pip, a lymphoid restricted IRF, contains a regulatory domain that is important for autoinhibition and ternary complex formation with the Ets factor PU.1. Genes & Development **10:**2335-2347.

16. **Brass, A. L., A. Q. Zhu, and H. Singh.** 1999. Assembly requirements of PU.1/Pip (IRF-4) activator complexes - Inhibiting function in vivo using fused dimers. EMBO Journal **18:**977-991.

17. **Brown, T. R., P. H. Scott, T. Stein, A. G. Winter, and R. J. White.** 2000. RNA polymerase III transcription: its control by tumor suppressors and its deregulation by transforming agents. Gene Expr **9:**15-28.

18. **Bruick, R. K.** 2000. Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia. Proc Natl Acad Sci U S A **97:**9082-7.

19. **Campbell, M., G. Chan, and T. Yen.** 2001. Mitotic checkpoint proteins HsMAD1 and HsMAD2 are associated with nuclear pore complexes in interphase. J Cell Sci **114:**953-63.

20. **Cann, A., and I. Chen.** 1996. Human T cell Leukemia Virus Types I and II., p. 1849. *In* B. Fields (ed.), Fields Virology, 3rd ed. Lippincott-Raven, Philadelphia.

21. **Carbone, A., A. Gloghini, M. R. Cozzi, D. Capello, A. Steffan, P. Monini, L. De Marco, and G. Gaidano.** 2000. Expression of MUM1/IRF4 selectively clusters with primary effusion lymphoma among lymphomatous effusions: implications for disease histogenesis and pathogenesis. Br J Haematol **111:**247-57.

22. **Carbone, A., A. Gloghini, L. M. Larocca, D. Capello, F. Pierconti, V. Canzonieri, U. Tirelli, R. Dalla-Favera, and G. Gaidano.** 2001. Expression profile of MUM1/IRF4, BCL-6, and CD138/syndecan-1 defines novel histogenetic subsets of human immunodeficiency virus-related lymphomas. Blood **97:**744-51.

23. **Casseb, J., and A. C. Penalva-de-Oliveira.** 2000. The pathogenesis of tropical spastic paraparesis/human T-cell leukemia type I-associated myelopathy. Braz J Med Biol Res **33:**1395-401.

24. **Chang, L., and M. Karin.** 2001. Mammalian MAP kinase signalling cascades. Nature **410:**37-40.

25. **Chen, G., J. Cizeau, C. Vande Velde, J. H. Park, G. Bozek, J. Bolton, L. Shi, D. Dubik, and A. Greenberg.** 1999. Nix and Nip3 form a subfamily of pro-apoptotic mitochondrial proteins. J Biol Chem **274:**7-10.

26. **Chu, Z. L., Y. A. Shin, J. M. Yang, J. A. DiDonato, and D. W. Ballard.** 1999. IKKg mediates the interaction of cellular IkB kinases with the tax transforming protein of HTLV-1. Journal of Biological Chemistry **274:**15297-15300.

27. **Czar, M. J., J. K. Oewns-Grillo, A. W. Yem, K. L. Leach, M. R. Deibel, M. J. Weish, and W. B. Pratt.** 1994. The hsp56 immunophilin component of unstranformed steroid receptor complexes is localized both to microtubules in the cytoplasm and the same nonrandom regions within the nucleus as the steroids receptor. Molecular Endocrinology **8:**1731-1741.

28. **Daenke, S., S. A. McCracken, and S. Booth.** 1999. Human T-cell leukaemia/lymphoma virus type 1 syncytium formation is regulated in a cell-specific manner by ICAM-1, ICAM-3 and VCAM-1 and can be inhibited by antibodies to integrin beta2 or beta7. J Gen Virol **80:**1429-36.

29. **Daly, C., and N. C. Reich.** 1993. Double-stranded RNA activates novel factors that bind to the interferon stimulated response element. Mol.Cell.Biol. **13:**3756-3764.

30. **Darnell Jr., J. E., I. M. Kerr, and G. R. Stark.** 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science **264:**1415-1421.

31. **DeKoter, R. P., and H. Singh.** 2000. Regulation of B lymphocyte and macrophage development by graded expression of PU.1. Science **288:**1439-41.

32. **Dent, A. L., J. Hu-Li, W. E. Paul, and L. M. Staudt.** 1998. T helper type 2 inflammatory disease in the absence of interleukin 4 and transcription factor STAT6. Proc Natl Acad Sci U S A **95:**13823-8.

33. **Dianov, G. L., B. R. Jensen, M. K. Kenny, and V. A. Bohr.** 1999. Replication protein A stimulates proliferating cell nuclear antigen- dependent repair of abasic sites in DNA by human cell extracts. Biochemistry **38:**11021-5.

34. **Dib, K.** 2000. BETA 2 integrin signaling in leukocytes. Front Biosci **5:**D438-51.

35. **Doucas, V., and R. M. Evans.** 1999. Human T-cell leukemia retrovirus-Tax protein is a repressor of nuclear receptor signaling. Proc Natl Acad Sci U S A **96:**2633-8.

36. **Driggers, P. H., D. L. Ennist, S. L. Gleason, W.-H. Mak, M. S. Marks, B.-Z. Levi, J. R. Flanagan, E. Appella, and K. Ozato.** 1990. An interferon g-regulated protein that binds the interferon-inducible enhancer element of major histocompatibility complex class I genes. Proceedings of the National Academy of Sciences USA **87:**3743-3747.

37. **Edlich, R. F., J. A. Arnette, and F. M. Williams.** 2000. Global epidemic of human T-cell lymphotropic virus type-I (HTLV-I). J Emerg Med **18:**109-19.

38. **Eisenbeis, C., H. Singh, and U. Storb.** 1993. PU.1 is a component of a multiprotein complex which binds an essential site in the murine immunoglobulin l2-4 enhancer. Molecular and Cellular Biology **13:**6452-6461.

39. **Eisenbeis, C. F., H. Singh, and U. Storb.** 1995. Pip, a novel IRF family member, is a lymphoid-specific, PU.1-dependent transcriptional activator. Genes & Development **9:**1377-1387.

40. **Eklund, E. A., A. Javala, and R. Kakar.** 1998. PU.1, IRF-1, and ICSBP cooperate to increase gp91phox expression. Journal of Biological Chemistry **273:**13957-13965.

41. **El-Sabban, M. E., R. Nasr, G. Dbaibo, O. Hermine, N. Abboushi, F. Quignon, J. C. Ameisen, F. Bex, H. de The, and A. Bazarbachi.** 2000. Arsenic-interferon-alpha-triggered apoptosis in HTLV-I transformed cells is associated with tax down-regulation and reversal of NF-kappa B activation. Blood **96:**2849-55.

42. **Emmel, E. A., C. L. Verweij, D. B. Durand, K. M. Higgins, E. Lacy, and G. R. Crabtree.** 1989. Cyclosporin A specifically inhibits function of nuclear proteins involved in T cell activation. Science **246:**1617-1620.

43. **Falini, B., M. Fizzotti, A. Pucciarini, B. Bigerna, T. Marafioti, M. Gambacorta, R. Pacini, C. Alunni, L. Natali-Tanci, B. Ugolini, C. Sebastiani, G. Cattoretti, S. Pileri, R. Dalla-Favera, and H. Stein.** 2000. A monoclonal antibody (MUM1p) detects expression of the MUM1/IRF4 protein in a subset of germinal center B cells, plasma cells, and activated T cells. Blood **95:**2084-92.

44. **Fang, D., Y. Haraguchi, A. Jinno, Y. Soda, N. Shimizu, and H. Hoshino.** 1999. Heat shock cognate protein 70 is a cell fusion-enhancing factor but not an entry factor for human T-cell lymphotropic virus type I. Biochem Biophys Res Commun **261:**357-63.

45. **Fisher, G., H. Bang, and C. Mech.** 1984. Determination of enzymatic catalysis for cis-trans-isomerization of peptide binding in proline-containing peptides. Biomedica Biochimia Acta **43:**1101-1111.

46. **Franchini, G., I. J. Mulloy, A. Koralnik, N. Nonico, J. J. Sparkowski, T. Andresson, D. J. Goldstein, and R. Schlegel.** 1993. The human T-cell leukemia/lymphotropic virus type I p12 protein cooperates with E5 oncoprotein of bovine papillomavirus in cell transformation and binds the 16 kilodalton subunit of the vacuolar H+ ATPase. Journal of Virology **67:**7701-7704.

47. **Fu, H., R. R. Subramanian, and S. C. Masters.** 2000. 14-3-3 proteins: structure, function, and regulation. Annu Rev Pharmacol Toxicol **40:**617-47.

48. **Fujita, T., Y. Kimura, M. Miyamoto, E. L. Barsoumian, and T. Taniguchi.** 1989. Induction of endogenous IFN genes by a regulatory transcription factor IRF-1. Nature **337:**270-272.

49. **Fujita, T., J. Sakakibara, Y. Sudo, M. Miyamoto, Y. Kimura, and T. Taniguchi.** 1988. Evidence for a nuclear factor(s), IRF-1, mediating induction and silencing properties to human IFN- gene regulatory elements. EMBO Journal **7:**3397-3405.

50. **Gaidano, G., and A. Carbone.** 2000. MUM1: a step ahead toward the understanding of lymphoma histogenesis. Leukemia **14:**563-6.

51. **Garrity, P. A., and B. Wold.** 1992. Effects of different DNA polymerases in ligation-mediated PCR: enhanced genomic sequencing and in vivo footprinting. Proceedings of the National Academy of Sciences USA **89:**1021-1025.

52. **Geleziunas, R., S. Ferrell, X. Lin, Y. Mu, E. T. Cunningham, Jr., M. Grant, M. A. Connelly, J. E. Hambor, K. B. Marcu, and W. C. Greene.** 1998. Human T-cell leukemia virus type 1 Tax induction of NF-kappaB involves activation of the IkappaB kinase alpha (IKKalpha) and IKKbeta cellular kinases. Mol Cell Biol **18:**5157-65.

53. **Genin, P., J. Braganca, N. Darracq, J. Doly, and A. Civas.** 1995. A novel PRD I and TG binding activity involved in virus-induced transcription of IFN-A genes. Nucleic Acids Res. **23:**5055-5063.

54. **Gessain, A., and R. Mahieux.** 2000. [A virus called HTLV-1. Epidemiological aspects]. Presse Med **29:**2233-9.

55. **Gessain, A., and R. Mahieux.** 2000. [A virus called HTLV-1. Virological and molecular aspects]. Presse Med **29:**2228-32.

56. **Gill, P., W. Harrington, M. Kaplan, and e. al.** 1995. Treatment of adult T-cell leukemin-lymphoma with a combination of interferon alpha and zidovudine. New England Journal of Medicine **332:**1744-1748.

57. **Gilmore, T. D.** 1991. Malignant transformation by mutant Rel proteins. Trends in Genetics **7:**318-322.

58. **Gilmore, T. D.** 1996. Role of rel family genes in normal and malignant lymphoid cell growth. Cancer Surveys **15:**69-87.

59. **Glimcher, L. H., and H. Singh.** 1999. Transcription factors in lymphocyte development--T and B cells get together. Cell **96:**13-23.

60. **Glynne, R., S. Akkaraju, J. I. Healy, J. Rayner, C. C. Goodnow, and D. H. Mack.** 2000. How self-tolerance and the immunosuppressive drug FK506 prevent B-cell mitogenesis. Nature **403:**672-6.

61. **Gonda, T.** 1998. The c-Myb oncoprotein. International Journal of Biochemistry and Cell Biology **30:**517-551.

62. **Gonda, T. J., R. G. Ramsay, and G. R. Johnson.** 1989. Murine myeloid cell lines derived by in vitro infection with recombinant c-myb retroviruses express myb rearranged vector proviruses. EMBO Journal **8:**1767-1775.

63. **Good, L., S. B. Maggirwar, E. W. Harhaj, and S. C. Sun.** 1997. Constitutive dephosphorylation and activation of a member of the nuclear factor of activated T cells, NF-AT1, in Tax-expressing and type I human T-cell leukemia virus-infected human T cells. J Biol Chem **272:**1425-8.

64. **Goren, I., E. Tavor, and A. Honigman.** 1999. Gene regulation mediated by interaction between HTLV-1 promoter elements and transcription factors Tax and CREB. Virology **256:**303-12.

65. **Gross, P., A. A. Yee, C. H. Arrowsmith, and R. B. Macgregor, Jr.** 1998. Quantitative hydroxyl radical footprinting reveals cooperative interactions between DNA-binding subdomains of PU.1 and IRF4. Biochemistry **37:**9802-11.

66. **Grumont, R. J., and S. Gerondakis.** 2000. Rel induces interferon regulatory factor 4 (IRF-4) expression in lymphocytes: modulation of interferon-regulated gene expression by rel/nuclear factor kappaB. J Exp Med **191:**1281-92.

67. **Grumont, R. J., I. J. Rourke, and S. Gerondakis.** 1999. Rel-dependent induction of A1 transcription is required to protect B cells from antigen receptor ligation-induced apoptosis. Genes & Development **13:**400-411.

68. **Gupta, S., A. Anthony, and A. B. Pernis.** 2001. Stage-specific modulation of Interferon-regulatory factor 4 function by kruppel-type zinc finger proteins. J Immunol **166:**6104-11.

69. **Gupta, S., M. Jiang, A. Anthony, and A. B. Pernis.** 1999. Lineage-specific modulation of interleukin 4 signaling by interferon regulatory factor 4. J Exp Med **190:**1837-48.

70. **Hakata, Y., T. Umemoto, S. Matsushita, and H. Shida.** 1998. Involvement of human CRM1 (exportin 1) in the export and multimerization of the Rex protein of human T-cell leukemia virus type 1. J Virol **72:**6602-7.

71. **Harada, H., T. Fujita, M. Miyamoto, Y. Kimura, M. Maruyama, A. Furia, T. Miyata, and T. Taniguchi.** 1989. Structurally similar but functionally distinct factors, IRF-1 and IRF-2, bind to the same regulatory elements of IFN and IFN-inducible genes. Cell **58:**729-739.

72. **Harada, H., M. Matsumoto, M. Sato, Y. Kashiwazaki, T. Kimura, M. Kitagawa, T. Yokochi, R. S. P. Tan, T. Takasugi, Y. Kadokawa, C. Schindler, R. D. Schreiber, S. Noguchi, and T. Taniguchi.** 1996. Regulation of IFN genes: evidence for a dual function of the transcription factor complex ISGF3 in the production and action of IFN·. Genes to Cells **1:**995-1005.

73. **Harada, H., K. Willison, J. Sakakibara, M. Miyamoto, T. Fujita, and T. Taniguchi.** 1990. Absence of type I IFN system in EC cells: transcriptional activator (IRF-1) and repressor (IRF-2) genes are developmentally regulated. Cell **63:**903-913.

74. **Harhaj, E. W., L. Good, G. Xiao, M. Uhlik, M. E. Cvijic, I. Rivera-Walsh, and S. C. Sun.** 2000. Somatic mutagenesis studies of NF-kappa B signaling in human T cells: evidence for an essential role of IKK gamma in NF-kappa B activation by T-cell costimulatory signals and HTLV-I Tax protein. Oncogene **19:**1448-56.

75. **Harhaj, E. W., and S. C. Sun.** 1999. IKKgamma serves as a docking subunit of the IkappaB kinase (IKK) and mediates interaction of IKK with the human T-cell leukemia virus Tax protein. J Biol Chem **274:**22911-4.

76. **Hatanaka, H., T. Kino, S. Miyata, N. Inamura, A. Kuroda, T. Goto, H. Tanaka, and M. Okuhara.** 1988. FR-900520 and FR-900523, novel immunosuppressants isolated from a Streptomyces. II. Fermentation, isolation and physico-chemical and biological characteristics. J Antibiot (Tokyo) **41:**1592-601.

77. **Henis-Korenblit, S., N. L. Strumpf, D. Goldstaub, and A. Kimchi.** 2000. A novel form of DAP5 protein accumulates in apoptotic cells as a result of caspase cleavage and internal ribosome entry site-mediated translation. Mol Cell Biol **20:**496-506.

78. **Himmelmann, A., A. Riva, G. L. Wilson, B. P. Lucas, C. Thevenin, and J. H. Kehrl.** 1997. PU.1/Pip and basic helix loop helix zipper transcription factors interact with binding sites in the CD20 promoter to help confer lineage- and stage-specific expression of CD20 in B lymphocytes. Blood **90:**3984-95.

79. **Hiscott, J., L. Petropoulos, and J. Lacoste.** 1995. Molecular interactions between HTLV-1 Tax protein and the NF-kB/IkB transcription complex. Virology **214:**3-11.

80. **Hixon, M. L., and A. Gualberto.** 2000. The control of mitosis. Front Biosci **5:**D50-7.

81. **Holtschke, T., J. Lîhler, Y. Kanno, T. Fehr, N. Giese, F. Rosenbauer, J. Lou, K.-P. Knobeloch, L. Gabriele, J. F. Waring, M. F. Bachmann, R. M. Zingernagel, H. C. Morse III, K. Ozato, and I. Horak.** 1996. Immunodeficiency and chronic myelogenous leukemia-like syndrome in mice with a targeted mutation of the ICSBP gene. Cell **87:**307-317.

82. **Hou, X., S. Foley, M. Cueto, and M. A. Robinson.** 2000. The human T-cell leukemia virus type I (HTLV-I) X region encoded protein p13(II) interacts with cellular proteins. Virology **277:**127-35.

83. **Hunter, T.** 1998. Prolyl isomerases and nuclear function. Cell **92:**141-143.

84. **Iida, S., P. H. Rao, M. Butler, P. Corradini, M. Boccadoro, B. Klein, R. S. K. Chaganti, and R. Dalla-Favera.** 1997. Deregulation of MUM1/IRF-4 by chromosomal translocation in multiple myeloma. Nature Genetics **17:**226-230.

85. **Ivery, M. T.** 2000. Immunophilins: switched on protein binding domains? Med Res Rev **20:**452-84.

86. **Iwai, K., N. Mori, M. Oie, N. Yamamoto, and M. Fujii.** 2001. Human T-cell leukemia virus type 1 tax protein activates transcription through AP-1 site by inducing DNA binding activity in T cells. Virology **279:**38-46.

87. **Janowski, B., S. Wollner, M. Schutkowski, and G. Fischer.** 1997. A protease-free assay for peptidyl prolyl cis/trans isomerases using standard peptide substrates. Anal Biochem **252:**299-307.

88. **Jeffery, K. J., K. Usuku, S. E. Hall, W. Matsumoto, G. P. Taylor, J. Procter, M. Bunce, G. S. Ogg, K. I. Welsh, J. N. Weber, A. L. Lloyd, M. A. Nowak, M. Nagai, D. Kodama, S. Izumo, M. Osame, and C. R. Bangham.** 1999. HLA alleles determine human T-lymphotropic virus-I (HTLV-I) proviral load and the risk of HTLV-I-associated myelopathy. Proc Natl Acad Sci U S A **96:**3848-53.

89. **Jiang, H., H. Lu, R. L. Schiltz, C. A. Pise-Masison, V. V. Ogryzko, Y. Nakatani, and J. N. Brady.** 1999. PCAF interacts with tax and stimulates tax

transactivation in a histone acetyltransferase-independent manner. Mol Cell Biol **19:**8136-45.

90. **Jin, D.-Y., F. Spencer, and K.-T. Jeang.** 1998. Human T-cell Leukemia Virus type 1 oncoprotein Tax targets the human mitotic checkpoint protein MAD1s. Cell **93:**81-91.

91. **Jin, D. Y., V. Giordano, K. V. Kibler, H. Nakano, and K. T. Jeang.** 1999. Role of adaptor function in oncoprotein-mediated activation of NF-kB. HTLV-1 Tax interacts directly with IKKg. Journal of Biological Chemistry **274:**17402-17405.

92. **Jing, Y., L. Wang, L. Xia, G. Q. Chen, Z. Chen, W. H. Miller, and S. Waxman.** 2001. Combined effect of all-trans retinoic acid and arsenic trioxide in acute promyelocytic leukemia cells in vitro and in vivo. Blood **97:**264-9.

93. **Johnson, D. G., and C. L. Walker.** 1999. Cyclins and cell cycle checkpoints. Annu Rev Pharmacol Toxicol **39:**295-312.

94. **Karin, M.** 1999. The beginning of the end: IkappaB kinase (IKK) and NF-kappaB activation. J Biol Chem **274:**27339-42.

95. **Karin, M.** 1999. How NF-kappaB is activated: the role of the IkappaB kinase (IKK) complex. Oncogene **18:**6867-6874.

96. **Kay, J.** 1996. Structure-function relationships in the FK506-binding protein (FKBP) family of the peptidylprolyl cis-trans isomerases. Biochem.J. **314:**361-385.

97. **Keller, A. D., and T. Maniatis.** 1991. Identification and characterization of a novel repressor of beta- interferon gene expression. Genes Dev **5:**868-79.

98. **Kiani, A., A. Rao, and J. Aramburu.** 2000. Manipulating immune responses with immunosuppressive agents that target NFAT. Immunity **12:**359-72.

99. **Kim, Y. M., H. S. Kang, S. G. Paik, K. H. Pyun, K. L. Anderson, B. E. Torbett, and I. Choi.** 1999. Roles of IFN consensus sequence binding protein and PU.1 in regulating IL-18 gene expression. J Immunol **163:**2000-7.

100. **Kino, T., H. Hatanaka, M. Hashimoto, M. Nishiyama, T. Goto, M. Okuhara, M. Kohsaka, H. Aoki, and H. Imanaka.** 1987. FK-506, a novel immunosuppressant isolated from a Streptomyces. I. Fermentation, isolation, and physico-chemical and biological characteristics. J Antibiot (Tokyo) **40:**1249-55.

101. **Kino, T., H. Hatanaka, S. Miyata, N. Inamura, M. Nishiyama, T. Yajima, T. Goto, M. Okuhara, M. Kohsaka, H. Aoki, and et al.** 1987. FK-506, a novel immunosuppressant isolated from a Streptomyces. II. Immunosuppressive effect of FK-506 in vitro. J Antibiot (Tokyo) **40:**1256-65.

102. **Koromilas, A. E., C. Cantin, A. W. Craig, R. Jagus, J. Hiscott, and N. Sonenberg.** 1995. The interferon-inducible protein kinase PKR modulates the transcriptional activation of immunoglobulin kappa gene. J Biol Chem **270:**25426-34.

103. **Kusuhara, K., M. Anderson, S. M. Pettiford, and P. L. Green.** 1999. Human T-cell leukemia virus type 2 Rex protein increases stability and promotes nuclear to cytoplasmic transport of gag/pol and env RNAs. J Virol **73:**8112-9.

104. **Kwok, R. P. S., M. E. Laurance, R. J. Lundblad, P. S. Goldman, H.-M. Shih, L. M. Connor, S. J. Marriott, and R. H. Goodman.** 1996. Control of cAMP-regulated enhancers by the viral transactivator Tax through CREB and the co-activator CBP. Nature **380:**642-646.

105. **La Grenade, L.** 1996. HTLV-I-associated infective dermatitis: past, present, and future. J Acquir Immune Defic Syndr Hum Retrovirol **13:**S46-9.

106. **Lang, P., L. Guizani, I. Vitte-Mony, R. Stancou, O. Dorseuil, G. Gacon, and J. Bertoglio.** 1992. ADP-ribosylation of the ras-related, GTP-binding protein RhoA inhibits lymphocyte-mediated cytotoxicity. J Biol Chem **267:**11677-80.

107. **Lau, J. F., J. P. Parisien, and C. M. Horvath.** 2000. Interferon regulatory factor subcellular localization is determined by a bipartite nuclear localization signal in the DNA-binding domain and interaction with cytoplasmic retention factors. Proc Natl Acad Sci U S A **97:**7278-83.

108. **Lawson, V. A., J. Y. Lee, J. C. Doultree, J. A. Marshall, and D. A. McPhee.** 2000. Visualisation of phenotypically mixed HIV-1 and HTLV-I virus particles by electron microscopy. J Biomed Sci **7:**71-4.

109. **Lebeau, M. C., N. Massol, J. Herrick, L. E. Faber, J. M. Renoir, C. Radanyi, and E. E. Baulieu.** 1992. P59, an hsp 90-binding protein. Cloning and sequencing of its cDNA and preparation of a peptide-directed polyclonal antibody. J Biol Chem **267:**4281-4.

110. **Lenzmeier, B. A., E. E. Baird, P. B. Dervan, and J. K. Nyborg.** 1999. The tax protein-DNA interaction is essential for HTLV-I transactivation in vitro. J Mol Biol **291:**731-44.

111. **Lenzmeier, B. A., H. A. Giebler, and J. K. Nyborg.** 1998. Human T-cell leukemia virus type 1 Tax requires direct access to DNA for recruitment of CREB binding protein to the viral promoter. Mol Cell Biol **18:**721-31.

112. **Lessor, T. J., J. Y. Yoo, X. Xia, N. Woodford, and A. W. Hamburger.** 2000. Ectopic expression of the ErbB-3 binding protein ebp1 inhibits growth and induces differentiation of human breast cancer cell lines. J Cell Physiol **183:**321-9.

113. **Leverson, J. D., and S. A. Ness.** 1998. Point mutations in v-Myb disrupt a cyclophilin-catalyzed negative regulatory mechanism. Molecular Cell **1:**203-211.

114. **Levy, D. E.** 1995. Interferon induction of gene expression through the Jak-Stat pathway. Seminars in Virology **6:**181-190.

115. **Li, H. C., T. Fujiyoshi, H. Lou, S. Yashiki, S. Sonoda, L. Cartier, L. Nunez, I. Munoz, S. Horai, and K. Tajima.** 1999. The presence of ancient human T-cell lymphotropic virus type I provirus DNA in an Andean mummy. Nat Med **5:**1428-32.

116. **Li, W., C. N. Nagineni, B. Efiok, A. B. Chepelinsky, and C. E. Egwuagu.** 1999. Interferon regulatory transcription factors are constitutively expressed and spatially regulated in the mouse lens. Dev Biol **210:**44-55.

117. **Lin, R., C. Heylbroeck, P. M. Pitha, and J. Hiscott.** 1998. Virus dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation, transactivation potential and proteasome mediated degradation. Molecular and Cellular Biology **18:**2986-2996.

118. **Lin, R., Y. Mamane, and J. Hiscott.** 1999. Structural and functional analysis of interferon regulatory factor 3: localization of the transactivation and autoinhibitory domains. Molecular and Cellular Biology **19:**2465-2474.

119. **Lin, T. S., S. Mahajan, and D. A. Frank.** 2000. STAT signaling in the pathogenesis and treatment of leukemias. Oncogene **19:**2496-504.

120. **Lipsick, J. S.** 1996. One billion years of Myb. Oncogene **13:**223-235.

121. **Liu, A., G. J. Cerniglia, E. J. Bernhard, and G. C. Prendergast.** 2001. RhoB is required to mediate apoptosis in neoplastically transformed cells after DNA damage. Proc Natl Acad Sci U S A **98:**6192-7.

122. **Low, K. G., L. F. Dorner, D. B. Fernando, J. Grossman, K.-T. Jeang, and M. J. Comb.** 1997. Human T-cell leukemia virus type 1 Tax releases cell cycle arrest induced by p16INK4a. Journal of Virology **71:**1956-1962.

123. **Lu, H. T., D. D. Yang, M. Wysk, E. Gatti, I. Mellman, R. J. Davis, and R. A. Flavell.** 1999. Defective IL-12 production in mitogen-activated protein (MAP) kinase kinase 3 (Mkk3)-deficient mice. Embo J **18:**1845-57.

124. **Majone, F., O. J. Semmes, and K. T. Jeang.** 1993. Induction of micronuclei by HTLV-I Tax: a cellular assay for function. Virology **193:**456-9.

125. **Mamane, Y., C. Heylbroeck, P. Genin, M. Algarte, M. J. Servant, C. LePage, C. DeLuca, H. Kwon, R. Lin, and J. Hiscott.** 1999. Interferon regulatory factors: the next generation. Gene **237:**1-14.

126. **Marecki, S., M. L. Atchison, and M. J. Fenton.** 1999. Differential expression and distinct functions of IFN regulatory factor 4 and IFN consensus sequence binding protein in macrophages. J Immunol **163:**2713-22.

127. **Marecki, S., and M. J. Fenton.** 2000. PU.1/Interferon Regulatory Factor interactions: mechanisms of transcriptional regulation. Cell Biochem Biophys **33:**127-48.

128. **Marecki, S., C. J. Riendeau, M. D. Liang, and M. J. Fenton.** 2001. PU.1 and multiple ifn regulatory factor proteins synergize to mediate transcriptional activation of the human IL-1beta gene. J Immunol **166:**6829-38.

129. **Marks, A.** 1996. Cellular functions of immunophilins. Physiological Reviews **76:**631-649.

130. **Matikainen, S., T. Ronni, A. Lehtonen, T. Sareneva, K. Melen, S. Nordling, D. E. Levy, and I. Julkunen.** 1997. Retinoic acid induces signal transducer and activator of transcription (STAT) 1, STAT2, and p48 expression in myeloid leukemia cells and enhances their responsiveness to interferons. Cell Growth Differ **8:**687-98.

131. **Matsuyama, T., H. Grossman, D. Mittrucker, D. P. Siderovski, F. Kiefer, T. Kawakami, C. D. Richardson, T. Tanaguchi, S. K. Yoshinaga, and T. W. Mak.** 1995. Moleculare cloning of LSIRF, a lymphoid-specific member of the interferon regulatory factor family that binds the interferon-stimulated response element (ISRE). Nucleic Acids Research **23:**2127-2136.

132. **Matsuyama, T., T. Kimura, M. Kitagawa, N. Watanabe, T. Kundig, R. Amakawa, K. Kishihara, A. Wakeham, J. Potter, C. Furlonger, A. Narendran, H. Suzuki, P. Ohashi, C. Paige, T. Taniguchi, and T. Mak.** 1993. Targeted disruption of IRF-1 or IRF-2 results in abnormal type I IFN induction and aberrant lymphocyte development. Cell **75:**83-97.

133. **Mattila, P. S.** 1996. The actions of cyclosporin A and FK506 on T lymphocyte activation. Biochem.Soc.Trans. **24:**45-49.

134. **Mattila, P. S., K. S. Ullman, S. Fiering, E. A. Emmel, M. McCutcheon, G. R. Crabtree, and L. A. Herzenberg.** 1990. The actions of cyclosporin A and FK506 suggest a novel step in the activation of T lymphocytes. EMBO Journal **9:**4425-4433.

135. **Meraro, D., S. Hashmueli, B. Koren, A. Azriel, A. Oumard, S. Kirchhoff, H. Hauser, S. Nagulapalli, M. L. Atchison, and B. Z. Levi.** 1999. Protein-protein and DNA-protein interactions affect the activity of lymphoid-specific IFN regulatory factors. J Immunol **163:**6468-78.

136. **Mercurio, F., B. W. Murray, A. Shevshenko, B. L. Bennet, D. B. Young, J. W. Li, G. Pascual, A. Motiwala, H. Zhu, M. Mann, and A. M. Manning.** 1999. I kappa B kinase (IKK)-associated protein 1, a common component of the heterogeneous IKK complex. Mol.Cell.Biol. **19:**1526-1538.

137. **Mittrucker, H.-W., T. Matsuyama, A. Grossman, T. M. Kundig, J. Potter, A. Shahinian, A. Wakeham, B. Patterson, P. Ohashi, and T. W. Mak.** 1997. Requirement for the transcription factor LSIRF/IRF4 for mature B and T lymphocyte function. Science **275:**540-543.

138. **Miyake, H., T. Suzuki, H. Hirai, and M. Yoshida.** 1999. Trans-activator Tax of human T-cell leukemia virus type 1 enhances mutation frequency of the cellular genome. Virology **253:**155-61.

139. **Miyamoto, M., T. Fujita, Y. Kimura, M. Maruyama, H. Harada, Y. Sudo, T. Miyata, and T. Taniguchi.** 1988. Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to the IFN- gene regulatory elements. Cell **54:**903-913.

140. **Miyamoto, S., P. J. Chiao, and I. M. Verma.** 1994. Enhanced IkB degradation is responsible for constitutive NF-kB activity in mature murine B-cell lines. Molecular and Cellular Biology **14:**3276-3282.

141. **Miyata, Y., B. Chambraud, C. Radanyi, J. Leclerc, M. Lebeau, J. Renoir, M. Catelli, I. Yahara, and E. Baulieu.** 1997. Phoshorylation of the

immunosuppressant FK506-binding protein FKBP52 by casein kinase II: Regulation of HSP90-binding activity of FKBP52. Proceedings of the National Academy of Sciences USA **94:**14500-14505.

142. **Moore, P. S., S. J. Gao, G. Dominguez, E. Cesarman, O. Lungu, D. M. Knowles, R. Garber, P. E. Pellett, D. J. McGeoch, and Y. Chang.** 1996. Primary characterization of a herpesvirus agent associated with Kaposi's sarcomae [published erratum appears in J Virol 1996 Dec;70(12):9083]. Journal of Virology **70:**549-58.

143. **Mori, N., M. Morishita, T. Tsukazaki, C. Z. Giam, A. Kumatori, Y. Tanaka, and N. Yamamoto.** 2001. Human T-cell leukemia virus type I oncoprotein Tax represses Smad-dependent transforming growth factor beta signaling through interaction with CREB-binding protein/p300. Blood **97:**2137-44.

144. **Moriuchi, H., M. Moriuchi, and A. S. Fauci.** 1998. Factors secreted by human T lymphotropic virus type I (HTLV-I)-infected cells can enhance or inhibit replication of HIV-1 in HTLV-I-uninfected cells: implications for in vivo coinfection with HTLV-I and HIV-1. J Exp Med **187:**1689-97.

145. **Moriuchi, M., H. Moriuchi, and A. S. Fauci.** 1999. HTLV type I Tax activation of the CXCR4 promoter by association with nuclear respiratory factor 1. AIDS Res Hum Retroviruses **15:**821-7.

146. **Morris, R.** 1994. Modes of action of FK506, cyclosporin A, and rapamycin. Transplantation Proceeding **26:**3272-3275.

147. **Mulloy, J. C., R. W. Crowley, J. Fullen, W. J. Leonard, and G. Franchini.** 1996. The human T cell leukemia/lymphotropic virus type 1 p12 protein binds the interleukin-2 receptor b and g c chains and affects their expression on the cell surface. Journal of Virology **70:**3599-3605.

148. **Murdoch, C.** 2000. CXCR4: chemokine receptor extraordinaire. Immunol Rev **177:**175-84.

149. **Nagulapalli, S., and M. L. Atchison.** 1998. Transcription factor Pip can enhance DNA binding by E47, leading to transcriptional synergy involving multiple protein domains. Molecular and Cellular Biology **18:**4639-4650.

150. **Nair, S. C., R. A. Rimerman, E. J. Toran, S. Chen, V. Prapapanich, R. N. Butts, and D. F. Smith.** 1997. Molecular cloning of human FKBP51 and comparisons of immunophilin interactions with Hsp90 and progesterone receptor. Mol Cell Biol **17:**594-603.

151. **Nakamura, T.** 2000. Immunopathogenesis of HTLV-I-associated myelopathy/tropical spastic paraparesis. Ann Med **32:**600-7.

152. **Nakazawa, M., T. Hasunuma, T. Ohshima, Y. Tanaka, K. Nishioka, and T. Nakajima.** 2000. CBP: A target molecule of HTLV-1 Tax in synoviocyte activation. Biochem Biophys Res Commun **269:**584-90.

153. **Nelson, N., M. S. Marks, P. H. Driggers, and K. Ozato.** 1993. Interferon consensus sequence binding protein, a member of the interferon regulatory factor family, suppresses interferon-induced gene transcription. Molecular and Cellular Biology **13:**588-599.

154. **Neuveut, C., K. G. Low, F. Maldarelli, I. Schmitt, F. Majone, R. Grassmann, and K. T. Jeang.** 1998. Human T-cell leukemia virus type 1 Tax and cell cycle progression: role of cyclin D-cdk and p110Rb. Mol Cell Biol **18:**3620-32.

155. **Newmyer, S. L., and S. L. Schmid.** 2001. Dominant-interfering Hsc70 mutants disrupt multiple stages of the clathrin-coated vesicle cycle in vivo. J Cell Biol **152:**607-20.

156. **Nguyen, H., J. Hiscott, and P. M. Pitha.** 1997. The growing family of IRF transcription factors. Cytokine and Growth Factor Reviews **8:**293-312.

157. **Okuma, K., M. Nakamura, S. Nakano, Y. Niho, and Y. Matsuura.** 1999. Host range of human T-cell leukemia virus type I analyzed by a cell fusion-dependent reporter gene activation assay. Virology **254:**235-44.

158. **Ortiz, M. A., J. Light, R. A. Maki, and N. Assa-Munti.** 1999. Mutation analysis of the Pip interaction domain reveals critical residues for protein-protein interactions. Proceedings of the National Academy of Sciences USA **96:**2740-2745.

159. **Pahl, A., and U. Keller.** 1992. FK-506-binding proteins from Streptomyces producing immunosuppressive macrolactones of the FK-506 type. Journal of Bacteriology **174:**5888-5894.

160. **Pahl, H. L.** 1999. Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene **18:**6853-6866.

161. **Paige, C. J., P. W. Kincade, and P. Ralph.** 1981. Independent control of immunoglobulin heavy and light chain expression in a murine pre-B-cell line. Nature **292:**631-3.

162. **Panem, S., E. V. Prochownik, F. R. Reale, and W. H. Kirsten.** 1975. Isolation of type C virions from a normal human fibroblast strain. Science **189:**297-9.

163. **Pawlak, G., and D. M. Helfman.** 2001. Cytoskeletal changes in cell transformation and tumorigenesis. Curr Opin Genet Dev **11:**41-7.

164. **Peattie, D., M. Harding, M. Fleming, M. DeCenzo, J. Lippke, G. Livingston, and M. Benasutti.** 1992. Expression and characterization of human

FKBP52, an immunophilin that associates with 90 KDa heat shock protein and is a component of steroid receptor complexes. Proceedings of the National Academy of Sciences USA **89:**10974-10978.

165. **Perkel, J. M., and M. L. Atchison.** 1998. A two-step mechanism for recruitment of Pip by PU.1. Journal of Immunology **160:**241-252.

166. **Perrot-Applanat, M., C. Cibert, G. Geraud, J.-M. Renoir, and E.-E. Baulieu.** 1995. The 59kDa FK506-binding protein, a 90 kDa heat shock protein binding immunophilin (FKBP59-HBI), is associated with the nucleus, the cytoskeleton and the mitotic apparatus. Journal of Cell Science **108:**2037-2051.

167. **Philpott, S. M., and G. C. Buehring.** 1999. Defective DNA repair in cells with human T-cell leukemia/bovine leukemia viruses: role of tax gene. J Natl Cancer Inst **91:**933-42.

168. **Pique, C., and M. C. Dokhelar.** 2000. In vivo production of Rof and Tof proteins of HTLV type 1: evidence from cytotoxic T lymphocytes. AIDS Res Hum Retroviruses **16:**1783-6.

169. **Pise-Masison, C. A., K. S. Choi, M. Radonovich, J. Dittmer, S. J. Kim, and J. N. Brady.** 1998. Inhibition of p53 transactivation function by the human T cell lymphotropic virus type 1 Tax protein. Journal of Virology **72:**1165-1170.

170. **Ploubidou, A., and M. Way.** 2001. Viral transport and the cytoskeleton. Curr Opin Cell Biol **13:**97-105.

171. **Poiez, B. F., F. W. Ruscetti, P. A. Gazadar, P. A. Bunn, J. D. Minna, and R. C. Gallo.** 1980. Detection and isolation of type C retrovirus particles from fresh cultured lymphocytes of a patient with cutaneous T-cell lymphoma. Proceedings of the National Academy of Sciences USA **77:**7415-7419.

172. **Portis, T., W. J. Grossman, J. C. Harding, J. L. Hess, and L. Ratner.** 2001. Analysis of p53 inactivation in a human T-cell leukemia virus type 1 Tax transgenic mouse model. J Virol **75:**2185-93.

173. **Radanyi, C., B. Chambraud, and E. Baulieu.** 1994. The ability of the immunophilin FKBP59-HBI to interact with the 90-kDa heat shock protein is encided by its tetratricopeptide repeat. Proceedings of the National Academy of Sciences USA **91:**11197-11201.

174. **Rao, S., A. Matsumura, J. Yoon, and M. C. Simon.** 1999. SPI-B activates transcription via a unique proline, serine, and threonine domain and exhibits DNA binding affinity differences from PU.1. J Biol Chem **274:**11115-24.

175. **Ratajczak, T., A. Carrello, P. J. Mark, B. J. Warner, R. J. Simpson, R. L. Moritz, and A. K. House.** 1993. The cyclophilin component of the unactivated estrogen receptor contains a tetratricopeptide repeat domain and shares identity with p59 (FKBP59). J Biol Chem **268:**13187-92.

176. **Reddy, T. R., W. D. Xu, and F. Wong-Staal.** 2000. General effect of Sam68 on Rev/Rex regulated expression of complex retroviruses. Oncogene **19:**4071-4.

177. **Rieske, P., and J. M. Pongubala.** 2000. AKT induces transcriptional activity of PU.1 through phosphorylation-mediated modifications within its transactivation domain. J Biol Chem **22:**22.

178. **Rivera, I., E. W. Harhaj, and S. C. Sun.** 1998. Involvement of NF-AT in type I human T-cell leukemia virus Tax-mediated Fas ligand promoter transactivation. J Biol Chem **273:**22382-8.

179. **Robek, M. D., F. H. Wong, and L. Ratner.** 1998. Human T-cell leukemia virus type 1 pX-I and pX-II open reading frames are dispensable for the immortalization of primary lymphocytes. J Virol **72:**4458-62.

180. **Rosenbauer, F., J. F. Waring, J. Foerster, M. Wietstruk, D. Philipp, and I. Horak.** 1999. Interferon consensus sequence binding protein and interferon regulatory factor-4/Pip form a complex that represses the expression of the interferon-stimulated gene-15 in macrophages. Blood **94:**4274-81.

181. **Rothwarf, D. M., E. Zandi, G. Natoli, and M. Karin.** 1998. IKKg is an essential regulatory subunit of the IkB kinase complex. Nature **395:**297-300.

182. **Ruediger, R., H. T. Pham, and G. Walter.** 2001. Disruption of protein phosphatase 2A subunit interaction in human cancers with mutations in the A alpha subunit gene. Oncogene **20:**10-5.

183. **Ruff, V. A., A. W. Yem, P. L. Munns, L. D. Adams, I. M. Reardon, M. R. Deibel, and K. L. Leach.** 1992. Tissue distribution and cellular localization of hsp56, an FK506-binding protein. Journal of Biological Chemistry **267:**21285-21288.

184. **Ruhlmann, A., and A. Nordheim.** 1997. Effects of the immunosuppressive drugs CsA and FK506 on intracellular signalling and gene regulation. Immunobiology **198:**192-206.

185. **Russo, J. J., R. A. Bohenzky, M.-C. Chien, J. Chen, M. Yan, D. Maddalena, J. P. Parry, D. Peruzzi, I. S. Edelman, Y. Chang, and P. Moore.** 1996. Nucleotide sequence of the kaposi sarcoma-associated herpesvirus (HHV8). Proceedings of the National Academy of Sciences USA **93:**14862-14867.

186. **Rybicki, B. A., M. J. Maliarik, E. Malvitz, R. G. Sheffer, M. Major, J. Popovich, Jr., and M. C. Iannuzzi.** 1999. The influence of T cell receptor and

cytokine genes on sarcoidosis susceptibility in African Americans. Hum Immunol **60:**867-74.

187. **Sagawa, K., M. Mochizuki, K. Katagirl, I. Tsuboi, S. Sugita, N. Mukaida, and K. Itoh.** 1996. In vitro effects of immunosuppressive agents on cytokine production by HTLV-I-infected T cell clones derived from the ocular fluid of patients with HTLV-I uveitis. Microbiol Immunol **40:**373-9.

188. **Saggioro, D., M. D'Agostino D, and L. Chieco-Bianchi.** 1999. Analysis of Tax-expressing cell lines generated from HTLV-I tax-transgenic mice: correlation between c-myc overexpression and neoplastic potential. Exp Cell Res **247:**525-33.

189. **Saito, T., T. Yamagata, T. Takahashi, H. Honda, and H. Hirai.** 1999. ICSAT overexpression is not sufficient to cause adult T-cell leukemia or multiple myeloma. Biochem Biophys Res Commun **260:**329-31.

190. **Sato, M., H. Suemori, N. Hata, M. Asagiri, K. Ogasawara, K. Nakao, T. Nakaya, M. Katsuki, S. Noguchi, N. Tanaka, and T. Taniguchi.** 2000. Distinct and Essential Roles of Transcription Factors IRF-3 and IRF-7 in Response to Viruses for IFN-alpha/beta Gene Induction. Immunity **13:**539-548.

191. **Schmidt, M., A. Hochhaus, S. A. Konig-Merediz, C. Brendel, J. Proba, G. J. Hoppe, B. Wittig, G. Ehninger, R. Hehlmann, and A. Neubauer.** 2000. Expression of interferon regulatory factor 4 in chronic myeloid leukemia: correlation with response to interferon alpha therapy [In Process Citation]. J Clin Oncol **18:**3331-8.

192. **Schmidt, M., S. Nagel, J. Proba, C. Thiede, M. Ritter, J. F. Waring, F. Rosenbauer, D. Huhn, B. Wittig, I. Horak, and A. Neubauer.** 1998. Lack of ICSBP transcripts in human myeloid leukemias. Blood **91:**22-29.

193. **Schonthal, A. H.** 1998. Role of PP2A in intracellular signal transduction pathways. Front Biosci **3:**D1262-73.

194. **Seasholtz, T. M., M. Majumdar, and J. H. Brown.** 1999. Rho as a mediator of G protein-coupled receptor signaling. Mol Pharmacol **55:**949-56.

195. **Semmes, O. J., F. Majone, C. Cantemir, L. Turchetto, B. Hjelle, and K. T. Jeang.** 1996. HTLV-I and HTLV-II Tax: differences in induction of micronuclei in cells and transcriptional activation of viral LTRs. Virology **217:**373-9.

196. **Sharf, R., D. Meraro, A. Azriel, A. M. Thornton, K. Ozato, E. F. Petricoin, A. C. Larner, F. Schaper, H. Hauser, and B.-Z. Levi.** 1997. Phosphorylation events modulate the ability of interferon consensus sequence binding protein to interact with interferon regulatory factors and to bind DNA. Journal of Biological Chemistry **272:**9785-9792.

197. **Siebert, R., C. P. Willers, and B. Opalka.** 1996. Role of the cyclin-dependent kinase 4 and 6 inhibitor gene family p15, p16, p18 and p19 in leukemia and lymphoma. Leuk Lymphoma **23:**505-20.

198. **Silverstein, A. M., M. D. Galigniana, M. S. Chen, J. K. Owens-Grillo, M. Chinkers, and W. B. Pratt.** 1997. Protein phosphatase 5 is a major component of glucocorticoid receptor-hsp90 complexes with properties of an FK506-binding immunophilin. J Biol Chem **272:**16224-30.

199. **Silverstein, A. M., M. D. Galigniana, K. C. Kanelakis, C. Radanyi, J. M. Renoir, and W. B. Pratt.** 1999. Different regions of the immunophilin FKBP52 determine its association with the glucocorticoid receptor, hsp90, and cytoplasmic dynein. J Biol Chem **274:**36980-6.

200. **Slattery, J. P., G. Franchini, and A. Gessain.** 1999. Genomic evolution, patterns of global dissemination, and interspecies transmission of human and simian T-cell leukemia/lymphotropic viruses. Genome Res **9:**525-40.

201. **Smith, D. F., B. A. Baggenstoss, T. N. Marion, and R. A. Rimerman.** 1993. Two FKBP-related proteins are associated with progesterone receptor complexes. J Biol Chem **268:**18365-71.

202. **Song, Y., C. Wong, and D. D. Chang.** 2000. Overexpression of wild-type RhoA produces growth arrest by disrupting actin cytoskeleton and microtubules. J Cell Biochem **80:**229-40.

203. **Stark, G. R., I. M. Kerr, B. R. G. Williams, R. H. Silverman, and R. D. Schreiber.** 1998. How cells respond to interferons. Annual Review of Biochemistry **67:**227-264.

204. **Sugiyama, Y., K. Tomoda, T. Tanaka, Y. Arata, N. Yoneda-Kato, and J. Kato.** 2001. Direct binding of the signal-transducing adaptor Grb2 facilitates down-regulation of the cyclin-dependent kinase inhibitor p27Kip1. J Biol Chem **276:**12084-90.

205. **Sun, S. C., and D. W. Ballard.** 1999. Persistent activation of NF-kappaB by the Tax transforming protein of HTLV-1: hijacking cellular IkappaB kinases. Oncogene **18:**6948-6958.

206. **Suzuki, T., S. Kiato, H. Matsushime, and M. Yoshida.** 1996. HTLV-1 tax protein interacts with cyclin-dependent kinase inhibitor p16INK4A and counteracts its inhibitory activity towards CDK4. EMBO Journal **15:**1607-1614.

207. **Suzuki, T., T. Narita, M. Uchida-Toita, and M. Yoshida.** 1999. Down-regulation of the INK4 family of cyclin-dependent kinase inhibitors by tax protein of HTLV-1 through two distinct mechanisms. Virology **259:**384-91.

208. **Takemoto, S., J. C. Mulloy, A. Cereseto, T. S. Migone, B. K. Patel, M. Matsuoka, K. Yamaguchi, K. Takatsuki, S. Kamihira, J. D. White, W. J. Leonard, T. Waldmann, and G. Franchini.** 1997. Proliferation of adult T cell leukemia/lymphoma cells is associated with the constitutive activation of JAK/STAT proteins. Proc Natl Acad Sci U S A **94:**13897-902.

209. **Tanaka, N., T. Kawakami, and T. Taniguchi.** 1993. Recognition DNA sequences of interferon regulatory factor 1 (IRF-1) and IRF-2, regulators of cell growth and the interferon system. Molecular and Cellular Biology **13:**4531-4538.

210. **Tanaka, Y., S. Mine, C. G. Figdor, A. Wake, H. Hirano, J. Tsukada, M. Aso, K. Fujii, K. Saito, Y. van Kooyk, and S. Eto.** 1998. Constitutive chemokine production results in activation of leukocyte function-associated antigen-1 on adult T-cell leukemia cells. Blood **91:**3909-19.

211. **Taniguchi, T., H. Harada, and M. Lamphier.** 1995. Regulation of interferon system and cell growth by the IRF transcription factors. J.Cancer.Res.Clin.Oncol. **121:**516-520.

212. **Taniguchi, T., K. Ogasawara, A. Takaoka, and N. Tanaka.** 2001. Irf family of transcription factors as regulators of host defense. Annu Rev Immunol **19:**623-55.

213. **Taniguchi, T., N. Tanaka, and S. Taki.** 1998. Regulation of the interferon system, immune response and oncogenesis by the transcription factor interferon regulatory factor-1. Eur Cytokine Netw **9:**43-8.

214. **Taylor, W. R., S. E. DePrimo, A. Agarwal, M. L. Agarwal, A. H. Schonthal, K. S. Katula, and G. R. Stark.** 1999. Mechanisms of G2 arrest in response to overexpression of p53. Mol Biol Cell **10:**3607-22.

215. **Tirnauer, J. S., and B. E. Bierer.** 2000. EB1 proteins regulate microtubule dynamics, cell polarity, and chromosome stability. J Cell Biol **149:**761-6.

216. **Trejo, S. R., and L. Ratner.** 2000. The HTLV receptor is a widely expressed protein. Virology **268:**41-8.

217. **Tsuboi, K., S. Iida, H. Inagaki, M. Kato, Y. Hayami, I. Hanamura, K. Miura, S. Harada, M. Kikuchi, H. Komatsu, S. Banno, A. Wakita, S. Nakamura, T. Eimoto, and R. Ueda.** 2000. MUM1/IRF4 expression as a frequent event in mature lymphoid malignancies. Leukemia **14:**449-56.

218. **Tsukasaki, K., P. Koeffler, and M. Tomonaga.** 2000. Human T-lymphotropic virus type 1 infection. Baillieres Best Pract Res Clin Haematol **13:**231-43.

219. **Tsukasaki, K., J. Krebs, K. Nagai, M. Tomonaga, H. P. Koeffler, C. R. Bartram, and A. Jauch.** 2001. Comparative genomic hybridization analysis in adult T-cell leukemia/lymphoma: correlation with clinical course. Blood **97:**3875-3881.

220. **Tsurimoto, T.** 1999. PCNA binding proteins. Front Biosci **4:**D849-58.

221. **Uchiyama, T.** 1997. Human T cell leukemia virus type I (HTLV-I) and human diseases. Annu Rev Immunol **15:**15-37.

222. **Uhlik, M., L. Good, G. Xiao, E. Harhaj, E. Zandi, M. Karin, and S.-C. Sun.** 1998. NF-kB-inducing kinase and IkB kinase participate in human T-cell

leukemia virus 1 Tax-mediated NF-kB activation. Journal of Biological Chemistry **273:**21132-21136.

223. **Uittenbogaard, M. N., A. P. Armstrong, A. Chiaramello, and J. K. Nyborg.** 1994. Human T-cell leukemia virus type I Tax protein represses gene expression through the basic helix-loop-helix family of transcription factors. J Biol Chem **269:**22466-9.

224. **Valentin, H., S. Hamaia, S. Konig, and L. Gazzolo.** 2001. Vascular cell adhesion molecule-1 induced by human T-cell leukaemia virus type 1 Tax protein in T-cells stimulates proliferation of human T-lymphocytes. J Gen Virol **82:**831-5.

225. **Van Dooren, S., M. Salemi, and A. M. Vandamme.** 2001. Dating the origin of the african human t-cell lymphotropic virus type-I (htlv-I) subtypes. Mol Biol Evol **18:**661-71.

226. **Vandamme, A. M., U. Bertazzoni, and M. Salemi.** 2000. Evolutionary strategies of human T-cell lymphotropic virus type II. Gene **261:**171-80.

227. **Wakui, H., A. P. Wright, J. Gustafsson, and J. Zilliacus.** 1997. Interaction of the ligand-activated glucocorticoid receptor with the 14-3-3 eta protein. J Biol Chem **272:**8153-6.

228. **Wang, L., H. Zhang, P. A. Solski, M. J. Hart, C. J. Der, and L. Su.** 2000. Modulation of HIV-1 replication by a novel RhoA effector activity. J Immunol **164:**5369-74.

229. **Watanabe, T., K. Yamaguchi, K. Takatsuki, M. Osame, and M. Yoshida.** 1990. Constitutive expression of parathyroid hormone-related protein gene in human T cell leukemia virus type 1 (HTLV-1) carriers and adult T cell leukemia patients that can be trans-activated by HTLV-1 tax gene. J Exp Med **172:**759-65.

230. **Weaver, B. K., K. P. Kumar, and N. C. Reich.** 1998. Interferon regulatory factor 3 and CREB-binding protein/p300 are subunits of double-stranded RNA-activated transcription factor DRAF1. Molecular and Cellular Biology **18:**1359-1368.

231. **Weihua, X., V. Kolla, and D. V. Kalvakolanu.** 1997. Interferon gamma-induced transcription of the murine ISGF3gamma (p48) gene is mediated by novel factors. Proc Natl Acad Sci U S A **94:**103-8.

232. **Weisz, A., P. Marx, R. Sharf, E. Appella, P. H. Driggers, K. Ozato, and B.-Z. Levi.** 1992. Human interferon consensus sequence binding protein is a negative regulator of enhancer elements common to interferon-inducible genes. Journal of Biological Chemistry **267:**25589-25596.

233. **Whitehead, I. P., I. E. Zohn, and C. J. Der.** 2001. Rho GTPase-dependent transformation by G protein-coupled receptors. Oncogene **20:**1547-55.

234. **Whittaker, G. R., and A. Helenius.** 1998. Nuclear import and export of viruses and virus genomes. Virology **246:**1-23.

235. **Wu, C. Y., H. Maeda, C. Contursi, K. Ozato, and R. A. Seder.** 1999. Differential requirement of ICSBP for the production of IL-12 and induction of Th1-type cells in response to IFNg. Journal of Immunology **162:**807-812.

236. **Wurster, A. L., T. Tanaka, and M. J. Grusby.** 2000. The biology of Stat4 and Stat6. Oncogene **19:**2577-84.

237. **Xiao, G., E. W. Harhaj, and S. C. Sun.** 2000. Domain-specific interaction with the ikappa B kinase (IKK) regulatory subunit IKKgamma is an essential step in tax-mediated activation of IKK [In Process Citation]. J Biol Chem **275:**34060-7.

238. **Xiao, G., and S. C. Sun.** 2000. Activation of IKKalpha and IKKbeta through their fusion with HTLV-I tax protein. Oncogene **19:**5198-203.

239. **Yamagata, T., J. Nishida, T. Tanaka, R. Sakai, K. Mitani, M. Yoshida, T. Taniguchi, Y. Yazaki, and H. Hirai.** 1996. A novel interferon regulatory factor family transcription factor, ICSAT/Pip/LSIRF, that negatively regulates the activity of interferon-regulated genes. Molecular and Cellular Biology **16:**1283-1294.

240. **Yamaoka, S., G. Courtois, C. Bessia, S. T. Whiteside, R. Weil, F. Agou, H. E. Kirk, R. J. Kay, and A. Israel.** 1998. Complementation cloning of NEMO, a component of the IkB kinase complex essential for NF-kB activation. Cell **93:**1231-1240.

241. **Yan, J. P., J. E. Garrus, H. A. Giebler, L. A. Stargell, and J. K. Nyborg.** 1998. Molecular interactions between the coactivator CBP and the human T-cell leukemia virus Tax protein. J Mol Biol **281:**395-400.

242. **Yao, J., and B. Wigdahl.** 2000. Human T cell lymphotropic virus type I genomic expression and impact on intracellular signaling pathways during neurodegenerative disease and leukemia. Front Biosci **5:**D138-68.

243. **Yee, A. A., P. Yin, D. P. Siderovski, T. W. Mak, D. W. Litchfield, and C. H. Arrowsmith.** 1998. Cooperative interaction between the DNA-binding domains of PU.1 and IRF4. J Mol Biol **279:**1075-83.

244. **Yin, M.-J., L. B. Christerson, Y. Yamamoto, Y.-T. Kwak, S. Xu, F. Mercurio, M. Barbosa, M. H. Cobb, and R. B. Gaynor.** 1998. HTLV-I Tax protein binds to MEKK1 to stimulate IkB kinase activity and NF-kB activation. Cell **93:**875-884.

245. **Yoneyama, M., W. Suhara, Y. Fukuhara, M. Sato, K. Ozato, and T. Fujita.** 1996. Autocrine amplification of type I interferon gene expression mediated by interferon stimulated gene factor 3 (ISGF3). Journal of Biochemistry **120:**160-169.

246. **Yoshida, M.** 1993. HTLV-1 tax: regulation of gene expression and disease. Trends in Microbiology **1:**131-135.

247. **Yoshida, M.** 1996. Molecular biology of HTLV-I: Recent progress. Journal of Acquired Immune Deficiency Syndromes **13 s.1:**s63-s68.

248. **Yoshida, M.** 2001. Multiple viral strategies of htlv-1 for dysregulation of cell growth control. Annu Rev Immunol **19:**475-96.

249. **Yoshida, M., and T. Suzuki.** 2000. HTLV type 1 Tax oncoprotein binds to DNA topoisomerase I and inhibits its catalytic activity. AIDS Res Hum Retroviruses **16:**1639-45.