NOTE TO USERS

THE FUNCTIONAL CHARACTERIZATION OF THE ALTERNATIVELY-SPLICED QUAKING RNA-BINDING ISOFORMS IN OLIGODENDROCYTES

by

Julie Pilotte

Division of Experimental Medicine, Department of Medicine

McGill University, Montréal

August 2003

A thesis submitted to the Faculty of Graduate Studies in partial fulfillment of the requirements for the degree of Doctorate of Philosophy

Your file Votre référence
ISBN: 0-612-98348-X
Our file Notre référence
ISBN: 0-612-98348-X

To my family and my friends for all their support.

ABSTRACT

The *quaking* viable mice have myelination defects and develop a tremor in their hind limbs 10 days after birth. This is caused by a deletion in the promoter/enhancer region of the *quaking* gene. The *quaking* gene is alternatively spliced, producing QUAKING isoforms that differ in their C-terminal 8-30 amino acid sequence. The QKI-5 isoform is nuclear, whereas the QKI-6 and QKI-7 isoforms are predominantly cytoplasmic. The QKI isoforms contain a single KH RNA-binding domain suggesting a role in RNA metabolism. Although the dysmyelinating phenotype of the mutant mouse suggests a role in myelination, the function of the protein still remains unknown. The goal of this thesis is to characterize the possible functions of the *quaking* protein. We first explored the ability of the QKI-7 isoform to induce apoptosis in fibroblast cells and confirmed this event in oligodendrocytes, where most of the QUAKING isoforms are present. We mapped the domain responsible for this event to the alternatively spliced C-terminus, which we named 'KILLER' sequence. Heterodimerization of the other QKI isoforms with QKI-7 causes its relocalization into the nucleus, suppressing its ability to induce apoptosis. Therefore we hypothesize that the balance between the different isoforms dictates whether the cell will live or die. We were also interested in finding a RNA binding substrate for this protein, therefore we looked at potential proteins that are altered in the *quaking* viable mouse. We found that the mRNA myelin basic protein (MBP) is in fact a target for the QKI proteins. We show that this interaction occurs through the 3'-UTR of MBP, in a 110 nucleotide region named QRE for Quaking Responsive Element. In addition, we have recreated a defect of the *quaking* viable

phenotype by over-expressing QKI-5 into the brain of mice. This causes retention of the MBP mRNA into the nucleus of oligodendrocytes. As a result, MBP expression is significantly reduced. These observations show that the QKI proteins are actively involved with the MBP mRNA and expression of MBP in myelination. This thesis also shows the importance of the different QKI isoforms in the localization of their physiological targets.

RÉSUMÉ

La souris viable *quaking* a des anomalies de myélinisation. Elle développe un tremblement au niveau des jambes arrières 10 jours après sa naissance. Ce phénotype est causé par une délétion génétique de la région promoteur/amplificateur du gène *quaking*. *Quaking* est sujet à un épissage alternatif, produisant ainsi des isoformes de la protéine QUAKING qui se distinguent de 8 à 30 acides aminés seulement dans la région C-terminal. QKI-5 est nucléaire, tandis que QKI-6 et QKI-7 sont principalement cytoplasmique. Les isoformes QUAKING contiennent un domaine KH responsable de lier l'ARN, suggèrant un rôle dans le métabolisme de l'ARN. Même si le phénotype dysmyélinisant de la souris viable *quaking* suggère un rôle dans la myélinisation, la fonction de la protéine est toujours inconnue. Le but de cette thèse est d'identifier et de caractériser les fonctions possibles du gène *quaking*. Nous avons premièrement exploré l'habileté de QKI-7 à induire l'apoptose dans des fibroblastes, qui a été confirmé dans les oligodendrocytes, où on retrouve la plupart des QUAKINGs. Nous avons déterminé le domaine responsable de ce phénotype à la région C-terminal de la protéine. Nous avons surnommé cette séquence 'KILLER'. C'est l'hétérodimérisation de QKI-7 avec les autres isoformes de QUAKING qui entraîne la relocalisation de QKI-7 dans le noyeau. Cet évènement empêche l'induction de apoptose. C'est le niveau relatif entre les différents isoformes qui dictent si la cellule vit ou si elle meure. Afin de trouver des substrats d'ARN pour QUAKING, nous avons regardé des candidats potentiels dont leur expression est altérée chez la souris viable *quaking*. Nous avons établi que QUAKING est capable de se lier à la région de 3'-UTR de l'ARN messager de MBP (myelin basic

protein). Nous démontrons que cette interaction se produit précisément dans une région de 110 nucléotides nommé QRE (Quaking Responsive Element). Nous avons aussi rétabli le phénotype de la souris viable *quaking* en surexprimant QKI-5 dans le cerveau de souris saine. Ceci cause une rétention de l' ARN messager de MBP au niveau du noyeau et prévient ainsi l'expression de la protéine MBP. Ces observations démontrent que les protéines QKI sont activement impliquées dans le métabolisme de l'ARN messager de MBP ainsi qu'avec l'expression de la protéine dans la myélinisation. Cette étude démontre aussi l'importance des différents isoformes QUAKING dans la localisation de leurs cibles physiologiques.

PREFACE

This Ph.D. thesis was written in accordance with the Guidelines for Thesis Preparation from the Faculty of Graduate Studies and Research at McGill University. This thesis is based on the option of writing the thesis as a manuscript-based thesis. The guidelines state: "Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.)..."

Papers included in this thesis:

Chapter 2 **Pilotte, J.**, Larocque, D., and Richard, S. (2001). Nuclear translocation controlled by alternatively spliced isoforms inactivates the QUAKING apoptotic inducer. Genes Dev. 15, 845-858.

Chapter 3 Larocque, D., **Pilotte, J.**, Chen, T., Cloutier, F., Massie B., Pedraza, L., Couture, R., Lasko, P., Almazan, G., and Richard, S. (2002). Nuclear retention of MBP mRNAs in the *Quaking Viable* mice. Neuron. 36, 815-829.

Contribution of authors:

The candidate performed most of the research. Contributions of other authors are described below:

In Chapter 2, the experiments described in Figure 2-9 were performed by Daniel Larocque.

Chapter 3 was a collaborative effort, where Figure 3-1 and 3-2 were performed by Taiping Chen, with the exception of the last panel in Figure 3-1A completed by the candidate, and Figure 3-1B that were done by Stéphane Richard. Daniel Larocque performed figure 3-5, as well as Figures 3-7 and 3-8. Frank Cloutier and Réjean Couture supervised the brain injections, the MBP exon II antibody was provided by Liliana Pedraza, and the adenoviral vectors were given by Bernard Massie. Guillermina Almazan provided us with her expertise with oligodendrocytes, and the microscopy was done in the laboratory of Paul Lasko.

All studies were conducted under the supervision of Dr. Stéphane Richard.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Stéphane Richard, for teaching me the ropes. His great vision and undying enthusiasm, as well as support and knowledge, has made this a very successful Ph.D. experience.

There is so many to thank who have shaped the path of this journey. Of course I am extremely grateful to past and present technicians, graduate students and postdoctoral fellows, great friends who have made the laboratory an absolute joy to come to everyday. You gave me strength when I really needed it, and I am here today because of you. Thank you.

Thank you to my family and very special friends who have always been there for me, even if I was 'too busy' for them on a number of occasions. Knowing that you were always there supporting me helped me get through some stressful times. And a special thank you to my love, Ryan.

I would like to thank the following for the financial support of my studies: The Canadian Institute for Health Research (CIHR) for the Doctoral Research Award (2000-2003), as well as the Multiple Sclerosis Society of Canada for providing me with my first student fellowship (1998-2000).

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

4E-BPs	4E-binding proteins
a.a	amino acids
APAF-1	apoptotic protease-activating factor-1
APP	amyloid precursor protein
ARE	AU-rich element
Bcl	B-cell lymphoma
BH	Bcl-2 homology
BIR	baculovirus inhibitor
CA	carbonic anhydrase
CAD	caspase-activated deoxyribonuclease
CaMKII-α	calcium-calmodulin-dependent kinase II α
CARD	caspase recruitment domain
CBC	cap binding complex
CK	C-terminal of the KH
CNP	2'3'-cyclic nucleotide 3'-phosphodiesterase
CNS	central nervous system
CPSF	cleavage and polyadenylation specificity factor
CTD	C-terminal domain
DD	death domain
DED	death effector domain
EAE	experimental allergic encephalomyelitis
endoG	endonuclease G
EJC	exon-exon junction complex
ENU	ethyl-N-nitrosourea
EST	expressed sequence tags
FADD	Fas-associated death domain
FG	phenylalanine/glycine
FMR1	fragile X mental retardation
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GLD-1	germ-line defective
Golli-MBP	gene expressed in the oligodendrocyte lineage-MBP
GSG	GRP33, Sam68, GLD-1
GT	guanylyltransferase
hnRNP K	heterogeneous nuclear ribonucleoprotein K
How	held-out-wings
Hqk	human quaking
IAP	inhibitor of apoptosis
IBM	IAP binding motif
ICAD	inhibitor of CAD
IRE	iron-responsive element
IRES	internal ribosomal entry site
kb	kilobases

KEP1	KH encompassing protein
KH	hnRNP K homology
MAG	myelin associated glycoprotein
MBP	myelin basic protein
MBPexII	MBP containing exon II
MOBP	myelin-associated oligodendrocyte basic protein
MOG	myelin/oligodendrocyte glycoprotein
MOSP	myelin/oligodendrocyte specific protein
MS	multiple sclerosis
msd	myelin synthesis deficiency
MT	7-methyltransferase
NCAM	neural cell adhesion molecule
NK	N-terminal of the KH
NLS	nuclear localization signal
NMD	nonsense-mediated decay
NMR	nuclear magnetic resonance
NPC	nuclear pore complex
NTF2	nuclear transport factor 2
OMgp	oligodendrocyte-myelin glycoprotein
OSP	oligodendrocyte-specific protein
PAP	poly(A) polymerase
PARN	poly(A) specific ribonuclease
p.c.	post coitum
PLP	proteolipid protein
PML	promyelocytic nuclear bodies
PNS	peripheral nervous system
PSA-NCAM	polysialyated neural cell adhesion molecule
PTB	polypyrimidine tract-binding protein
PYD	pyrin domain
QASE	quaking alternative splicing element
QKI	quaking
qk^v	quaking viable
QRE	quaking response element
RGH	reaper, grim, hid
RNP	ribonucleoprotein
RRMs	RNA-recognition motifs
RTP	RNA 5' triphosphatase
RTS	RNA trafficking signal
Sam68	Src-associated-in-mitosis
SF1	splicing factor
SLM	Sam68-like mammalian proteins
SMAC	second mitochondrial activator of caspases
SNB	Sam68 nuclear bodies
STAR	signal transduction and activator of RNA
SVZ	subventricular zone
TNF-α	tumor necrosis factor-α

TGE	tra-2 and GLI element
TRAIL	TNF-α related apoptosis-inducing ligand
UTR	untranslated region
VDAC	voltage-dependent anion channel
vz	ventricular zone
Xqua	Xenopus *quaking*
ZD	zebrafish specific domain

Chapter 1

Introduction and literature review

1.1 General introduction

Myelination, responsible for the ensheathment of the axon, is essential in the function and development of the nervous system, allowing for rapid signaling. The glial cells are major players in myelinogenesis and constitute 90% of the cells in our brain (Pfrieger and Barres, 1995). Although a lot of research has been done in this particular area, complete understanding of myelination still remains obscure. Damage or loss of the myelin sheath usually slows down or completely blocks the signaling, and this phenomenon has attracted a lot of interest in the field of neurobiology.

There have been a number of animal models used to study the pathologic processes associated with the myelin lesions. In my project, we have used the *quaking* viable mice to study hypomyelination. A spontaneous mutation in these mice causes a trembling mostly pronounced in the hind limbs around 10 days after birth (Sidman et al., 1964). The dysmyelination is not caused by a mutation in one of the known components of myelin, but from a deletion of the promoter/enhancer region of the *quaking* gene (Ebersole et al., 1996). This gene generates three major isoforms, which possess a KH domain known for its ability to bind RNA. Closer inspection of the sequence reveals that this protein belongs to the GSG family of proteins, which have an extended single KH region. Studies have indicated that GSG family members as well as *quaking* itself are involved in RNA metabolism, which comprises pre-mRNA processing in the nucleus, export, transport, stabilization and translational events in the cytoplasm.

The objective of this thesis is to understand the function of *quaking* in oligodendrocytes. Chapter 2 will focus on *quaking*'s role in apoptosis, extending previous observations in our laboratory that QKI-7 induces apoptosis in a fibroblast cell line (Chen and Richard, 1998). Chapter 3 will concentrate on the ability of *quaking* to bind to the myelin basic protein mRNA, and will bring further insights in the molecular events leading to the dysmyelination of the *quaking* viable mouse. In the introduction, I will cover the background material needed for Chapters 2 and 3, starting with a review on the *quaking* viable mouse and the background on the *quaking* gene. In order to complement the research covered in this thesis, the topics of myelination, as well as apoptosis and RNA metabolism will be generally reviewed, pertaining to the possible outcomes of *quaking* in RNA-binding.

1.2 The *quaking* viable mouse

1.2.1 Phenotype and general features

The *quaking* viable (qk^v) mouse was first discovered in 1961 in a DBA/2 subline and characterized by Sidman's group (Guenet, 1980; Sidman et al., 1964). The clinical symptoms characteristic of this mutant first become apparent at 10 days of age then increase progressively to reach their maximum intensity after four weeks (reviewed in Hogan and Greenfield, 1984a). The outstanding feature is a generalized rapid trembling affecting the trunk and all four limbs, with increased severity in the hind limbs. Most of the animals survive to adulthood, but they have a reduced size and frequently display tonic seizures, with the occasional animal developing hydrocephalus (reviewed in Hogan and Greenfield, 1984a). The qk^v mouse has a recessive spontaneous mutation where

heterozygotes are unaffected (Bennett et al., 1971). The females can breed, but the males are sterile (reviewed in Hogan and Greenfield, 1984a).

Morphological studies of the *qk*^{v} brain have shown that there is little myelin in the central nervous system (CNS), and only minor effects seen in the peripheral nervous system (PNS) (reviewed in Hogan and Greenfield, 1984a). The myelin deficiency is most pronounced in the brain, less severe in the spinal cord. There is a marked reduction in number of myelin lamellae per axon diameter, a lack of compaction of the myelin sheath, a tendency to form cytoplasmic loops and the atypical formation of Schmidt-Lanterman incisures (reviewed in Hogan and Greenfield, 1984a). The oligodendrocytes appear normal, but their density is increased in fiber tracts (Friedrich, 1974). The oligodendrocyte perikaryon often contains lamellar inclusions, dense bodies or vacuoles, which probably represent lysosomal degradation of myelin. Axons in the CNS are largely spared, but there is mild astrogliosis (Suzuki and Zagoren, 1975).

1.2.2 Affected lipids and proteins

Some of the major deficiencies observed in the *qk*^{v} mouse involve certain brain lipids. Cerebrosides, sulfatides and major phosphoglycerides are highly affected (Baumann et al., 1968; Hogan and Joseph, 1970). Furthermore, some myelination specific proteins are also deficient in the *qk*^{v} adult. For example, there is a two-third reduction of the proteolipid protein (PLP), as well as a reduction of the myelin basic protein (MBP) 14K and 17.5K isoforms in myelin fractions (Fagg et al., 1979; Nussbaum and Mandel, 1973). Some enzymes involved in myelination are also decreased, especially in cholesterol hydrolase, 2'3'-cyclic nucleotide 3'-phosphodiesterase (CNP) and carbonic

anhydrase II (CA) (Hogan and Greenfield, 1984a; Sapirstein, 1982). Conversely, neurotransmitters such as acetylcholine, dopamine and norepinephrine are increased in the qk^v mouse and this may be related to increased activity and stress caused by the tremors. Glial fibrillaric protein (GFAP), the S100 proteins, and the synaptic vesicle antigen C1 were also markedly increased (Jacque et al., 1974; Kempf et al., 1973).

Studies have shown that most of the major myelin components affected are synthesized and accumulated at a relatively normal rate in qk^v mice, supporting the hypothesis that the mutation causes an arrest in myelin assembly (Brostoff et al., 1977; Carnow et al., 1984; Delassalle et al., 1981; Sorg et al., 1986). There is a significant decrease in the incorporation of MBPs as well as PLP into the myelin, even though MBP protein levels are decreased, mRNA levels are relatively similar (Carnow et al., 1984; Greenfield et al., 1977; Sorg et al., 1987; Sorg et al., 1986). Both PLP and CNP RNA levels and localization have been shown to be altered (Sorg et al., 1987; Sorg et al., 1986). In situ hybridization in cultured oligodendrocytes demonstrated that MBP mRNA is mostly located in the processes (~80%), but the amount is reduced to 23% in qk^v oligodendrocytes (Barbarese, 1991). However, the MBP mRNAs are distributed over the oligodendrocyte cell body and myelin after 3 weeks postnatal (Campagnoni et al., 1991).

Alternative splicing defects in the myelin associated glycoprotein (MAG), PLP, and the neural cell adhesion molecule (NCAM) are also seen in the qk^v mice (Bartoszewicz et al., 1995; Frail and Braun, 1985; Fujita et al., 1988). There are two regulated spliced isoforms of MAG, the large (L-MAG) and small (S-MAG) isoform (Arquint et al., 1987). In the qk^v mouse, L-MAG mRNA levels are severely reduced, whereas S-MAG mRNA levels are increased. Even with increased mRNA levels, the S-

MAG protein levels remain low (Bartoszewicz et al., 1995; Frail and Braun, 1985; Fujita et al., 1988; Fujita et al., 1990). Both MAG isoforms are abnormally glycosylated in the qk^v mouse, leading to a higher molecular weight (Bartoszewicz et al., 1995). The expression of L-MAG becomes more severely reduced with maturation and is thought to be depleted by endocytosis from the periaxonal membrane (Bo et al., 1995). Immunostaining of the L-MAG protein also reveals a mislocalization to the perinuclear area (Bo et al., 1995).

1.3 ENU-induced mutations

The qk^v mutation resides on chromosome 17 in the mouse, but for many years it was not known whether this mutation affected other genes as well. Therefore mutations were introduced into the affected region of the chromosome with the chemical ethyl-N-nitrosourea (ENU). Justice and Bode were the first to demonstrate ENU-induced mutations in the *qk* locus (Bode, 1984). These point mutation are unable to complement themselves and are recessive lethal (Justice and Bode, 1988). Embryological studies have shown that the somite pair number ranges between 15-26 and that most develop forelimb buds but not hind limb buds. Over 50% of the defective embryos also had hemorrhaging in the mesencephalon. A lot of embryos exhibited problems with closure of the head fold, and there were problems as well with general neural and heart development, starting as early as day 9 p.c. Fatalities started occurring at around day 8-9.5 of gestation (Justice and Bode, 1988).

Shedlovsky and Dove strengthened Justice and Bode's findings and also characterized an extra mutation, called the qk^{l-1} mutant for *lethal-1* (Shedlovsky et al.,

1988). When crossed to the qk^{v} mouse, it creates a transient phenotype less severe than qk^{v} homozygotes. The typical *quaking* phenotype can be observed after 2-3 weeks, but then disappears by 5-6 weeks. This study also showed that the sterility observed in qk^{v} homozygotes was not caused by *qk*, as crosses with the *qk* ENU-induced mutations does not result in sterility (Shedlovsky et al., 1988). The qk^{1-1} strain consists of an AT to GC transition at nucleotide 2783, which is immediately downstream of the coding sequence of isoform QKI-6 (see section 1.4.1 for the QKI isoforms) (Cox et al., 1999). This creates a potential exon-intron splice-site, leading to the loss of the QKI-5 specific exons, eliminating the QKI-5 isoform. The other QKI isoform are still produced at similar levels to the wild-type (Cox et al., 1999).

A knockout of *qk* has also been accomplished by homologous recombination, but has yet to be published (unpublished observations) (Vernet and Artzt, 1997). As expected, homozygotes die early and qk^{v}/qk^{KO} heterozygotes exhibit even more pronounced phenotypes than qk^{v}/qk^{ENU}. They are unable to support their own body weight and only attain 60% body weight compared to qk^{v}/qk^{v}, which are already smaller in size than control littermates (Vernet and Artzt, 1997).

The mutation in qk^{kt4} (or qk^{kt3}) has been shown to be a non-conserved glutamic acid to glycine change at position 48 in the NK domain caused by an AT to GC transition at nucleotide 650 (Cox et al., 1999; Ebersole et al., 1996). The qk^{k2} mutation results in a TA to AT transversion at nucleotide 977 that changes a valine at position 157 into a glutamic acid, which is in the conserved KH RNA-binding domain. The defect in qk^{kt1} remains elusive (Cox et al., 1999).

1.4 The *quaking* gene

1.4.1 Sequence arrangement

Positional cloning of the *qk* locus was used initially to understand the defect in the qk^v mouse. A 1.2 Mb YAC contig spanning the *quaking* region was found, and this led to the realization that the deletion in the qk^v mouse was at least a minimum of 910 kb in length (Cox et al., 1994). The gene was cloned using a cosmid walk in a qk^v/qk^v library, which used a CpG island at the proximal deletion breakpoint (Ebersole et al., 1996). Sequence analysis of the *qk* gene revealed the presence of a single KH RNA-binding domain, very similar to the KH domain of Sam68 (Fumagalli et al., 1994). It had an even higher homology to *C. elegans* GLD-1, where 55% of the protein sequence is identical and 77% is similar over 208 amino acids (Ebersole et al., 1996; Jones and Schedl, 1995). The QUAKING (QKI) protein also has high homology to additional regions near the KH domain, which were named QUA1 and QUA2 or NK (N-terminal of KH) and CK (C-terminal of KH), a particularity to the GSG family of proteins (see Figure 1-1) (Ebersole et al., 1996; Jones and Schedl, 1995). The protein also contains two RG clusters, known to be associated with RNA binding (Ebersole et al., 1996). The presence of multiple proline-rich regions similarly to Sam68, as well as five tyrosines in the carboxyl end and seven consensus SH3-binding sites suggest a common role of QKI in signal transduction. This is how QKI obtained its designation as a signal transduction and activator of RNA (STAR) protein (Ebersole et al., 1996). The *qk* gene possesses at least 6 exons common to all transcripts, and alternative splicing of the last exons produce 5 different isoforms, QKI-5a and b, QKI-6, QKI-7, and QKI-G (Cox et al., 1999). Another group reported at

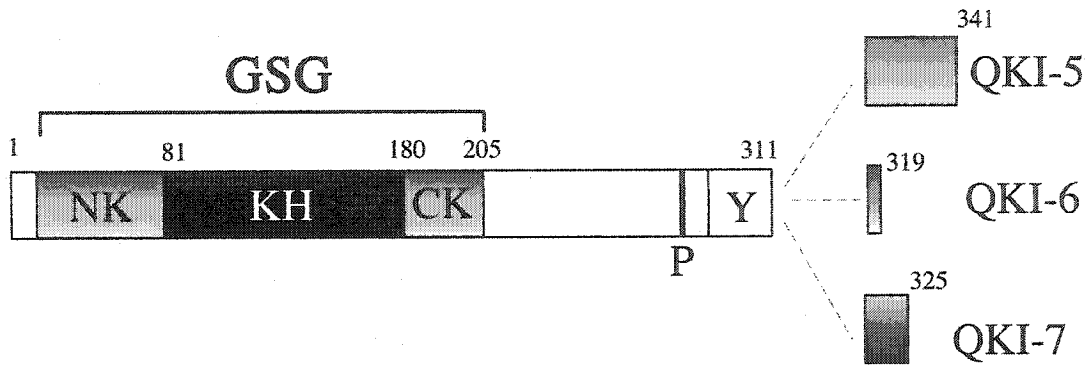

Figure 1-1. Schematic diagram of the major QKI isoforms.

The GSG, KH, NK, and CK domains are indicated above, as well as the proline (P) and tyrosine-rich (Y) regions. The extra boxes at the C-terminal denote the unique sequences of the major QKI alternatively spliced isoforms.

least 9 exons spanning ~65 kb of DNA, giving rise to six transcripts encoding five different protein isoforms. The additional isoform has a truncated KH domain, perhaps acting antagonistically in vivo (Kondo et al., 1999). Exons 1-4 are common to all transcripts, with two distinct 3'UTR downstream of exon 4. Sharing of 3'UTRs lead to similar patterns of expression, suggesting a regulatory role for 3'UTRs in cellular localization (Kondo et al., 1999). Several AUUUA putative mRNA-destabilizing sequences are present in all of the 3'UTRs (Sachs and Wahle, 1993).

1.4.2 Localization of *quaking*

Whole-mount in situ hybridization in embryos has revealed that *qk* expression is first detected in the neuroepithelium of the head folds at 7.5 days, with strong expression detected ventrally in the nascent brain and neural tube of 8.5-9.5 day-old embryos

(Ebersole et al., 1996). The 5-kb transcript is the isoform mostly detected in testis, whereas the 6- and 7-kb transcripts are detected in brain and lung (Ebersole et al., 1996). Some of the 6-kb transcript is also detected in the heart. Further analysis by northern have shown that the 5-kb transcript is highly expressed in the first two postnatal weeks, and decreases dramatically after day 14, whereas the 6- and 7-kb transcripts are expressed all throughout postnatal development and adulthood, with a strong peak at ~day 14, coinciding with the active timing of myelination (Hardy et al., 1996). Additional 5- and 7-kb transcripts, 5-kb-B and 7B-kb respectively, have been identified. These transcripts showed similar patterns of expression as their same sized transcripts, although the 7-kb-B isoform is barely detectable in any tissue (Kondo et al., 1999). An interesting observation is the splicing of two different messages (5-kb-B and 6-kb) for the QKI-6 isoform. It seems that the long and short forms are both expressed in the brain, whereas the short form is expressed more prominently in the heart (Kondo et al., 1999). Although all of the identified transcripts presumably produced lead to different isoforms, their expression has yet to be shown. It is also interesting to note that the ΔKH transcript is mostly expressed on postnatal day 3, with a sharp decrease thereafter. All the major transcripts were detected in *quaking* viable mice and were reduced to ~30% of normal levels (Kondo et al., 1999). Therefore the decrease in expression might be due to the large deletion in the *qk* alleles.

Immunostaining on mouse brain sections identified QKI positive cells in white matter tracks (Hardy et al., 1996). QKI staining was especially remarkable in myelinating oligodendrocytes and Schawnn cells. It was also detected in other CNS cell types, including Bergmann glia of the Purkinje cell layer in the cerebellum, as well as in

moderate amounts in astrocytes of the hippocampus, cerebral cortex and all white matter tracts, including the optic nerve (Hardy et al., 1996). Neurons on the other hand are devoid of any QKI proteins (Hardy et al., 1996).

QKI-5 localization is restricted to the nucleus, whereas both QKI-6 and QKI-7 are located in the perikaryal cytoplasm with lower levels in the nucleus (Hardy et al., 1996). Although the QKI isoforms are expressed in the cytoplasm of myelinating cells, none were detected in the myelin sheaths themselves (Hardy et al., 1996). The localization of QKI-5 is different in astrocytes, where some staining in the proximal processes of the astrocytes has been observed (Hardy et al., 1996). A 7-amino acid novel nuclear localization sequence, RVHPYQR, was found in the carboxyl terminus of QKI-5, which seems to be a sequence subclass for GSG proteins such as Sam68 and ETLE/SLM-2 (Wu et al., 1999). This was termed STAR-NLS and is also found in non-STAR proteins. This nuclear transport seems to be dependent on transcription, as transcription inhibition with actinomycin D resulted in trapping of QKI-5 in the cytoplasm (Wu et al., 1999). QKI-5 has been shown to shuttle between the nucleus and the cytoplasm, a function unique to this protein (Wu et al., 1999).

When qk^{v} mouse brains were immunostained for QKI, Artzt's group found that QKI-6 and QKI-7 were absent from myelin-forming cells, whereas QKI-5 levels were comparable to normal mice (Hardy et al., 1996). These results were similar in the PNS. This was cell specific, as astrocytes and Bergmann glia still expressed QKI levels comparable to normal mice. The *quaking* viable mice exhibit a rostral-caudal gradient in the severity of dysmyelination, with the greatest severity in the forebrain tracts, and in areas such as the corpus collosum and anterior commissure, no QKI isoforms can be

detected (Hardy et al., 1996). Other dysmyelinating mice such as the *shiverer* and *jimpy* mice were also observed for changes in QKI levels, but at P14, no changes in the levels or the localization of QKI proteins have been observed (Hardy et al., 1996).

1.4.3 The QUAKING proteins dimerize

Most proteins possess multiple KH RNA-binding domains, suggesting that more than one KH domain is required for stable and specific RNA binding (Gibson et al., 1993; Siomi et al., 1994). Since QKI only possesses one KH domain, it has been postulated that oligomerization optimizes RNA binding. Indeed it has been shown that QKI-7 can homodimerize, and that it can associate to close family members such as *C.elegans* GLD-1, as well as another KH containing protein, *Drosophila's* BicC (Chen et al., 1997). Further mapping experiments showed that the NK region (18-57a.a.) is necessary for dimerization (Chen and Richard, 1998). Unlike Sam68, this interaction did not require RNA, as RNase treatment beforehand had no effect on dimerization of QKI-7 (Chen and Richard, 1998). Computer analysis of the GSG domain has revealed that this interaction is probably dependent on a predicted coiled-coil region. Strengthening this hypothesis is the fact that the qk^{kt4} lethal mouse mutation falls into the domain abolishing this predicted coiled-coil interaction (Chen and Richard, 1998). Treating C6 Glioma cells with the irreversible cross-linker bis(maleimido) hexane resulted in QKI complexes ranging from 80-90 kDa, suggesting that these proteins exist as heterodimers (Chen and Richard, 1998). Another group confirmed that QKI homodimerizes and can be disrupted with the qk^{kt4} mutation (Wu et al., 1999).

1.5 The possible functions of *quaking*

1.5.1 Neurogenesis

The presence of QKI-5 during embryogenesis suggests a role for the protein in the development of the nervous system. Indeed, QKI has been found to be expressed in neural progenitors of the ventricular zone (vz) during CNS development (Hardy, 1998). In mammals, the neural and glial subtypes of the mature CNS are generated from a morphologically homogeneous population of neuroepithelial cells emerging from the neural tube (Lumsden and Krumlauf, 1996). During the beginning of embryogenesis, proliferating neural progenitors become restricted to the vz, whereas neural progenitors differentiating into neurons migrate centrifugally away from the vz (Hardy, 1998). Once most of neurogenesis is completed, the major glial subtypes of the CNS are able to emerge. Little is known about these events, but it is known that NeuroD and the *achaete-scute* complex are needed for neuronal determination, whereas inactivation of other genes such as Neuron Restrictive Silencer Factor are important for the negative regulation of neuronal phenotype (Schoenherr and Anderson, 1995; Tanabe and Jessell, 1996). Therefore gliogenesis probably requires some silencing of neural determinant genes in conjunction with glial activating genes. QKI expression in the vz seems to be specific for cells of glial lineage, as its expression is down-regulated during neuronal differentiation. All three QKI isoforms are reported to be expressed at E10.5 to E15.5, but it is mostly the QKI-5 isoform that is detected. Interestingly, QKI-5 was also observed in the cytoplasm of migrating cells at E13.5 and E14.5 (Hardy, 1998). Expression of the QKI proteins after birth can be detected in residual vz as well as in the posterior subventricular zone (SVZ). Differentiated oligodendrocytes first appear in the cervical spinal cord and hindbrain at

E14.5-E16.5, which coincides with QKI positive glial progenitors observed emerging from the vz at around E13.5 (Hardy, 1998).

1.5.2 Blood vessel development

Although evidence thus far points toward a role for QKI in glial development, it was recently suggested that QKI is involved in blood vessel development (Noveroske et al., 2002). These authors provide evidence that there is no defect in cardiac muscle differentiation or function in qk^{k2}/qk^{k2} mice, and that these mutants lack a well-developed vessel network in the yolk sac. QKI-5 immunostaining was detected at this early stage, but in the cytoplasm of the endodermal cells adjacent to the vascular cells in the yolk sac. In qk^{k2}/qk^{k2} embryos, the endothelial tube structures are immature causing a failure to remodel and as a result, endothelial tubes are not invested by mural cells, causing an unstable capillary plexus insufficient to sustain the developing embryo (Noveroske et al., 2002).

1.5.3 Translational repression

The QKI homologue GLD-1 causes translational repression of the sex determination gene TRA-2 (Jan et al., 1999). This is accomplished by GLD-1 binding to the regulatory element TGE (tra-2 and GLI element) in the 3'UTR. Mutation of the glycine 227 to an asparagine in the KH motif resulted in loss of GLD-1 binding to the TGEs (Jan et al., 1999). It has been demonstrated both in vitro and in vivo that QKI-6, as well as the other QKI isoforms, can behave in a similar manner and replace GLD-1

(Saccomanno et al., 1999). QKI-6 was able to bind to TGEs and specifically repress translation of reporter constructs, causing 40-50% decrease in activity, like GLD-1. The qk^{kt4} mutation did not abolish binding to the TGEs although this interaction was weaker, but it was unable to repress translation of a reporter RNA containing the tra-2 wild-type 3'UTR. Northern blot analysis showed that there were no decreases in RNA levels, therefore this effect was not due to RNA decay. This was also confirmed in vivo by functionally replacing GLD-1 by QKI-6 in hermaphrodites (Saccomanno et al., 1999). It is thought that repression of tra-2 translation by GLD-1 probably occurs by either binding directly or indirectly to translational initiation factors, mask the tra-2 transcripts from the translational machinery, or that it may influence the length of the poly(A) tail, suggesting a similar role for QKI (Jan et al., 1999).

1.5.4 QKI mRNA targets

During our quest to find RNA targets for QKI, Feng's group observed that QKI-7 bound to MBP mRNA (Li et al., 2000). Using the streptavidin-biotin pull down assay, they showed that QKI associated with the 3'UTR of MBP. This binding seemed to require part of the coding region as well, since a preferential isoform binding was observed, M14>M18.5>M21.5 (Li et al., 2000). An RNase protection assay was developed to directly measure the MBP mRNA isoforms, which suggested that reduction of MBP expression is due to isoform-preferential destabilization of MBP mRNAs in the cytoplasm of qk^{v} brain during early myelination. There was a decrease of MBP mRNA in the qk^{v} myelin fraction, but with an accumulation of MBP mRNA associated with the membrane-free polyribosomes, providing evidence of their mislocalization (Li et al.,

2000). The levels of MBP mRNA were significantly reduced during the time of myelination from P12-20, and it was more pronounced in mRNA isoforms missing the exon 2 (M18.5 and M14) (Li et al., 2000). The total MBP expression was more severely reduced at all developmental stages compared to the decrease in mRNA levels previously reported (Carnow et al., 1984; Sorg et al., 1987; Sorg et al., 1986). This did not seem to be due to differential splicing as no changes in the other isoforms were detected, and transcription levels were unaffected as well (Li et al., 2000). MBP mRNAs in the brainstem were ~80% of the levels detected in qk^v/wt littermate controls, but the MBP protein levels were <10% of normal levels, similarly to previous reports (Barbarese, 1991; Li, 2000). Since MBP mRNAs are able to associate with free ribosomes, it suggests that they are actively translated and that protein degradation occurs afterward. It is interesting to note that the MBP mRNA M14 was reduced as well in *jimpy* mice, suggesting a more global regulation of this isoform during demyelination (Zhang and Feng, 2001).

CNP mRNA was also shown to be weakly associated with QKI (Zhang and Feng, 2001). The enzymatic activity of CNP is abnormally low in the qk^v brain (Macklin et al., 1991). Only a slight reduction in the CNP mRNA levels was detected in the qk^v mouse (Zhang and Feng, 2001). This is in contrast to the *jimpy* mouse where both levels of MBP and CNP mRNAs are reduced (Carnow et al., 1984; Nave et al., 1987). The level of CNP expression peaks during myelination (P20) and gradually decline after, but in the qk^v mouse, CNP protein levels are reduced (Zhang and Feng, 2001). This decrease in protein expression was not due to low mRNA levels or even in a failure in translation, as CNP mRNA levels associated normally to polyribosomes.

1.5.5 QKI and splicing

The MAG pre-mRNA contains 13 exons, and alternative splicing of exon 12 by inclusion or skipping gives rise to the L- (inclusion) and S- (skipping) MAG isoforms, respectively (Lai et al., 1987; Tropak et al., 1988). Using a MAG minigene to transfect cells, it was shown that QKI-5 can repress the inclusion of exon 12, therefore suggesting that QKI-5 is a negative regulator participating in splicing of the L-MAG isoform (Wu et al., 2002). A 53-nt region in the downstream intron was identified as the QKI-5 splicing element named QASE (Quaking alternative splicing element). Splicing of other myelin components were also affected in qk^{v} mice such as PLP exon 3b and MBP exon6, whereas MBP exon 5 seemed to be affected at later times during adulthood. On the other hand, the polypyrimidine tract-binding protein (PTB), a well-characterized negative regulator of alternative splicing, gave results similar to QKI-5 (Wu et al., 2002). No direct interaction was shown to the QASE with QKI-5, but UV-crosslinking showed a protein complex made of four prominent bands of 50, 42, 40 and 38 kDa, with the latter corresponding to the QKI-5 protein translated in vitro (Wu et al., 2002).

1.5.6 QKI and remyelination

Supporting the role of QKI in oligodendrocyte differentiation is a study where QKI proteins are observed to be upregulated dramatically in cells that are transitioning from premyelinating oligodendrocytes to myelin-bearing cells (Wu et al., 2001). They have found that cytoplasmic QKIs are dramatically reduced in mature oligodendrocytes, similar to levels found in astrocytes (Wu et al., 2001). There is some growing evidence that new myelin is generated into adulthood, and this study provided evidence that ~12%

of oligodendrocytes seem to be actively myelinating in adult rat brain (Sturrock, 1987; Wu et al., 2001). Elevation of MAP1B and of the QKI isoforms in actively myelinating cells of the adult is observed, suggesting a possible role for these proteins in remyelination (Wu et al., 2001). MAP1B is thought to play a role in altering the shape of oligodendrocytes (Vouyiouklis and Brophy, 1993). They also demonstrated that an intact cytoskeleton seems to be important for QKI-6 and QKI-7 localization, as disruption of microtubules with nocadozole resulted in mislocalization (Wu et al., 2001). Elevated levels of QKI-6 and QKI-7 in oligodendrocytes in vivo were found mostly in the perikaryal cytoplasm with some presence in connecting processes as well (Wu et al., 2001).

1.6 *QUAKING* family members

Other than in the mouse, homologues of QKI have been found in humans, chickens, *Xenopus*, *Zebrafish*, *Drosophila*, as well as in *C. elegans*, some with almost complete sequence homology (Jones and Schedl, 1995; Li et al., 2002; Mezquita et al., 1998; Tanaka et al., 1997; Zaffran et al., 1997; Zorn et al., 1997). Understanding the function of QKI in other organisms is helpful in determining its actions in higher organism such as humans.

1.6.1 Human *quaking*

It has been shown that the human *quaking* gene, *Hqk*, maps to the chromosome region 6q25-q26 (Li et al., 2002). No neurological diseases have been mapped to this

region, but cytogenic alterations are associated with a variety of human malignancies, including gliomablastomas and astrocytomas (Mitclman et al., 1997; Miyakawa et al., 2000; Trent et al., 1990). Analysis of the sequence show that the *Hqk* gene contains 8 exons spanning ~200 kb genomic region, generating at least four alternatively spliced transcripts similar to the mouse, thus *Hqk*-5, -6, -7 and -7B (Li et al., 2002). The amino acid sequence is identical and there is 96% identity at the nucleotide level. There is also very high sequence similarity between human and mouse 3'UTRs, ranging from 73%-85%. Northern blot analysis on various human tissues from MTN blots revealed that high levels of the 5-kb *Hqk* message were detected in the thymus, testis, ovary and heart. The 7-kb message was mostly detected in the brain, with some in peripheral blood leukocytes (Li et al., 2002). The levels of expression observed in 18 week old human fetal heart and brain revealed low levels of the 5-kb message but high levels of 7-kb. As in the mouse, the 7B-kb message was not detected anywhere (Li et al., 2002).

Analysis of primary tumors demonstrated a high incidence of alterations in *Hqk* transcripts in gliomas (~30%) (Li et al., 2002). In three of the samples, the 7-kb transcript was not detected. Two of the samples had none of the transcripts and another had downregulated levels of the 5-kb message. No alterations were found in schawnnomas or meningiomas (Li et al., 2002).

1.6.2 Chicken *quaking*

The coding region of the chicken *quaking* orthologue is highly conserved to the mouse, with 90% identity in the cDNA (Mezquita et al., 1998). Three transcripts of 5-, 6-, and 7-kb are also detected in 6 day old embryo heart, brain, and testis. The 6-kb form

was predominant in prepuberal testis enriched in spermatogenesis, with lower amounts of the 5- and 7-kb transcripts. In adult testis, the predominant form is the 5-kb message (Mezquita et al., 1998).

1.6.3 Xenopus *Xqua*

Zorn and Krieg were the first to isolate and characterize a cDNA encoding the Xenopus *quaking* homologue (*Xqua*) and to assemble the human sequence from expressed sequence tags (ESTs) (Zorn et al., 1997; Zorn and Krieg, 1997). *Xqua* is 94% identical at the protein level to the mouse and human *qkI* (Zorn et al., 1997). Two *Xqua* transcripts of 5-kb and 5.5-kb in length correspond to the 5- and 6-kb transcripts in mice, respectively. The 5-kb message was detected in embryo at the gastrula stage, whereas the 6-kb transcript was detected at the later tailbud stage embryo. Whole mount in situ hybridization has detected *Xqua* in chordamesoderm of the dorsal blastomere lip of the midgastrula embryo (stage 11), which is analogous to the node in the mouse embryo that differentiates into the notochord (Zorn and Krieg, 1997). High levels of *Xqua* mRNA are found in the brain and the neural tube, primarily where active differentiation takes place, and it is found as well in branchial arches and developing heart. These mRNAs give rise to two different proteins, named $Xqua^{357}$ and $Xqua^{365}$, which are identical except for an 8-amino acid insertion located just carboxy-terminal to the GSG domain. $Xqua^{357}$ has been shown to reside in both cytoplasm and nucleus by microinjected mRNAs into oocytes (Zorn and Krieg, 1997).

Since this is a KH containing protein, RNA binding was confirmed by using total RNA, and homodimerization was observed as well (Chen et al., 1997; Zorn et al., 1997;

Zorn and Krieg, 1997). This RNA-binding is dependent on the KH domain as well as the carboxy terminal, as deletion of both domains resulted in severe loss of RNA-binding activity (Zorn and Krieg, 1997). *Xqua*^{357}, the embryonic form, can enhance notochord development, where overexpression of a dominant negative missing the KH RNA-binding domain led to the blocking of this event. This caused microencephaly and acephaly with truncation of the anterior notochord, to losses of the entire anterior half of the embryo and a complete loss of all head structures at higher dosages. Spina bifida was also observed in cases showing severe anterior truncation (Zorn and Krieg, 1997). The normal function of *Xqua*^{357} seems to be necessary for the accumulation of important developmental mRNAs such as *Xnot*, *Xbra* and *goosecoid* (gsc), as they are all down-regulated when injected with the dominant negative *Xqua* (Cho et al., 1991; Gont et al., 1996; Smith et al., 1991; Zorn and Krieg, 1997). These results demonstrate that *Xqua* is acting very early during development and is required for the accumulation of several essential mRNAs (Zorn and Krieg, 1997).

1.6.4 Zebrafish *zqk*

A QKI-5 orthologue was also found in zebrafish named *zqk* (Tanaka et al., 1997). It has an additional 123 nucleotides insertion, called the zebrafish specific domain (ZD). RT-PCR was used to identify two isoforms of *zqk* with and without the ZD and the splice site corresponds to the alternative splice site in mouse *qk* (Tanaka et al., 1997). Whole mount in situ hybridization of zebrafish embryos showed that *zqk* mRNA is expressed maternally in the blastula, then gradually becomes restricted to the dorsal mesoderm during gastrulation. *Zqk* is expressed in the head portion of the neural keel and putative

head neural crest cells. Although myelination takes place in zebrafish, *zqk* expression dropped to low levels in the brain when differentiation of myelin takes place, suggesting another role for this protein (Tanaka et al., 1997).

1.6.5 *C. elegans* GLD-1

Other homologues with high sequence identity to the *quaking* KH domain include the *Drosophila* How protein and the *C. elegans* GLD-1 (Jones and Schedl, 1995; Zaffran et al., 1997). *Caenorhabditis elegans* GLD-1 is a germ-line specific tumor suppressor gene that is essential for oogenesis (Jones and Schedl, 1995). *C. elegans* either are diploid animals with a single X chromosome developing only as sperm producing males, or hermaphrodites with two X chromosomes, producing sperm before switching to the production of oocytes (Jones and Schedl, 1995). GLD-1 is required for oogenesis in XX hermaphrodites (Francis et al., 1995). In null *gld-1* hermaphrodites, germ cells re-enter a mitotic cell cycle and proliferate to produce a germ-line tumor (Francis et al., 1995). It is thought that GLD-1 participates in negative regulation of mitotic proliferation in germ-line cells, and promotes the male germ cell fate in hermaphrodites (Francis et al., 1995). Sixteen out of the 32 mutations analyzed in GLD-1 occur in the region of the GSG. Of particular interest is a class A missense mutation, known for being null or a strong loss-of-function mutation. This mutation, a glycine to a serine or aspartate at position 227, affects a single residue in the KH motif (Jones and Schedl, 1995).

The sex determination gene *tra-2* is translationally regulated by elements in the 3'-UTR called TGEs for tra-2 and GLI elements (Jan et al., 1999). It is a requirement for female cell fates, and is necessary to inhibit downstream male determinants (Kuwabara

and Kimble, 1992). GLD-1 specifically binds to the TGEs and represses translation of the TRA-2 protein in the germline (Jan et al., 1999). Mutation of the Gly227Asp in the KH motif results in loss of GLD-1 binding to the TGEs (Jan et al., 1999).

Immunoprecipitation/substractive hybridization/cloning strategies have identified a series of potential mRNA targets for GLD-1 (Lee and Schedl, 2001). One of them is a yolk receptor needed for yolk uptake by late-stage oocytes called *rme-2*. It is thought that GLD-1 represses *rme-2* by its translation regulation rather than causing its degradation, as no differences in stability can be found in the *gld-1* null mutant. GLD-1 also seems to be involved in the regulation of the *pumilio* gene family, as well as *lin-45* RAF kinase, which could be accountable for the tumor formation (Lee and Schedl, 2001).

1.6.6 *Drosophila How*

The *Drosophila who/how* or *struthio* has a 43 % identity with the *quaking*. However, the maxi-KH and GSG domains are 93% and 66% identical, respectively (Baehrecke, 1997; Lo and Frasch, 1997; Zaffran et al., 1997). *Who/How* mutants die at the end of embryogenesis. Other mutants survive as adults and develop defects in wing position that extend at a 90° angle horizontal from the body axis appropriately named 'held-out-wings' (Baehrecke, 1997; Zaffran et al., 1997). Their wings do not fold properly, and also develop blisters (Baehrecke, 1997). In *How* mutants although development of the heart seems normal, the heart rate is slower than in wild-type (Zaffran et al., 1997). A missense mutation, who^{e44}, which results in an arginine to a cysteine change was identified as the culprit (Baehrecke, 1997).

Developmental northern showed two mRNA species, where expression of the 4.5-kb mRNA increases to high levels in 4- to 8-hr embryos until the end of embryogenesis (Lo and Frasch, 1997). The 4.0-kb message is only seen postembryonically in adult females, indicating that it is maternally transcribed (Lo and Frasch, 1997). The 4.0-kb cDNA encodes a protein similar to QKI-5 called How(L) for its longer six amino acids, whereas the 4.5-kb cDNA does not have any similarity to the alternatively spliced QKI-6 or QKI-7, encoding a shorter protein called How(S) (Lo and Frasch, 1997; Nabel-Rosen et al., 1999).

Recent studies have indicated that How controls tendon cell differentiation. This was observed in *how* loss-of-function mutant embryos, where tendon precursors undergo premature differentiation (Nabel-Rosen et al., 1999). The balance between the two How isoforms is responsible for the regulation of the transcription factor Stripe. The long, nuclear specific protein How(L) downregulates the Stripe protein levels by binding Stripe mRNA and inhibiting its nuclear export. The short isoform How(S), is expressed later in both compartments of the cell and is thought to counteract this inhibition (Nabel-Rosen et al., 1999). How(S) is upregulated in the muscle-bound tendon cell following Vein secretion, which activates the EGF receptor pathway (Nabel-Rosen et al., 1999). How(S) elevates Stripe levels through binding to the 3'UTR and counteracts the repression of How(L) when both isoforms are expressed (Nabel-Rosen et al., 2002). It is thought that How(S) also stabilizes its mRNA target (Nabel-Rosen et al., 1999).

This group also showed that krox20, the EGR2 homologue involved in the terminal differentiation of Schawnn cells, may function in a similar manner to Stripe (Nabel-Rosen et al., 2002). QKI-5 repressed both gfp-krox20 and gfp-stripe 3'UTR. In

contrast, QKI-6 and QKI-7 led to an increase of these fused proteins. Interestingly, a sequence motif similar to TGE is present in both Stripe and krox20 (Nabel-Rosen et al., 2002).

1.7 The GSG domain and family members

The GSG domain contains a region of ~170 amino acid which was termed GSG on the basis of the significant homology between GLD-1, GRP33 and Sam68 (GRP33/Sam68/GLD-1) (Cruz-Alvarez and Pellicer, 1987; Fumagalli et al., 1994; Jones and Schedl, 1995). The conserved structural motif called the KH domain is found within the GSG domain, suggesting an RNA binding function (Siomi et al., 1993). The GSG domain contains a single KH motif as opposed to other KH containing proteins, and extends approximately 75 amino acids into the N-terminus (NK) and 25 amino acids into the C-terminus (CK, see Figure 1-1).

1.7.1 The KH domain

The K homology (KH) domain which contains approximately 50 conserved amino acids was originally described in the pre-mRNA binding heterogeneous nuclear ribonucleoprotein K (hnRNP K) protein (Siomi et al., 1993). The domain also occurs in a diverse group of proteins (Siomi et al., 1993). The hnRNPs bind pre-RNA directly and appear to participate in pre-mRNA processing and mRNA transport from the nucleus to the cytoplasm (Siomi et al., 1993). The three dimensional structure of one of the KH domains of vigilin was determined by nuclear magnetic resonance (NMR), which showed that the domain consists of a stable βααββα fold (Musco et al., 1996). It is suggested that

this is a potential RNA binding surface, where two helices are connected by a highly conserved glycine-lysine-X-glycine (GkxG) motif (Musco et al., 1996).

1.7.2 GSG family members

One of the original proteins in the GSG alignment is the 33 kDa glycine rich protein GRP33, identified in the brine shrimp *Artemia salina* during initial characterization of hnRNP proteins and their function (Cruz-Alvarez and Pellicer, 1987). This protein has 39 glycine residues in its C-terminal flanked by glycines, typical of glycine-dimethylarginine clusters present in other RNA binding proteins. It is closely related to the major protein component of *Artemia* heterogeneous nuclear ribonucleoprotein particles, but its function remains unknown (Cruz-Alvarez and Pellicer, 1987).

Interest in Sam68 first appeared when it was discovered that it becomes tyrosine phosphorylated and physically interacts with $p60^{src}$ during mitosis, hence the name: Src-associated-in-mitosis (Taylor and Shalloway, 1994). Phosphorylation and binding are dependent on SH2 as well as SH3 domains. The SH3 domain also binds hnRNP K (Taylor and Shalloway, 1994). For a while, Sam68 was thought to be related to the RasGAP-associated p62 protein, but it is in fact encoded by the hump62 human cDNA and has no relation to the previous (Lock et al., 1996). Sam68 has been shown to interact via its SH3 and/or SH2 domains with several signaling molecules including Grb2, phospholipase Cγ-1, Ras GTPase and p85α (Richard et al., 1995), (Taylor et al., 1995). Sam68 has also been identified as a substrate for a series of tyrosine kinases from the Src

family kinases including $p56^{lck}$, $p59^{fyn}$, BRK/SIK, and ZAP70 (Derry et al., 2000; Lang et al., 1997; Richard et al., 1995; Vogel and Fujita, 1995).

Sam68 binds homopolymeric RNA poly(U) and poly(A) and tyrosine phosphorylation by $p59^{fyn}$ severely inhibits this association, suggesting a link between RNA metabolism and signal transduction (Wang et al., 1995). SH3 binding to Sam68 also decreased its ability to bind to poly(U) in vitro (Taylor et al., 1995). More specifically, Sam68 binds the RNA sequence UAAA with high affinity as deduced by SELEX (Lin et al., 1997). No physiological RNA targets have been found to date, rendering the real function of Sam68 a mystery.

Sam68 is a ubiquitously expressed nuclear protein. The integrity of the KH domain seems to be necessary for its nuclear localization (McBride et al., 1998). Transcription also seems to be essential in localization of the protein, as treatment with transcription inhibitors and deletion of the KH domain resulted in punctate nuclear structures (McBride et al., 1998). Sam68 concentrates in novel nuclear structures called SNBs for Sam68 Nuclear Bodies predominantly found in transformed cells (Chen et al., 1999). These SNBs are distinct from typical structures such as coiled bodies, gems, and promyelocytic nuclear bodies (PML) (Chen et al., 1999; Lamond and Earnshaw, 1998).

Sam68 has also been implicated in viral processes. Sam68 associates with the poliovirus RNA-dependent RNA polymerase 3D upon viral infection and relocalizes to the plasma membrane (McBride et al., 1996). In the HIV virus, Sam68 can functionally replace Rev by interacting with the RRE and participate in RRE-mediated gene expression and virus replication (Reddy et al., 1999). In the Mason-Pfizer monkey virus, Sam68 increased viral mRNA nuclear export by binding to the transport via the

constitutive transport element (CTE). However, RNA-binding defective point mutant, G178E, as well tyrosine-phosphorylated Sam68 are unable to mediate nuclear export (Coyle et al., 2003).

Recent evidence shows that Sam68 is involved in splicing, as Sam68 co-localizes with splicing-associated factors in SNBs (Chen et al., 1999; Coyle et al., 2003). A link between signal transduction and splice regulation is possible since Sam68 could act as a new extracellular signal-regulated kinase (ERK) target (Matter et al., 2002). Sam68 binds to splice-regulatory elements of the CD44 v5-exon and enhances inclusion of the v5-exon sequence. This process is impaired by phosphorylation (Matter et al., 2002).

Two mammalian proteins of high homology to Sam68 were identified and named SLM-1 and SLM-2 for Sam68-like mammalian proteins (Di Fruscio et al., 1999). These proteins also localize to the nucleus and have ~70% sequence identity with Sam68 in the GSG domain. They heterodimerize with Sam68, but only SLM-1 is tyrosine phosphorylated by Src kinases during mitosis like Sam68 (Di Fruscio et al., 1999). SLM-1 and SLM-2 colocalize to SNBs as well (Chen et al., 1999).

Database searches for expressed sequence tags (EST) similar to Sam68 led to the identification of two Drosophila KH domain proteins, named KEP1 (KH encompassing protein) and Sam50 for its high homology to the mammalian Sam68 (Di Fruscio et al., 1998). KEP1 is nuclear whereas Sam50 is cytoplasmic. These two proteins activate apoptosis in Drosophila S2 cells (Di Fruscio et al., 1998), and recently KEP1 was identified as a splicing regulator of the Drosophila caspase homologue *dredd* (Di Fruscio et al., 2003).

Another GSG protein is the splicing factor SF1 protein, which differs somewhat from other GSG family members. SF1 is a 75 kDa protein required for the formation of the first ATP-dependent spliceosomal complex A (Arning et al., 1996; Kramer, 1992). This interaction appears to be largely sequence-independent with a preference for guanosine and uridine rich sequences (Arning et al., 1996). It is also known as ZFM1, reported to be a transcription factor in the brain (Toda et al., 1994). Sequences N-terminal to the KH domain mediate the interaction between hSF1 and the splicing factor U2AF65, which binds to the polypyrimidine tract upstream of the 3' splice site (Rain et al., 1998).

1.8 Myelination

1.8.1 General introduction

Myelination is an important biological process that ensures proper nerve signaling over long distances, and is also an efficient space saver (reviewed in Baumann, 2001). The myelin is responsible for ensheathing the axons and is produced at the distal tips of oligodendrocytes and Schawnn cells (Barbarese, 1991; Campagnoni et al., 1990). This process occurs in a multitude of steps. In the central nervous system (CNS), there first is a migration of the oligodendrocyte precursor cell to the axons that are to be myelinated. Second, there is adhesion of the oligodendrocyte process to the axon. Thirdly, there is a spiraling of the processes around the axons with a predetermined area to be myelinated and space to remain unmyelinated (i.e. Nodes of Ranvier). A length of myelin sheath is known as an internode, delineated at either end by nodes of Ranvier (reviewed in Hogan and Greenfield, 1984b). The myelin sheath is rich in lipids and has a low water content, and its high resistance and low capacitance ensures that the current flows down the fiber

to the next node rather than leak back across the membrane (reviewed in Baumann, 2001). The impulse is generated through sodium channels densely packed into regularly spaced tracts of the fiber at the nodes of Ranvier. Consequently, the impulse is conducted along the axon about 10 times more rapidly than in the absence of myelin, and since excitation occurs only at the nodes of Ranvier, less energy is spent in conduction of the action potential (reviewed in Hogan and Greenfield, 1984c).

1.8.2 The nervous system

Myelination first starts in the spinal cord and then progresses to the brainstem and cerebral cortex. In vertebrates, myelin is associated with the nervous system, which exists in two forms: the central and peripheral nervous system (CNS and PNS) (reviewed in Hogan and Greenfield, 1984b). Myelination first appears in the PNS, where the Schawnn cell is responsible for the formation and maintenance of myelin. Oligodendrocytes are the cells responsible for myelin formation in the CNS, which is initiated when the axons to be myelinated acquire a diameter of approximately 1 μm. In most laboratory rodents, PNS and CNS myelination is fairly complete by 3-4 weeks of age (reviewed in Hogan and Greenfield, 1984b). Schawnn cells and oligodendrocytes have similar function, but there are a few differences. For example, Schawnn cells only myelinate one axon, whereas one oligodendrocyte can myelinate many segments of axons simultaneously, up to 30-50 internodes of myelin. PNS myelin has a larger amount of cytoplasm around the PNS axon as compared to the CNS myelin. Also, the myelinated axons of the PNS are bathed in nodal-gap substance from the myelin sheath and are not exposed to the extracellular space, unlike the CNS myelin (reviewed in Hogan and Greenfield, 1984b).

1.8.3 Oligodendrocyte differentiation and maturation

Glial cells, which include the myelinating cells as well as astrocytes and microglia, constitute the majority of cells (90%) in the nervous system (Pfrieger and Barres, 1995). They are necessary for correct neuronal development and for the function of mature neurons (reviewed in Baumann, 2001). They first appear early in development at the time of neurogenesis during the early gestational period, and provide support for neurons during their migration to the cortex. Oligodendrocyte precursors originate from neuroepithelial cells of the ventricular zone, and migrate very far from the subventricular zone (SVZ) to populate the brain and form white matter (Doetsch et al., 1997). The earliest precursor cells recognized are proliferative cells expressing embryonic neural cell adhesion molecules (Pfeiffer et al., 1993). Oligodendrocyte progenitors are mostly recognized by their bipolar morphology and the presence of specific markers such as the glycolipid GD3, the recognized antigen A2B5, and the chondroitin sulfate proteoglycan NG2. Also expressed are nestin, PSA-NCAM (polysialyated neural cell adhesion molecule), PDGFα, DM-20 and CNP (Pfeiffer et al., 1993).

The proliferation and differentiation of oligodendrocyte precursor cells is dependent on electrical activity in vivo, as well as growth factors, cytokines, hormones and neurotransmitters (Barres and Raff, 1993). The growth factors PDGF and basic FGF (bFGF) are mitogenic, and PDGF is also chemotactic for oligodendrocyte progenitors and promote their short-term survival (Barres et al., 1992). Other growth factors include insulin-like growth factor (IGF-1), neurotrophin-3 (NT-3), brain-derived neurotrophic factor (BDNF), transforming growth factor-β (TGF-β), glial growth factor (GGF), ciliary neurotrophic growth factor (CNTF), IL-6 and IL-2 (McMorris et al., 1988; Barres et al.,

1994; McTigue et al., 1998; Canoll et al., 1996; Barres et al., 1993; Otero et al., 1997). Neurotransmitters are also involved in this process, another function besides nerve conduction. Some examples include glutamate, involved in shaping of the oligodendrocyte population, the main receptor being dl-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) (Yuan et al., 1998). Present as well are the dopamine D3 receptor (DR3), expressed in precursors and immature oligodendrocytes, and the $GABA_A$ receptors (Bongarzone et al., 1998; Berger et al., 1992).

After migration in the mammalian CNS, progenitors settle along fiber tracts of the future white matter and then transform into preoligodendrocytes, multiprocessed cells which keep the property of cell division and acquire the marker O4 (Hardy and Reynolds, 1993). At this stage, they are less motile and lose their mitogenic response to PDGF. In the absence of an environmental mitogenic stimulus, oligodendrocyte progenitors tend to cease proliferation and differentiate. The preoligodendrocyte becomes an immature oligodendrocyte and starts expressing the markers GalC, RIP, CAII, CNP and MBP while losing GD3 and A2B5 (reviewed in Pfeiffer et al., 1993). After one to two days, mature oligodendrocytes develop with the regulated expression of terminal markers and the synthesis of the myelin membrane. The appearance of MBP, MAG and PLP signify a mature oligodendrocyte, and MOG correlate with late stages of the maturation of myelinated oligodendrocytes (Solly et al., 1996). Once mature, oligodendrocytes cannot migrate, therefore preventing premature differentiation of progenitors is crucial for ensuring that they successfully make it to their final destination (Pfeiffer et al., 1993). The Notch receptors are present on oligodendrocytes and their precursors and are thought to control this process (Wang et al., 1998). Downregulation of Jagged1 expression by

retinal ganglion cells correlates with the onset of myelination in the optic nerve, as well as maturation of immature astrocytes.

Oligodendrocytes continue to increase in number for 6 weeks post natal in rodents (Barres and Raff, 1994). The final number of mature myelinating oligodendrocytes is determined by the proliferative rate of their progenitors as well as the process of apoptosis that occurs during development (Barres et al., 1992; Barres and Raff, 1994; Trapp et al., 1997). The cells that make contact with axons survive and begin myelination.

1.8.4 Components of myelin

Myelin is known for specific features such as its periodic structure, with alternating electron-dense and light layers (reviewed in Baumann, 2001). The myelin differs from other membranes in its very large lipid/protein ratio (80:20). The lipid composition is relatively similar to other cells, where the major lipid component of myelin is cholesterol, containing less phosphotidylcholine and an abundance of glycolipids (reviewed in Hogan and Greenfield, 1984d). The contents of water and salts in lamellar myelin are reduced, which make up ~35-45% of the total volume. The myelin proteins generally function as spacers to maintain normal membrane separations at the cytoplasmic and extracellular appositions (reviewed in Hogan and Greenfield, 1984d). The distance between the layers is due to a balance of attractive and repulsive forces, and molecular contact between components that protrude into the spaces between the membranes. The basic proteins have been proposed to be the structural cement in myelin formation and stabilization (reviewed in Hogan and Greenfield, 1984d).

The myelin basic protein (MBP) and proteolipid protein (PLP) account for the major portion of myelin proteins. PLP is the major myelin protein, constituting 50% of total protein. The predominant form is PLP, but another alternatively spliced variant called DM-20 has a 35 amino acid deletion and is mostly seen in the PNS (Nave et al., 1986). It has been suggested that PLP forms a stabilizing membrane junction after myelin compaction similar to a "zipper" (Klugmann et al., 1997).

MBPs comprise 30% of the protein content in the CNS myelin (Campagnoni and Macklin, 1988). The MBP gene is contained within a larger transcription unit called the Golli-MBP gene for gene expressed in the oligodendrocyte lineage, which is also found in the immune system and in some neurons (Campagnoni et al., 1993). There are four major protein isoforms, the 21.5 kDa, 17 kDa, 18.5 kDa and 14 kDa isoforms (de Ferra et al., 1985). The 21.5 kDa and 17 kDa isoforms contain exon II and appear earlier during development. Their relocalization from the cytoplasm to the nucleus suggests a role in the regulation of myelination (Allinquant et al., 1991; Pedraza et al., 1997). The expression of the MBP mRNAs can be detected as early as E14.5 in the spinal cord of the mouse (Peyron et al., 1997). It has been shown by in situ hybridization in cultured oligodendrocytes that MBP mRNA is mostly located in the processes (Barbarese, 1991). MBP is thought to be synthesized on free ribosomes at the distal end of the processes probably near the site of myelin assembly (Colman et al., 1982; Verity and Campagnoni, 1988). Once it has associated with the myelin sheath, little MBP can be detected in the oligodendrocyte cell body (Sternberger et al., 1978). Posttranslational modifications such as acetylation, phosphorylation and methylation can occur. Methylation has been suggested to be important in compaction of the membrane during myelin maturation

(Campagnoni and Macklin, 1988). In the PNS, MBP is present but is not thought to play a major role in myelin compaction, as it can be replaced by P0. The glycoprotein P0 is the major protein of the PNS, accounting for 50% of the total protein content. It is thought to participate in membrane separation at the extracellular surfaces (Ding and Brunden, 1994).

Oligodendrocyte-specific protein (OSP) is present in oligodendrocytes and is very similar to PMP-22 in the PNS (Bronstein et al., 1997). It is the third most abundant protein in the CNS after MBP, and is a tight junction protein. CNP represents 4% of the total number of myelin proteins, and it is a 2',3'-cyclic nucleotide hydrolase, but no 2',3'-nucleotides have been found in oligodendrocytes (Vogel and Thompson, 1988). This enzyme is not found in compact myelin, but in the cytoplasm of oligodendrocyte and in the paranodal loops.

Myelin-associated-glycoprotein (MAG) is an adhesion molecule enriched at the periaxonal membrane of myelin internodes, thought to be responsible for maintaining myelin-forming cell-axon contact and spiral growth of myelin (Bo et al., 1995). It is suggested that MAG may have a role in helping oligodendrocytes distinguish between myelinated and non-myelinated axons in the CNS. MAG is a large molecule of ~100 kDa and represents 1% of the total amount of myelin proteins (D'eustachio et al., 1988). There are two regulated spliced isoforms of MAG, L-MAG and S-MAG, for large and small MAGs respectively. These isoforms differ in their alternatively spliced C-terminal. The L-MAG and S-MAG isoforms are present in approximately equal amounts in the mice CNS. However, the large L-MAG isoform is predominant during early stages of myelination (Bo et al., 1995; Tropak et al., 1988). The L-MAG contains several putative

phosphorylation sites and the tyrosine kinase *fyn* has been identified as a downstream effector of this protein (Umemori et al., 1994; Jaramillo et al., 1994). Further evidence strengthening an association of *fyn* with MAG is revealed by the knock-out *fyn* mice which displays a dysmyelination phenotype (Sperber et al., 2001; Umemori et al., 1994). MAG belongs to a family of glycoproteins sharing a common L2/HNK1 carbohydrate epitope and is also part of the family of syalic acid binding lectins, suggesting that it may be involved in cell surface recognition (Quarles, 1997). Myelin/oligodendrocyte glycoprotein (MOG) is very similar to MAG, also expressing the L2/HNK1 epitope. MOG is located on the outer lamellae of compact myelin sheath in the CNS, and correlates with late stages of myelination (Solly et al., 1996).

Other proteins known to be important for proper myelination include the Myelin-Associated Oligodendrocyte Basic Protein (MOBP), a small highly basic protein that is located in the major dense line of myelin similarly to MBP. Oligodendrocyte-myelin glycoprotein (OMgp) is a minor protein that localizes to the paranodal areas and Cx32 is very important in gap junctions of oligodendrocytes (Habib et al., 1998; Scherer et al., 1995; Yamamoto et al., 1994). Myelin/oligodendrocyte specific protein (MOSP), RIP antigen, and NI-35/250 are all also found among a multitude of proteins in myelin (reviewed in Baumann, 2001).

1.8.5 Demyelination and dysmyelinating diseases

Demyelination can block or slow down signal conduction, as in the case with a number of demyelinating diseases. Loss of myelin, even from a single internode, is sufficient to block conduction (reviewed in Baumann, 2001). A number of genetic and/or

inflammatory diseases may affect myelin formation (dysmyelinating) or its maintenance (demyelinating). Myelin diseases are grouped in different classes determined by certain criterias (reviewed in Hogan and Greenfield, 1984e). The first class consists of acquired allergic (inflammatory) and infectious diseases of myelin. *Quaking* belongs to the second class of hereditary metabolic diseases of myelin, in which inborn errors of metabolism often become apparent in the first decade of life and are usually characterized by a diffuse loss of both myelin and axons. The third class consists of acquired toxic-metabolic disorders, secondary to the action of exogenous myelinotoxic compounds. The fourth class comprises of nutritional diseases of myelin. The fifth class consists of traumatic diseases of myelin brought on by events such as edema, compression by tumors, barbotage and pressure release (reviewed in Hogan and Greenfield, 1984e).

Multiple Sclerosis (MS) is a class one demyelinating disease and happens to be the most common in humans. MS is still not very well understood, but the common concept is that the fibers abruptly lose their myelin sheaths due to macrophages that localize to the myelin membrane and lyse the myelin from the axons (Trapp et al., 1998). MS is most prevalent in young adults between the ages of 20-40 with a male/female ratio of 1:1.5 (reviewed in Hogan and Greenfield, 1984e). There is a link with geographic distribution as well as a close association of histocompatability haplotypes. Most patients will initially experience a complete remission after each episode, however it gets worse with time and is followed by incomplete remissions. The patients eventually develop an increasing neurological deficit. MS white matter increases its water content and decreases total lipids. MBP is completely lost in the center of most plaques and is reduced at plaque margins, whereas PLP, MAG, and CNP proteins are reduced (reviewed in Hogan and

Greenfield, 1984e). These observations are not common to all demyelinating diseases, as certain diseases such as Krabbe's has relatively normal amounts of myelin components, but very low amounts of the enzymes CNP and carbonic anhydrase II (CA). Perlizaeus-Merzbacher disease caused by a mutation in the PLP gene is perhaps the human demyelinating disease most similar to the hypomyelinating mouse models *jimpy* and *quaking* (reviewed in Hogan and Greenfield, 1984e).

Dysmyelinating mutants have been useful for the study of myelin biology at a time when molecular biological techniques were not as powerful as they are today. They have contributed considerably to our knowledge of genetic myelin disorders, particularly from the morphological and biochemical standpoints. Not only have myelin mutants been important for the identification and cloning of genes possibly related to human diseases, but they are also useful in the analysis of myelin development and biology of myelin-forming cells. Furthermore, they are essential for the development of therapeutic strategies useful for human diseases. By comparing the *quaking* viable mouse to other dysmyelinating mice mutants, it is also possible to evaluate common dysmyelination effects in the brain and determine how these correlate with the *quaking* gene lesion.

The first recognized mutant animals used for studying myelin were the *jimpy*, *quaking* and *myelin synthesis deficiency (msd)* mice (reviewed in Hogan and Greenfield, 1984a). The *jimpy* disorder is a sex-linked recessive disorder discovered in 1954 and caused by a mutation in the PLP gene (Phillips, 1954). These animals develop weakness or paralysis of the hind limbs at 11-12 days postpartum, followed by tonic seizures and finally death by 30-35 days (Billings-Gagliardi et al., 1980; Duncan et al., 1989; Hogan and Greenfield, 1984a). They lack myelin in the entire CNS, while the PNS myelin

appears completely normal. The number of oligodendrocytes is reduced by 50% and there is a relative increase of large glial-cell precursors (Knapp et al., 1986). There is a deficiency of myelin-associated lipids, where the PLP reduction is the most pronounced and around half of the MBP is decreased in the *jimpy* brain. (reviewed in Hogan and Greenfield, 1984a). The *msd* mutant is very similar to *jimpy*, but the mutation is not as severe (Billings-Gagliardi et al., 1980). A similar mutation in the PLP gene has also been identified in the rat called *myelin deficiency (md)*, with generalized tremors followed by death at one month of age (Duncan et al., 1987). Other mutants of PLP/DM20 include the *canine shaking pup*, the *rumpshaker* mouse, and the *paralytic tremor* rabbit (reviewed in Baumann, 2001; Griffiths et al., 1981).

The *shiverer* mouse is an autosomal recessive mutation that was discovered in 1973 (Biddle et al., 1973). These mice have a generalized action tremor appearing at 12 days of age and become progressively worse in the hind limbs (Doolittle and Schweikart, 1977; Hogan and Greenfield, 1984a). After 30 days, frequent clonic seizures occur and they usually die four to five months after birth. The mutation occurs in the MBP gene whose product is hence almost completely absent from the CNS as well as the PNS, though to a lesser extent (Dupouey et al., 1979; Roach et al., 1985). The PNS appears normal, but there are anomalies most probably resulting from anoxia associated with the seizures. Hybridization studies have shown that there is reduced MBP mRNA as well (Roach et al., 1983), and also a deficit in PLP protein can be observed (reviewed in Hogan and Greenfield, 1984a). A less severe MBP mutant called the *myelin deficient mutant (mld)* has been described, and these mice survive to adulthood (Doolittle and

Schweikart, 1977). A reduced level of normal length MBP transcripts is observed comparable to approximately 5% of wild type mice.

The *twitcher* mutant is an autosomal recessive mutation, an animal model for human Krabbe's disease (Duchen et al., 1980). At around 30 days of age, there is the appearance of progressive muscle weakness, wasting of trunk and limbs, and death in the third month (reviewed in Hogan and Greenfield, 1984a). There is loss of myelin in both the CNS and PNS, with large multinucleated macrophages. There is no difference from control littermates at day 10, suggesting a chronological sequence of early normal myelination, hypomyelination and myelin breakdown (reviewed in Hogan and Greenfield, 1984a).

The *trembler* mutant is another mouse that has a spontaneous mutation producing convulsions in young animals and a generalized tremor (Falconer, 1951). Here, it is the PNS that is mainly affected and not the CNS. The PNS is hypomyelinated with an increase of up to 10 fold in Schawnn cell numbers (reviewed in Hogan and Greenfield, 1984a). This is caused by a missense mutation in the hydrophobic domain of PMP22 (Martini, 1997)

Some models of dysmyelination mutants can be induced, for instance the well characterized Experimental Allergic Encephalamyelitis (EAE) mouse. This experimental disease is inducible by sensitizing the animal with an inoculation of myelin proteins (Hogan and Greenfield, 1984a; Wekerle, 1993). After 2-3 weeks, animals become paralyzed. The acute lesions bear some morphological resemblances to acute MS, justifying the strong interest in this mouse model.

One dysmyelination mutant of interest in regards to *quaking* is the P0 knock-out mouse. It is interesting to note that in the P0-/- mice, neither the QKI-6 or QKI-7 proteins are expressed in Schawnn cells similarly to the *quaking* viable mouse (Xu et al., 2000). This suggests that QKI-6 and QKI-7 are probably regulated by some event absent in these two mice. As a result of mice lacking P0, Schawnn cells have a down-regulation of PMP22, and an up-regulation of MAG and PLP apparent after 4 weeks, which might be due to a compensatory mechanism to restore myelin. No apparent changes in MBP could be seen at 6 months of age (Xu et al., 2000). The P0-/- mouse displays abnormal formation of the nodes of Ranvier and Schmidt-Lantermann incisures, differing from those of the *quaking* and *trembler* mice, which are of normal appearance (Xu et al., 2000).

1.8.6 Remyelination

Remyelination is possible, as it has been shown that the PNS possesses the ability to remyelinate within 1-2 weeks after injury (reviewed in Hogan and Greenfield, 1984b). Schawnn cells undergo rapid division after the internodes degenerate, and the daughter cells migrate to occupy separate territories along the naked fiber. But this remyelination is not absolute given that axons have a reduced diameter and thinner than normal PNS myelin sheaths even months later. The CNS on the other hand does not remyelinate as easily since oligodendrocytes have poor mitotic activity and the impact of oligodendrocyte cell loss is more severe in the CNS, as one oligodendrocyte myelinates many internodes. More recent studies have shown that remyelination is possible in the CNS through immature oligodendrocytes (reviewed in Baumann, 2001; Capello et al.,

1997; Duncan, 1996; Scolding, 1997). Understanding how myelination occurs as well as the possibilities of remyelination will lead to great advances in the treatment of demyelinating diseases such as MS as well as understanding how damage is produced after other culprits such as spinal cord injury. It is also very important to understand the deeper role of myelin in its protecting and nurturing relationship with neurons, the basis of our life signaling.

1.9 Apoptosis

1.9.1 The KH proteins and apoptosis

Increasing amount of evidence suggests that KH containing proteins might have a role in apoptosis. A proteomic analysis of Jurkat cells identified 21 proteins, 15 of which contained RNA binding motifs that are modified during Fas-induced apoptosis, suggesting an important role for RNA binding proteins in the apoptotic process (Thiede et al., 2001). Of these KH containing proteins, ZMF1/SF1 has been shown by differential-comparative cDNA display on myeloid cells to be upregulated after p53-induced apoptosis (Vernet and Artzt, 1997). The MCG10 protein, which has two KH domains, can suppress proliferation of cells by inducing apoptosis and cell cycle arrest in G2/M (Zhu and Chen, 2000). Overexpression of the Drosophila dFMR1 leads to apoptotic cell loss, and KEP1 as well as Sam50 can induce apoptosis when transfected in S2 insect cell lines (Di Fruscio et al., 1998; Wan et al., 2000). Just recently, KEP1 was identified as an apoptotic regulator, where KEP1 binds to *dredd* mRNA, the Drosophila equivalent of caspase-8 (Di Fruscio et al., 2003). Removal of KEP-1 causes the inclusion of intron II in the *dredd* transcript. This creates the β-isoform of *dredd*, which lacks the

catalytic domain of the caspase and is therefore inactive.(Di Fruscio et al., 2003). Ultimately, the over-expression of QKI-7 into mouse fibroblast NIH 3T3 cells leads to chromosome condensation and membrane blebbing, typical characteristics of apoptosis (Chen and Richard, 1998). This will be the focus of the study in Chapter 2.

1.9.2 The apoptotic pathways

The term apoptosis was adopted by Currie and colleagues in 1972 to describe a common type of programmed cell death (Kerr et al., 1972). We know that apoptosis results in proteolytic cleavage of over 100 substrates in mammalian cells, usually mediated by aspartate-directed cysteine proteases called caspases (Hengartner, 2000). Two main pathways are described in programmed cell death: the death receptor pathway (extrinsic) and the mitochondrial pathway (intrinsic) (see Figure 1-2) (Ashkenazi and Dixit, 1998; Tartaglia and Goeddel, 1992).

The caspases involved in the apoptotic cascade were first identified in *Caenorhabditis elegans*, where a limited number of genes participate in this process and encapsulates a good model of the apoptotic pathway. *Ced-3* encodes a caspase, *ced-4* has homology to mammalian Apaf-1, *ced-9* is a homologue of Bcl-2, and *egl-1* corresponds to a Bcl-2 family member containing only the BH3 apoptotic domain (Hengartner et al., 1992; Metztein, 1998; Yuan and Horvitz, 1992; Yuan et al., 1993). According to the latest models, CED-9 binds to CED-4 maintaining it inactive. As a result, CED-4 is unable to activate CED-3 (Metztein, 1998). Expression of EGL-1 displaces CED-4 from the complex, consecutively allowing the activation of CED-3 (Conradt and Horvitz, 1998).

Drosophila has also been a well-studied apoptotic model. It has three pro-apoptotic proteins named Reaper, Hid and Grim, all located on the H99 chromosomal locus (Chen et al., 1996; Gozani et al., 2002). Their death-inducing domain resides in a four amino acid stretch in the N-terminal known as IBM (IAP binding motif) or RGH (reaper, grim, hid) (Gozani et al., 2002; Vucic et al., 1998). A fourth protein within this locus was recently cloned called Sickle, which seems to be sensitive to genotoxic stimuli (Srinivasula et al., 2002). These proteins are thought to displace the Inhibitors of Apoptosis (IAPs) from caspases, causing the cell to self-destruct (Gozani et al., 2002; Wang et al., 1999).

In the mammalian system, the death receptor pathway is activated by ligand binding to its receptor, which contain death domains (DD) and death effector domains (DED) (see Figure 1-2) (Ashkenazi and Dixit, 1998). Upon assembly of this complex, procaspase-8 is activated and can then cleave caspase-3, responsible for the cleavage of key substrates in the cell (Kaufmann and Hengartner, 2001). One of the most common members of this family include the Fas/CD95 ligand, which binds to its corresponding receptor known for its Fas-associated death domain (FADD) (Chinnaiyan et al., 1995). Other example include the tumor necrosis factor-α (TNF-α), which binds to its related TNF-αreceptor 1, and the DR4 and DR5 ligands that bind to the related TNF-α apoptosis-inducing ligand (TRAIL) (Ashkenazi and Dixit, 1998; Tartaglia et al., 1993). The pyrin domains (PYD) also constitute another family of death receptors (Martinon et al., 2001).

The mitochondrial pathway is usually stress-induced by factors such as DNA damage caused by radiation, chemical toxicity, and serum withdrawal. The electron

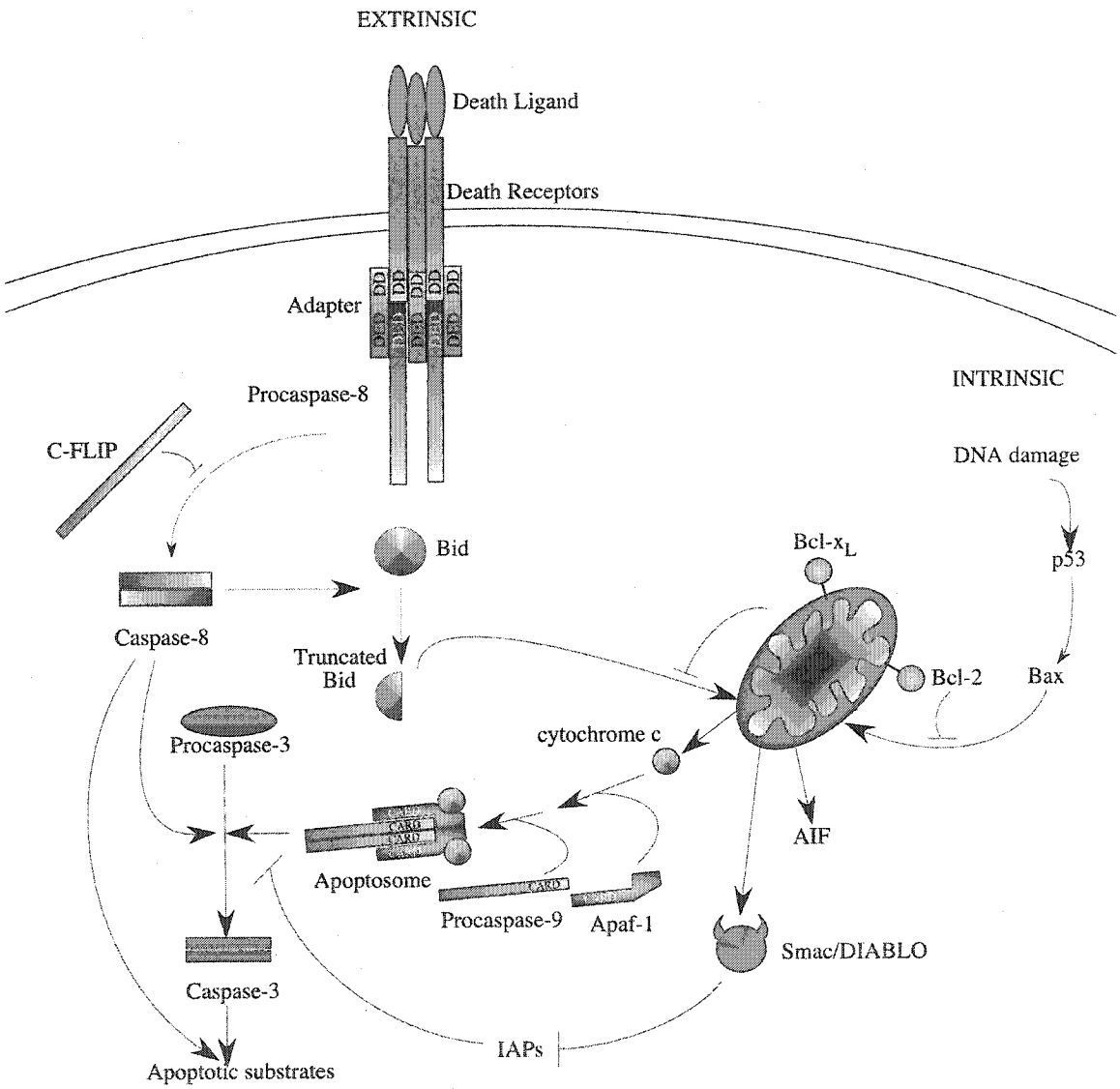

Figure 1-2. The two main pathways of apoptosis.

See the text of section 1.9 for a detailed description.

transport chain protein cytochrome c is first released out of the mitochondria and associates with APAF-1 (apoptotic protease-activating factor-1) resulting in its oligomerization. This permits recruitment and subsequent activation of procaspase-9 to the caspase recruitment domains (CARD) on APAF-1 the so called apoptosome (Li et al., 1997). Once caspase-9 is proteotically active, it is able to cleave caspase-3. The apoptosome is a large complex, with a relative molecular mass of ~700 kDa (Cain, 2000). Other proteins are also released from the mitochondria including the apoptosis inducing factor (AIF) and endonuclease G (endoG) (Li et al., 2001; Susin et al., 1999). The inhibitors of apoptosis (IAPs) are also involved in this pathway and are thought to protect the caspases from cleavage (Deveraux et al., 1997). The SMAC/Diablo protein can sequester these IAPs away from caspases, in turn facilitating cleavage of the latter (Du et al., 2000; Verhagen et al., 2000). There can also be cross-over from one pathway to the other. For example, active caspase-8 from the extrinsic pathway can cleave the pro-apoptotic Bcl-2 member Bid, facilitating the release of cytochrome c from the mitochondria (Li et al., 1998). Caspase activation can also occur via the aspartate-directed serine protease Granzyme B found in the granules of cytotoxic T-cells. Granzyme B is thought to cleave Bid and activate the mitochondrial pathway (Barry et al., 2000) .

1.9.3 The Bcl-2 family

The Bcl-2 protein was named for a gene product isolated from B-cell lymphoma (Tsujimoto et al., 1985). The Bcl-2 family members in the mitochondrial pathway seem to regulate the release of proteins such as cytochrome c out of the mitochondria (Gross et

al., 1999; Reed, 1998). They are divided in three groups. Group I, the modulators, consists of anti-apoptotic proteins such as Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Boo/Diva, A1/Bfl1, Nfr3, and are characterized by four Bcl-2 homology (BH) domains (Adams and Cory, 1998; Gross et al., 1999). Their principal role is to sequester pro-apoptotic group II and III Bcl-2 family members. Group II includes Bax, Bak and Bok/Mtd and they lack the BH4 domain. They are highly apoptotic and are needed for cytochrome c release. Group III, the sensor group, include Bid, Bad, Bik, Bim, Blk, Bmf, Hrk, Bnip3, Nix, Noxa and Puma, and only possess a single BH3 domain (Adams and Cory, 1998; Gross et al., 1999; Huang and A., 2000; Nakano and Woudsen, 2001). It is thought that Bax and Bak relocate from the cytoplasm to the outer mitochondrial membrane upon signalization and undergo conformational change. They then oligomerize and insert themselves in the membrane, forming pores that facilitate the release of cytochrome c (Green and Reed, 1998). Group III proteins such as Bid seem to facilitate this process. The anti-apoptotic proteins such as Bcl-2 and Bcl-x_L prevent the insertion of Bax into the outer mitochondrial membrane by selectively binding to the protein, but the mechanism is still unknown (Green and Reed, 1998). Some speculations predict that the voltage-dependent anion channel (VDAC) can bind to Bcl-2 family members and promote the formation of a large pore channel (Shimizu et al., 1999).

1.9.4 The Inhibitors of Apoptosis

The inhibitors of Apoptosis (IAP) protein family members include cIAP1, cIAP2, XIAP, NIAP, survivin and livin (Kaufmann and Hengartner, 2001). XIAP, cIAP1 and cIAP2 can block active caspases-8 and -9, as well as -3, -7 and -9 at high concentrations

(Deveraux and Reed, 1999; Kaufmann and Hengartner, 2001). They have multiple N-terminal BIR (baculovirus inhibitor) domains as well as a C-terminal RING domain. The BIR domains bind to the surface of caspases, blocking the catalytic grooves of target enzymes, and the RING domains act as ubiquitin ligases (Kaufmann and Hengartner, 2001). SMAC (second mitochondrial activator of caspases) or Diablo as well as Omi/HtrA2 are also released from the mitochondria during apoptosis and bind to IAPs in order to inactivate them (Du et al., 2000; Gozani et al., 2002; Verhagen et al., 2000).

1.9.5 The caspases and their substrates

Caspases are cysteine proteases that cleave their substrates on the carboxyl side of aspartate residues (Thornberry and Lazebnik, 1998). Caspases are synthesized as enzymatically inert zymogens, which are composed of three domains: an N-terminal prodomain, and the p20 and p10 domains (Hengartner, 2000). The mature enzyme is a heterotetramer containing two p20/p10 heterodimers and two active sites. Mammalian caspases-1, -4, -5, -11, -12 and -14 are thought to be involved in cytokine maturation, whereas caspases-2, -3, -6, -7, -, 8, -9 and -10 are the ones involved in apoptosis (Kaufmann and Hengartner, 2001). They are further sub-categorized as either 'initiator' or 'effector' caspases. The 'initiator caspases' (caspases-2, -8, -9, and -10) have long prodomains with recognition motifs such as DEDs or CARDs that contribute to the transduction of various signals into proteolytic activity (Kaufmann and Hengartner, 2001). The 'effector' caspases, (caspases-3, -6, -7) are known for their short prodomains, devoid of intrinsic activity (Kaufmann and Hengartner, 2001). Once active, they are able to cleave apoptotic substrates. Examples include the cleavage of the nuclear lamins

required for nuclear shrinking and budding, cleavage of cytoskeletal proteins such as gelsolin and fodrin leading to loss of overall shape, and PAK2 cleavage, a p21-activated kinase family member, which leads to membrane blebbing (Buendia et al., 1999; Kothakota et al., 1997; Rudel and Bokoch, 1997). Also native to this process is the caspase-activated deoxyribonuclease (CAD), which resides in the nucleus sequestered by its chaperone and inhibitor, ICAD (inhibitor of CAD) (Nagata, 2000). As cells undergo apoptosis, the nuclear envelope is damaged, permitting the entry of caspase-3. Caspase-3 subsequently cleaves ICAD releasing CAD, which can then digest the chromatin.

1.9.6 The tumor suppressor p53

Another important component of the apoptosis pathway is the tumor suppressor p53. It is induced in various forms of cellular stress, including DNA damage, hypoxia, nucleotide deprivation and genes that induce cell cycle arrest in G1 or cell death (Ko and Prives, 1996; Livingstone et al., 1992; Prives, 1998). p53 expression is normally maintained at low levels through as negative-feedback mechanism (Kubbutat et al., 1998; Yin et al., 2002). Its interaction with the MDM2 protein represses its transcriptional activity and mediates the degradation of p53 (Yin et al., 2002). DNA-damage induces phosphorylation of p53 attenuating the p53-MDM2 interaction, where ARF can then bind to MDM-2 and prevent p53 degradation (Prives, 1998).

Cell death is simply part of the circle of life. Imbalances in this process have been reported in a multitude of human diseases, such as neurodegenerative disorders, ischemias and cancers. Apoptosis is not always dependent on caspases, as Bax is capable of killing cells even in the presence of caspase inhibitors, releasing enough cytochrome c

and endoG to create toxic damage to the cell (Nylandsted et al., 2000). Accordingly, apoptosis is not necessarily an irreversible process, which offers optimism in finding possible treatments to prevent cell death of tissues after injury or specifically induce it in cancerous cells. A better understanding of the signals instructing a cell to commit suicide or repair itself as well as the pathways utilized will shed more light on the apoptotic process.

1.10 RNA metabolism

1.10.1 RNA processing

RNA is first transcribed into the nucleus by the RNA polymerase II (pol II) into mRNA precursors (pre-mRNAs) (Bentley, 2002). This process occurs through interactions of the processing factors with the C-terminal domain (CTD), the largest subunit of RNA pol II (Jensen et al., 2003). Before making its way to the cytoplasm, the pre-mRNA has to be modified by specific factors. These modifications include capping, splicing and processing of the 3' end by cleavage/polyadenylation. Once these events have taken place and the appropriate factors have assembled to the mRNA, the complex is exported out of the nucleus and into the cytoplasm where the translation machinery assembles itself on the mRNA. All of these events are cleverly organized and occur co-transcriptionally. As a matter of fact, the CTD is involved in sequestering most of these modification factors (Hirose and Manley, 2000). I will describe some of these events, but take note that they do overlap with each other and are not necessarily in the following order.

1.10.2 Capping

The first modification consists of the addition of a methylated guanosine cap structure to the 5' end of the pre-mRNA, which occurs after it is approximately 25 bases long. This is a three steps reaction, where RNA 5' triphosphatase (RTP) first hydrolyzes the triphosphate of the first nucleotide to a diphosphate. This is followed by a guanylyltransferase (GT) activity and finally 7-methyltransferase (MT) methylates the N7 position of the transferred GMP (Proudfoot et al., 2002). This initial cap structure is then recognized by the cap-binding complex (CBC), which is eventually replaced by the translation initiation factor eIF4E when translocated to the cytoplasm. These proteins are believed to play a major role in the stabilization of the mRNA since they represent an obstacle for 5'-3' exonucleases (Beelman and Parker, 1995).

1.10.3 Splicing

Once the RNA has been capped, it is thought to be ready for splicing. This is the action where the introns are excised by a complex called the spliceosome. It assembles onto each intron from a set of five small ribonucleoproteins (UsnRNPs; U1, U2, U4, U5, and U6) with corresponding small stable uridine-rich RNA (snRNAs) and numerous accessory proteins (Black, 2003). In fact, proteomics studies completed over the last few years have identified hundreds of splicing factors (Jurica and Moore, 2003). Intron excision occurs in two transesterification steps: first there is a 5' splice site cleavage and lariat formation, leading to two intermediates, then a 3' splice site cleavage and exon ligation with release of the lariat structure (Moore et al., 1993).

The pre-mRNA contains several consensus *cis*-elements essential for the splicing reaction within splice sites. The 5' splice site is marked by a GU dinucleotide within a larger less conserved sequence, and the 3' splice site region has a branching point, followed by a polypyrimidine tract and a terminal AG (Black, 2003). Interaction of the U1 snRNP with the 5' splice site sequence via base pairing between the splice site and the U1 snRNA initiates the spliceosome assembly (Black et al., 1985; Luhrmann et al., 1990). The dimeric splicing factor U2AF interacts with the 3' splice site and adjacent pyrimidine tract, along with a branching binding protein (SF1) (Berglund et al., 1997). This is defined as the E (early) or commitment complex. Recruitment of U2 snRNP, where its U2 snRNA base pairs with the branchpoint sequences, forms the A complex. This pairing causes a bulge, exposing the 2'OH of the branchpoint adenosine. The U4/U6 dimer joins the complex as a tri-snRNP with U5, forming the B complex. The complex is then rearranged into complex C, where RNA helicases remove U4, and U6 displaces U1 from the 5' splice site. U2 and U6 RNA-RNA interaction with the pre-mRNA seems to form a catalytic core placing the 5' splice site and branchpoint in close proximity (Madhani and Guthrie, 1992). The role of U5 is likely to position the exons for the second nucleophilic attack (Newman, 1997). The complex C can then catalyze the two chemical steps of splicing, where the 2'OH acts as a nucleophile to attack the 5'exon-intron border. *Trans*-esterification results in a free 5' exon and a lariat-shaped molecule consisting of the intron sequence and the 3' exon. In the second step, the 3'OH of the freed 5' exon attacks the intron-3' exon border. The subsequent *trans*-esterification results in the fusion of the two exon sequences and the release of the lariat-shaped intron (Moore et al., 1993). The spliceosome assembly is also stimulated by SR proteins that can bind

exonic splicing-enhancer elements and regulate splicing, which serve to bridge the 5'-3' splice site complexes (Proudfoot et al., 2002)

1.10.4 Polyadenylation

The last part of pre-mRNA processing consists of the 3'end modification. Two RNA sequence elements are recognized in this event: the highly conserved sequence AAUAAA, 10-30 nucleotides upstream of the cleavage site, and a poorly defined GU- or U-rich sequence at approximately the same distance downstream. The sequence AAUAAA is the binding site for the cleavage and polyadenylation specificity factor (CPSF), whereas the poly(A) binding protein PABPN1 binds to an 11-14 adenylate residue stretch. These proteins, along with other factors, stimulate the poly(A) polymerase (PAP) for the addition of adenosines to the 3' end of the pre-mRNA (Mangus et al., 2003). In the nuclei of mammalian cells, poly(A) tails grow to a length of 200-250 nucleotides before it is exported out into the cytoplasm (Sachs and Wahle, 1993). The multiple association of cytoplasmic PABP to the poly(A) requires a minimal binding site of 112 adenosines, and this interaction occurs via RNA-recognition motifs (RRMs) (Deo et al., 1999). Although PABP itself does not contain any catalytic activity, it seems to provide a scaffold for binding factors implicated in polyadenylation, as well as export, translation and turnover of the transcripts (Huang and Carmichael, 1996; Mangus et al., 2003).

1.10.5 Assembly of RNPs and nuclear export

Once the pre-mRNA has been processed into the mature mRNA, it is ready for export into the cytoplasm. This requires the assembly of a complex of proteins involved in the exporting of the mRNA. All nucleocytoplasmic transport events occur through the nuclear pore complex (NPC), a gigantic multiprotein complex of ~120 MDa, that penetrates the double membrane of the nuclear envelope (Weis, 2002). It is composed of 30-50 different proteins. The transport receptors shuttle between the cytoplasm and the nucleus and interact with a subset of nucleoporins that contain the characteristic phenylalanine/glycine (FG) rich repeat motif. There are three major classes of transport receptors that have been identified to date: the family of importin-β-like proteins, the small homodimeric nuclear transport factor 2 (NTF2)/p10 that imports small GTPase Ran into the nucleus, and the Tap/Mex67 family. The Ran proteins regulate the binding and release of importin-β-like transport factors. This process requires energy input, provided by the RanGTP cycle. In the current model, RanGTP binds to importins and induces cargo release in the nucleus as part of the import cycle. Export on the other hand depends on exportins and stable RanGTP complexes with their cargos. Once in the cytoplasm, RanGTP hydrolysis is induced by RanGTPase-activating protein (RanGAP) and RanBP1/2, which causes complex disassembly and terminates the export reaction. This pathway is mostly responsible for tRNAs and UsnRNAs export/import (Gorlich et al., 1996). Mex67/Tap does not belong to the importin-β protein family, and is thought to be the pathway utilized for the nuclear export of mRNA. This nuclear export complex assembles during splicing, where a key step consists of the deposition of the exon-exon junction complex (EJC) onto the mRNA, which consists of mRNA binding proteins such

as Y14, RNPS1, SRm160, DEK, Mago and Upf3 (Weis, 2002). Other factors that have a role in mRNA export besides Tap include shuttling hnRNPs such as hnRNP A1, HuR, CRM1/exportin and SR shuttling proteins (Dreyfuss et al., 2002; Michael et al., 1995).

1.10.6 The mechanism of translation

In order for the translation to begin, ribosomes need to be recruited to the mRNA in a multistep process. In eukaryotes, the small ribosomal subunit (40S subunit) is recruited to the mRNA, where it is placed at the initiation codon after scanning of the mRNA (Sachs et al., 1997). The large ribosomal subunit (60S) is then associated for completion of the ribosome. In order to recruit the ribosomes and initiate translation, assembly of initiation factors to the mRNA is required. The eIF4E binds the cap binding protein, which in turn recruits the 40S subunit to the mRNA via a network of protein interactions like eIF3. It is thought that eIF4A is an ATP-dependent RNA helicase that is stimulated in the presence of the RNA-binding protein eIF4B, unwinding the mRNA in preparation for the 40S subunit association (Rozen et al., 1990). The eIF4E/eIF4G complex (also known as the eIF4F) is regulated by the 4E-binding proteins (4E-BPs) (Mader et al., 1995). When non-phosphorylated, these proteins function as competitive inhibitors of the eIF4A-eIF4G complex. Phosphorylation of the 4E-BPs dissociates them from eIF4E/eIF4G and allows eIF4E to assemble to the translation complex. The poly(A) tail is also involved in the 40S subunit recruitment. It interacts with the 5' end of mRNA via eIF4G, which binds both the PABP-poly(A) and the cap binding factor eIF4E. This association promotes a 5'-3' interaction that stimulates initiation of translation by formation of the 'closed' loop (Wells et al., 1998). The PABP interacting protein Paip1

has also been shown to enhance translation, whereas Paip2 binding to PABP has an inhibitory effect (Craig et al., 1998; Khaleghpour et al., 2001; Roy et al., 2002). Finally translation termination occurs through the recognition of specific factors such as the termination factor eRF1, responsible for catalyzing polypeptide hydrolysis in response to recognition of the nonsense codons by the ribosome. This is activated by the GTPase eRF3, which directly interacts with the C-terminal domain of PABP. This interaction enhances the efficiency of termination and promotes ribosome recycling for multiple rounds of translation with the same mRNA. The eRF3 also seems to minimize the multimerization of PABP monomers on the poly(A), providing access of poly(A) shortening enzymes and linking translational termination to normal mRNA decay (Hoshino et al., 1999; Mangus et al., 2003; Uchida et al., 2002).

There are some cases, usually viral as well as the well-known iron-responsive elements (IREs), which bypass cap-dependent translation with the help of an internal ribosomal entry site (IRES) (Hentze and Kuhn, 1996). The ribosome can bind directly to the IRES and scan the mRNA for the initiator codon AUG, leading to translation of the transcript.

1.10.7 Translation regulation

Once in the cytoplasm, translation of the mRNA is not necessarily immediate, as a number of events can still occur before production of the protein. Translational control usually occurs through *cis*-regulatory elements, which are often found in the 3'UTR. The best-characterized example is polyadenylation, necessary for stabilization of the mRNA. Regulation is particularly important for proteins that are active only for brief periods,

such as growth factors, transcription factors and protein that control cell cycle progression, as half-lives of mRNAs may vary from a few minutes to more than 24 hours (Tourrière et al., 2002). Differential stability can be used to produce localized concentrations of RNA, where the use of *cis*-acting regulatory elements participate in protecting the transcripts from degradation at their final destinations.

Translational control mechanisms of localized RNAs can be categorized in four classes. First there is the simplest, consisting of active translation of the mRNA with the help of the poly(A) tail and the translation factors. The second mechanism involves repression by deadenylation of the poly(A) tail, which influences the stability of the transcript. Finally, repression of translation can occur either before or after translation initiation. In these two cases, the *trans*-acting factors somehow prevent ribosomal recruitment or alter it to prevent translation (Kloc et al., 2002).

1.10.8 Stabilization

The process of mRNA decay can be initiated by three distinct events: endonucleolytic cleavage, removal of the 5'cap, and poly(A) shortening. These events eliminate the closed-loop state of the mRNP by removing or separating the binding sites for the respective 5' and 3' proteins. The PABP as well as other proteins stabilize the poly(A) tail by protecting it from deadenylases and 3'-5' exonucleases (Bernstein et al., 1989).

The term mRNA surveillance is usually associated with the nonsense-mediated decay (NMD) of transcripts harboring premature termination codons. This occurs in the nucleus and is closely connected to the transcription process involving the RNA

exosome, a complex of 3'-5' exonucleases (Bousquet-Antonelli et al., 2000; Jacobs Anderson and Parker, 1998; Mitchell et al., 1997). It is thought that failure to produce a proper poly(A) tail results in retention of the mRNA to its site of transcription and destruction of the transcript.

Deadenylation usually occurs in the cytoplasm where the poly(A) tail is shortened after nuclear export, a process stimulated by AU-rich instability elements (AREs) (Couttet et al., 1997). AREs are found mostly in early response genes such as cytokines and proto-oncogenes. AU-BP (AU-rich binding proteins) binds to the ARE, which recruits the exosome and destabilizes the translation initiation complex at the cap structure (Gao et al., 2001). This allows interaction between the cap structure and the deadenylase PARN (Martinez et al., 2000). After deadenylation, PARN dissociates from the complex and the decapping enzyme can remove the cap structure by hydrolysis. The final step is degradation of the mRNA body by the exosome and eventually Xrn-1-like exonucleases (Tourrière et al., 2002).

1.10.9 Localization

The localization of RNA is an extremely efficient way of targeting gene products to individual subcellular compartments or specific regions of the cell or embryo. *Trans*-acting factors are often present in granules or RNP complexes, which are thought to contain the components necessary for RNA processing, transport, localization, anchoring and translation. The proteins and RNA elements responsible for this journey are beginning to be identified (Kiebler and DesGroseillers, 2000). It is thought that RNP proteins direct the RNA by means of associations with the cytoskeleton (Kloc et al.,

2002). There likely is an active transport mechanism involving a molecular motor such as kinesin, kinesin-like and dynein proteins. There are two classes of cytoskeletal networks that have been implicated in the transport of RNA cargo: the actin network associated with short-distances, and the microtubules, which have been associated with long distance transport (Kloc et al., 2002).

Translational regulation has been extensively studied in Drosophila, where localization and translational regulation of mRNA are coupled in development. Examples include Oskar, where unlocalized *oskar* mRNA is translationally repressed by Bruno until they are located to the posterior pole of the maturing oocyte (Kim-Ha et al., 1995). Similarly, the *trans*-acting factor Smaug is thought to interfere with the translating ribosome and repress Nanos translation by binding to its TCE element in the 3'UTR until it is properly localized (Bashirullah et al., 1999; Dahanukar et al., 1999). Another example includes Squid, the hnRNP A1 homologue in Drosophila, which is required for the localization of *gurken* mRNA in the oocyte (Norvell et al., 1999). Staufen has also been demonstrated to be important in the localization, anchoring and translation of *oskar*, and has been implicated in the localization of RNAs in vertebrate neurons (Jan, 2000; Micklem et al., 2000). The αsubunit of calcium-calmodulin-dependent kinase II (CaMKII-α), which is important for learning and memory, is an example of mRNA localization specific to the post-synaptic regions of the dendrites (Mayford et al., 1996). Similar mechanisms have been identified in oligodendrocytes, where hnRNP A2 was found to bind to the MBP mRNA localization element RTS (RNA trafficking signal) (Ainger et al., 1993; Carson et al., 2001; Hoek et al., 1998). HnRNP A2 contains a similar peptide sequence to the eIF4E binding motif, suggesting an involvement in eIF4E

binding and an increased translation efficiency (Kwon et al., 1999). Other localization RNA elements found in the untranslated region of MBP include the Y element and the RNA localization region (RLR) (Ainger et al., 1997; Han et al., 1995; Wu and Hecht, 2000). Other proteins containing localization elements found in oligodendrocytes include MOBP, tau, and the amyloid precursor protein (APP) (Barbarese et al., 2000; Carson et al., 2001). Data suggests that within these localization elements are regions that form stem-loop structures that are critical for proper localization of the mRNA (Chartrand et al., 1999). Localization of mRNA will be one of the topics discussed in Chapter 3.

Chapter 2

NUCLEAR TRANSLOCATION CONTROLLED BY ALTERNATIVELY SPLICED ISOFORMS INACTIVATES THE QUAKING APOPTOTIC INDUCER

2.1 Preface

Although the *quaking* viable mouse has been extensively studied since its discovery in 1964, the exact function of the protein still remains a mystery. We have previously observed that the QKI-7 isoform was able to induce apoptosis when overexpressed in mouse fibroblast cells (Chen et al., 1998). These results directed us to the characterization of mechanisms involved in cell-induced death by QUAKING. Our first objective was to map the region necessary for this event, and then to investigate QKI-7's ability to induce apoptosis in the myelinating oligodendrocyte.

2.2 Summary

The *quaking* viable mice have myelination defects and develop a characteristic tremor 10 days after birth. The *quaking* gene encodes at least five alternatively spliced QUAKING (QKI) isoforms that differ in their C-terminal 8-30 amino acid sequence. The reason for the existence of the different QKI isoforms and their function are unknown. Here we show that only one QKI isoform, QKI-7, can induce apoptosis in fibroblasts and primary rat oligodendrocytes. Heterodimerization of the QKI isoforms results in the nuclear translocation of QKI-7 and the suppression of apoptosis. The unique C-terminal

14 amino acids of QKI-7 confers the ability to induce apoptosis to heterologous proteins such as the green fluorescent protein and a QKI-related protein, *Caenorhabditis elegans* GLD-1. Thus, the unique C-terminal sequences of QKI-7 may function as a life-or-death 'sensor' that monitors the balance between the alternatively spliced QKI isoforms. Moreover, our findings suggest that nuclear translocation is a novel mechanism of inactivating apoptotic inducers.

2.3 Introduction

Apoptosis, or programmed cell death, is a physiological process characterized by a cascade of events culminating in the destruction of the cell. There are numerous protein families that have been shown to induce apoptosis including the TNF/Fas ligands (Tartaglia and Goeddel 1992; Ashkenazi and Dixit 1998), oncogenes (Evan and Littlewood 1998), the Bcl-2 family (Adams and Cory 1998; Reed 1998; Gross et al. 1999; Porter 1999), and p53 (Livingstone et al. 1992; Ko and Prives 1996). Heterogeneous nuclear ribonucleoprotein particle K (KH) homology domain containing RNA-binding proteins (Gibson et al. 1993; Siomi et al. 1993) are an emerging class of apoptotic inducers. We have shown that mouse QKI-7 and *Drosophila* KEP1 and Sam50 are potent inducers of apoptosis (Chen and Richard 1998; Di Fruscio et al. 1998). Two other KH domain-containing proteins, *Drosophila* FMRP and MCG10, have also been shown to induce apoptosis (Wan et al. 2000; Zhu and Chen 2000). The mechanism by which this family of RNA-binding proteins induce cell death is unknown.

The *quaking* viable (qk^v) mice have been studied for more than thirty years and represent an animal model for dysmyelination (Hogan and Greenfield 1984). Ten days after birth these animals develop a rapid tremor that is especially pronounced in the hind limbs (Hogan and Greenfield 1984). The gene responsible for the defect was cloned and termed the *quaking* (*qk*) gene (Ebersole et al. 1996). The mouse *qk* gene expresses at least five alternatively spliced mRNA including QKI-5, QKI-6, QKI-7, and QKI-G that differ in their C-terminal 30 amino acids (Ebersole et al. 1996; Cox et al. 1999; Kondo et al. 1999). The KH domain of the QKI proteins is embedded in a larger conserved domain of ~200 amino acids called the GSG (GRP33, Sam68, GLD-1) domain (Jones and Schedl

1995; Di Fruscio et al. 1998) or the STAR (signal transduction activator of RNA metabolism; Vernet and Artzt 1997) domain. The GSG domain of the QKI proteins is required for RNA binding and dimerization (Chen et al. 1997; Zorn and Krieg 1997; Chen and Richard 1998; Wu et al. 1999).

The cellular localization of the QKI isoforms differ in oligodendrocytes (OLs), the myelinating cells of the central nervous system. QKI-5 is predominantly nuclear; QKI-6 and QKI-7 are localized in the perikaryal cytoplasm with lower levels in the nucleus (Hardy et al. 1996). In the qk^{v} mice, part of the *quaking* enhancer/promoter is deleted (Ebersole et al. 1996) and, as a result, QKI-6 and QKI-7 isoforms are not expressed in OLs (Hardy et al. 1996). Several missense mutations have been generated in the *qk* gene by using ethylnitrosourea, and these mutations are known to be embryonic lethal in mice (Justice and Bode 1986, 1988; Shedlovsky et al. 1988). One allele, qk^{kt4}, was found to alter QKI glutamic acid 48 to glycine (Ebersole et al. 1996). This amino acid substitution disrupts a predicted coiled-coil region, prevents dimerization, and may be the underlying mechanism for the embryonic lethality (Chen and Richard 1998).

QKI homologs have been identified in many species including *Xenopus* (Zorn et al. 1997), chicken (Mezquita et al. 1998), zebrafish (Tanaka et al. 1997), and *Drosophila* (Baehrecke 1997; Zaffran et al. 1997). *Caenorhabditis elegans* does not have a QKI homolog but has a closely related protein called GLD-1 (germ-line defective; Francis et al. 1995) that has a high degree of sequence identity with the QKI GSG domain (Jones and Schedl 1995; Vernet and Artzt 1997). GLD-1 is required for germ cell differentiation and has been shown to function as a tumor suppressor (Jones and Schedl 1995) and as a translational repressor (Jan et al. 1999). The relatedness between the GSG domains of

QKI and GLD-1 suggest that these proteins may recognize similar RNA targets as dimers. We have shown that *C. elegans* GLD-1 is able to homodimerize and that QKI and GLD-1 associate with one another when ectopically expressed in HeLa cells (Chen et al. 1997). It was also shown that QKI-6 can replace GLD-1 in repressing the translation of a GLD-1-specific RNA target, tra-2 (Saccomanno et al. 1999). These observations demonstrate the functional similarity between the QKI and GLD-1 GSG domains. The overexpression of GLD-1 in NIH 3T3 cells did not induce apoptosis as was observed with QKI-7 (Chen and Richard 1998). These findings suggested that the QKI-7 GSG domain may not be required for the induction of apoptosis.

In this study, we identified a short region of 14 amino acids in QKI-7 that confers the ability to induce apoptosis to heterologous proteins. The other QKI isoforms were unable to induce apoptosis, but induced cell survival when expressed with QKI-7. Here we describe the possible mechanism by which the QKI-7 apoptotic inducer is suppressed by the QKI isoforms. Our data suggest that a balance between the alternatively spliced QKI isoforms is required for cell survival.

2.4 Materials and methods

2.4.1 DNA constructions.

The plasmid constructs myc-Bluescript QKI-7, QKI-7:EG, QKI-7:81-325, QKI:1-205, QKI:1-180, GFP-QKI-7, GFP-QKI-7:EG, and myc-pcDNA QKI-7 were described previously (Chen and Richard 1998). The corresponding GFP proteins were obtained by digesting the myc-Bluescript plasmids with *Eco*RI and subcloning in the *Eco*RI site of pEGFP-C1 (Clontech). The plasmids GFP-QKI:1-311, GFP-QKI-7:NLS, GFP-QKI-6, GFP--QKI-G were constructed by amplifying plasmid myc-QKI-7 with the T7 promoter primer and the following reverse primers: 5'-CAAGAATTCATAACACACCACTGG GTTC-3' (QKI:1-311), 5'-CGTGAATTCACACTTTCTTTTTCTTCTTTGGATGGGCT GAAATATCAGGCATG-3' (QKI-7:NLS), 5'-TTTGAATTCACCCTTTTGTCGGAAAA GCCATCCCTAACACACCACTGGGTTCAATAGG-3' (QKI-6), 5'-GACGAATTCAC ATTGTACACTATCATACTTCCCTAACACACCACTGGGTTCAATAGG-3' (QKI-G). Plasmid GFP-GLD1:14 was constructed by a two-step subcloning strategy: The GLD-1 DNA sequence was first subcloned into pEGFP-C1 using PCR amplification of myc-GLD-1 as the DNA template with the T7 promoter and the oligonucleotide 5'-TACAAGCTTGAAAGAGGTGTTGTTGAC-3' as primer. The amplified DNA fragment was digested with *Bgl*II and *Hin*dIII and subcloned into the corresponding sites of pEGFP-C1, generating GFP-GLD-1. The QKI-7 C-terminal tail was then amplified using GFP-QKI-7 as a DNA template using a GFP reverse primer and the forward primer 5'-CATAAGCTTGAGTGGATTGAAATGCCA-3'. The amplified DNA fragment was digested with *Hin*dIII and *Bam*HI and subcloned into the corresponding sites of GFP-GLD-1, generating GFP-GLD-1:14. The plasmid GFP:14 was constructed by inverse

PCR using GFP-QKI-7 as a DNA template with the following oligonucleotides as forward and reverse primers containing a *Bgl*II site: 5'-GGTAGATCTGAGTGGATTGA AATGGCCAGTC-3' and 5'-ATGGAATTCTATCTGTAGGTGCCATTCAG-3'. The amplified plasmid was digested with *Bgl*II and then ligated with T4 DNA ligase. Plasmid myc-QKI-5 was constructed by a two-step strategy: The C terminus of QKI-5 was amplified from mouse EST T83554 using the following primers 5'-CAATTCTAGAGTA TCCTATCCTATTGAACCTAGT-3' and 5'-CCAGAGCTCGAATTCAGCTCGCTGC ACTGACGA-3'. The amplified DNA fragment was subcloned directionally in *Xba*I and *Sac*I sites of Bluescript SK+ generating pXS. Subsequently, the N-terminal common sequences of QKI were amplified by using myc-QKI-7 as template and the T7 promoter primer and the reverse primer 5'-GTACTCTAGAATTGATGTAGCTGGTGCCA-3'. The DNA fragment flanked with *Xba*I sites (one from the polylinker and the other introduced by the reverse primer) was digested with *Xba*I and subcloned in pXS. The introduction of the *Xba*I site was such that no amino acid was altered. Moreover, at the 3' end of QKI-5 an *Eco*RI site was introduced before the *Sac*I site. Thus, the entire QKI-5 coding sequences were subcloned in pEGFP-C1 using *Eco*RI. Plasmid GFP-QKI-7:205-325 was constructed by amplifying sequences from GFP-QKI-7 using 5'-CATGAATTCACCAGCCCTTGCCTTTTC-3' and a GFP reverse primer. The amplified sequences were digested with *Eco*RI and subcloned in pEGFP-C1. Myc- and HA-epitope-tagged QKI isoforms were constructed by digesting the corresponding GFP-QKI isoform plasmid with *Eco*RI and subcloning the DNA fragments in myc-Bluescript or HA-Bluescript. The substitution of EG was introduced in the isoforms as follows: myc-QKI-7 contains an internal *Kpn*I site downstream of the EG mutation and a *Kpn*I site is

found in the polylinker at the 3' end of the cDNA. Thus, QKI-7:EG was digested with *Kpn*I and the different C termini from the other isoforms were introduced in the *Kpn*I. The myc-QKI:EG constructs were then subcloned into the pEGFP-C1 or HA-BS using *Eco*RI. The pCEP4-Bcl-2 expression vector was a gift of Walter Nishioka (Vical Inc., San Diego, CA). The transfer vectors expressing the tetracycline-inducible QKI-5, QKI-6, and QKI-7 proteins were constructed by subcloning the cDNAs encoding myc-tagged QKI isoforms from myc-BS QKI-5, QKI-6, and QKI-7 into the *Bgl*II site of pADTR5-K7-GFPq (Massie et al. 1998, 1999). These plasmids each express a different QKI isoform under the regulation of a tetracycline-inducible promoter and a second cassette that constitutively expresses GFP. The latter cassette serves as a marker for infection. Recombinant adenoviruses were generated, purified, and titered as described by the manufacturer (Quantum Biotechnology). The adenovirus that expresses constitutively the trans-acting factor (tTA) was a gift of Bernard Massie (Biotechnology Research Institute, Montréal).

2.4.2 Cell culture and transient transfection.

NIH 3T3 cells were transfected with DNA plasmids encoding GFP alone, GFP fusion proteins, or myc epitope QKI-7 using LipofectAMINE PLUS reagent (Life Technologies-BRL). The cells were plated 12 hours before transfection typically at a density of 10^5 cells/22 mm^2 cover slip. The transfection of HA- and myc-Bluescript QKI isoforms into HeLa cells was carried out with the T7 vaccinia virus system as described previously (Chen et al. 1997). For the experiments with the caspase-3 inhibitor, 50 μM Z-

DEVD.fmk (Calbiochem) or DMSO was added to the media after the LipofectAMINE transfection.

2.4.3 Apoptosis assays, transfection efficiency, and immunostaining.

After transfection (12 or 36 hours), the cells were fixed with 4% paraformaldehyde in 1x PBS for 10 min and permeabilized with 1% Triton X-100 in 1x PBS for 5 min, and the nuclei were stained with 3 μg/mL 4,6-diamidino-2-phenylindole (DAPI). The morphology of transfected cells and the localization of QKI isoforms were examined by fluorescence microscopy. Cells with characteristic morphological features such as nuclear condensation and fragmentation were considered apoptotic. The transfection efficiency was calculated as a percentage of transfected cells (green cells/total cells). For immunostaining, the fixed cells were incubated with the anti-myc 9E10 antibody (1:1000) at room temperature for 1 hour and then followed by incubation of a rhodamine-conjugated goat anti-mouse secondary antibody (Jackson Laboratories; 1:200) for 30 minutes. The nuclei were stained with DAPI.

2.4.4 Protein expression.

For protein expression, the cells were lysed in Laemmli buffer and the proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted. Immunoblotting was performed using anti-myc 9E10, or anti-hemagglutinin (HA), anti-pan QKI (Chen and Richard 1998), anti-QKI-5, anti-QKI-6, and anti-QKI-7 (Hardy et al. 1996) rabbit polyclonal antibodies, followed by a horseradish peroxidase-conjugated

secondary antibody and developed using chemiluminescence. Coimmunoprecipitations were performed as described previously (Chen and Richard 1998).

2.4.5 Primary rat OLs cultures and cell viabilities.

OLs progenitor cells were purified from newborn Sprague-Dawley rats essentially as described using a percoll gradient (Lubetzki et al. 1991). These cells were incubated for 2 days in media supplemented with 10% fetal calf serum, 2.5 ng/mL platelet-derived growth factor-AA, 2.5 ng/mL basic fibroblast growth factor, and 10 nM tri-iodothyronine. These OLs were infected with the indicated QKI expressing adenovirus and an adenovirus AdCMV-tTA that expresses the tTA. Each virus was added at an M.O.I. of 10.

OL cells survival assays were measured by staining with annexin V-phycoerythrin as described by the manufacturer (Pharmingen). Doxycyclin (1 μg/mL) was added at the same time as the adenoviruses to represent the uninduced cultures. The doxycyclin binds to the tTA and represses the tTA resulting in a noninduced culture. The induced and noninduced OLs were gated for GFP green expression and for annexin V-phycoerythrin red. The annexin V positive cells represented the cells undergoing early signs of programmed cell death and infection was calculated as the percentage of GFP positive cells.

2.5 Results

2.5.1 Characterization of the apoptosis induced by QKI-7.

The expression of QKI-7 in NIH 3T3 cells induces cell death (Chen and Richard 1998). To further assess the apoptosis and determine whether early signs of apoptosis were observed, we stained QKI-7-expressing cells with annexin V conjugated with fluorescein isothiocyanate (FITC) and propidium iodide. Abundant staining with annexin V-FITC was observed in NIH 3T3 cells transfected with QKI-7 compared with the control cells transfected with QKI-5 (Fig. 2-1A, top panels). Some of the QKI-7 transfected cells that stained with annexin V also stained with propidium iodide (Fig. 2-1A, bottom panels), implying that they represent later stages of cell death. To confirm that the transfected cells were staining with annexin V-FITC, we performed double staining by using annexin V-FITC and indirect immunofluorescence using anti-myc antibodies followed by a rhodamine-conjugated secondary antibody. Most of the myc epitope-tagged QKI-7 transfected cells that stained with annexin V-FITC also stained with anti-myc antibodies (Fig. 2-1B, right panels), demonstrating that the QKI-7 transfected cells were dying. In comparison, the myc-QKI-5 transfected cells that stained with annexin V-FITC did not stain with anti-myc antibodies (Fig. 2-1B, left panels), demonstrating that the cell death observed in these cells corresponds to background cell death and not from the presence of QKI-5. Two additional experiments were performed to assess whether classical apoptotic/survival pathways were being utilized. We examined the effect of overexpressing a known survival protein such as Bcl-2 (Adams and Cory 1998; Gross et al. 1999). NIH 3T3 cells were cotransfected with expression vectors encoding GFP alone and Bcl-2, GFP-QKI-7 with pCEP4 empty vector, and GFP-

QKI-7 and Bcl-2. The transfected green cells were counted and scored as apoptotic if they displayed irregular, condensed, or fragmented nuclei with the nuclear stain DAPI. The expression of GFP-QKI-7 with pCEP4 empty vector induced >50% apoptosis at 36 hours (Fig. 2-1C), consistent with our previous observations (Chen and Richard 1998). The expression of GFP alone and Bcl-2 had background levels of cell death (~15%) at 36 hours (Fig. 2-1C). The cotransfection of Bcl-2 suppressed the apoptosis mediated by GFP-QKI-7 in a dose-dependent manner (Fig. 2-1C, left panel). The percentage of green cells or the transfection efficiency ranged from 15%-20% 12 hours after transfection and decreased by 36 hours, in some cases to ~5%, indicating that some green cells had died and did not remain attached to the dish (Fig. 2-1C, right panel). To examine whether the apoptosis was caspase dependent, GFP-QKI-7 transfected cells were treated with 50 μM Z-DEVD.fmk, a known caspase 3 inhibitor (Nicholson et al. 1995), or dimethylsulfoxide as a control. The addition of Z-DEVD.fmk suppressed the apoptosis induced by QKI-7 to near background levels (Fig. 2-1D, left panel). In addition, the number of green cells was maintained at 15%, 36 hours post-transfection in the presence of Z-DEVD.fmk, consistent with cell survival. Taken together, our results suggest that QKI-7-expressing NIH 3T3 cells exhibit the hallmarks of apoptosis. The QKI-7 transfected cells stain by using the TUNEL assay (Chen and Richard 1998) and annexin V-FITC. The apoptotic process is caspase dependent and suppressed by the overexpression of the survival protein Bcl-2.

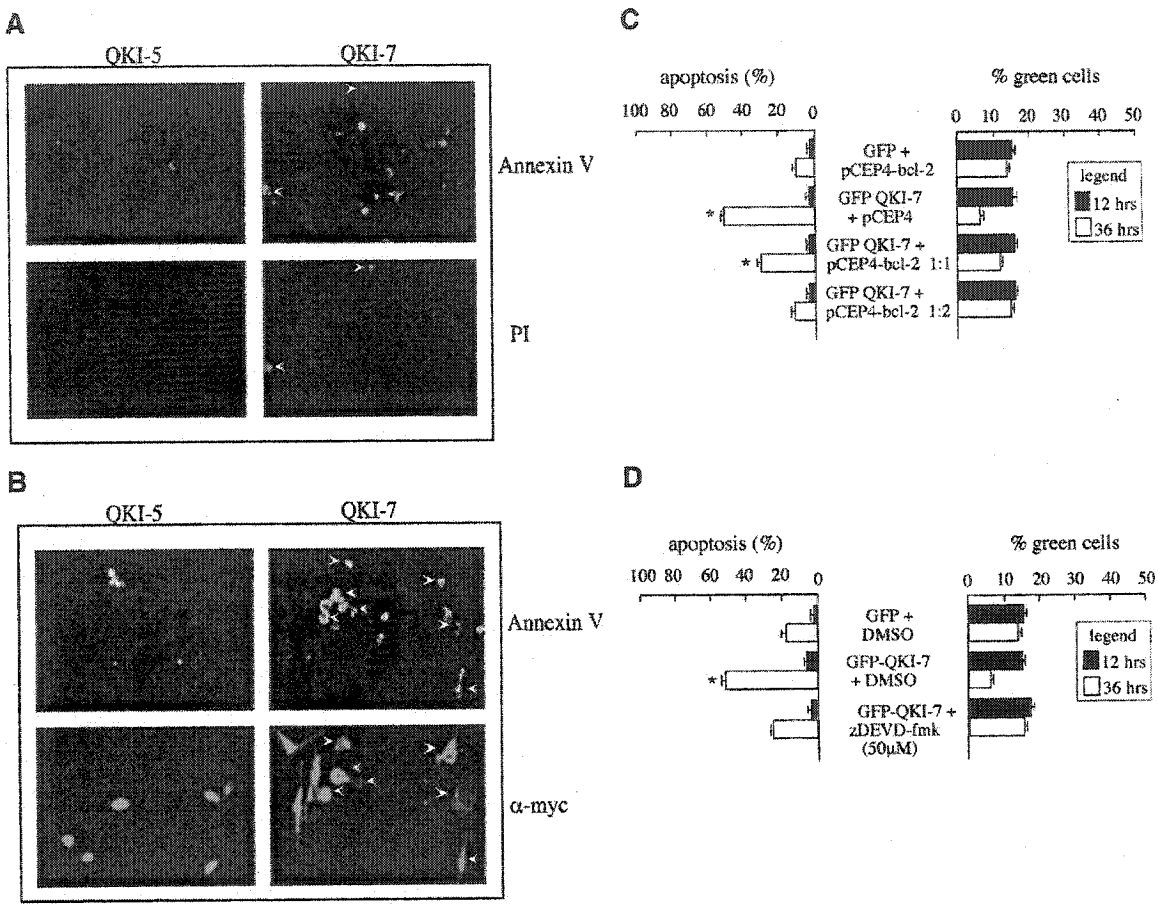

Figure 2-1. QKI-7 is a potent apoptotic inducer. (*A*) Detection of early apoptosis by annexin V. Myc-QKI-5 or myc-QKI-7 were transfected into NIH 3T3 cells and stained with annexin V-FITC and propidium iodide (PI). The cells were visualized by fluorescence microscopy. The arrowheads align the cells in *top* and *bottom* panels. (*B*) Myc-QKI-5 or myc-QKI-7 were transfected into NIH 3T3 cells stained live with annexin V-FITC and fixed, and the myc-tagged QKI-7 was visualized by indirect immunofluorescence with anti-myc antibody followed by a rhodamine-conjugated secondary antibody. (*C*) Suppression of apoptosis by Bcl-2 overexpression. GFP alone or GFP-QKI-7 were cotransfected into NIH 3T3 cells with increasing amounts of a Bcl-2 expression vector (pCEP4-Bcl-2) or empty plasmid (pCEP4). After 12 hours (solid bars) and 36 hours (open bars) the cells were fixed and stained with DAPI to visualize the apoptotic nuclei. (*D*) The caspase inhibitor Z-DEVD.fmk suppresses QKI-7-induced apoptosis. GFP or GFP-QKI-7 were transfected into NIH 3T3 cells and treated with either DMSO (control) or 50 μM Z-DEVD.fmk as indicated. After 12 hours (solid bars) and 36 hours (open bars) the cells were fixed and stained with DAPI to visualize the apoptotic nuclei. (*C,D*) The presence of apoptotic nuclei was scored as cells undergoing apoptosis and expressed as % green cells. Each bar represents the mean + S.E. of 3 experiments ($n > 450$, n = number of cells counted). Statistical evaluation was calculated by paired Student's *t*-test. (*) Values that differ significantly from GFP at 36 hours($P < 0.01$).

2.5.2 The C terminus of QKI-7 is necessary and sufficient to signal apoptosis.

A structure analysis of QKI-7 was performed to determine the regions or domains required for the induction of apoptosis. Truncations and chimeric proteins were engineered as GFP fusion proteins, transfected in NIH 3T3 cells, and analyzed for their ability to induce apoptosis. The QKI GSG domain is a tripartite protein module with a central KH domain flanked by the N terminus of KH region (NK) and the C terminus of KH region (CK). The NK and CK regions are also referred to as the QUA1 and the QUA2 regions (Vernet and Artzt 1997). To investigate the role of the NK region in QKI, known to mediate dimerization (Chen and Richard 1998), or the entire GSG domain in QKI-7-mediated apoptosis, deletion analyses were performed. The truncated proteins deleted for the NK region (QKI-7:81-325) or entire GSG domain (QKI-7:205-325) induced apoptosis in NIH 3T3 cells to the same extent as QKI-7 (Fig. 2-2A). These findings suggest that RNA binding and the GSG domain are not required for the induction of cell death.

The QKI proteins are known to be alternatively spliced at their C termini resulting in at least four different isoforms (Fig. 2-2B). The most common isoforms as detected by Northern blot analysis are QKI-5, QKI-6, and QKI-7 (Ebersole et al. 1996). The deletion of the C-terminal 14 amino acids, unique to isoform QKI-7, was sufficient to prevent cell death (Fig. 2-2A; QKI:1-311). Larger deletions as in proteins QKI:1-205 and QKI:1-180 were also unable to induce apoptosis (Fig. 2-2A). These data suggest that the induction of apoptosis may be isoform specific and that the QKI-7 C-terminal 14 amino acids are required. To investigate whether the C-terminal 14 amino acids of QKI-7 were able to

confer to heterologous proteins the ability to induce apoptosis, we fused the QKI-7 C-terminal 14 amino acids to nonapoptotic proteins such as GFP and *C. elegans* GLD-1 (Chen and Richard 1998). The expression of either GFP or GLD-1 with the C-terminal 14 amino acids of QKI-7 (GFP:14, GLD-1:14) was sufficient to induce apoptotic cell death to similar levels as QKI-7 (Fig. 2-2A). These findings demonstrate that the addition of amino acids EWIEMPVMPDISAH at the C terminus of cytoplasmic proteins is sufficient to induce apoptotic cell death. We next targeted QKI-7 to the nucleus with a strong nuclear localization signal (NLS) and asked whether nuclear QKI-7 could induce apoptosis in NIH 3T3 cells. The GFP fusion protein containing QKI-7:NLS was localized in the nucleus but was unable to induce cell death (Fig. 2-2A), suggesting that the C-terminal 14 amino acids of QKI-7 signal apoptotic cell death in the cytoplasm.

The ability of QKI-7 and GFP:14 to induce apoptosis in a cell line that expresses endogenous QKI proteins was investigated. We have shown previously that C6 glioma cells express three QKI isoforms (Chen and Richard 1998). C6 glioma cells were transfected with GFP-QKI-7 or GFP:14 and analyzed for apoptotic cell death. The transfection of GFP-QKI-7 induced apoptosis in a dose-dependent manner with minimal apoptosis observed with 1 μg of DNA (~25%) and a maximal response with 3 μg of DNA (~70%, Fig. 2-2C). In contrast, the expression of GFP:14 induced near maximal levels of apoptosis at 1 μg of DNA (~60%) and increased minimally with 3 μg of DNA (~70%). These data suggest that elevated concentrations of QKI-7 are required to induce apoptosis in cells expressing endogenous QKI isoforms. The fact that GFP:14 was more potent than QKI-7 at inducing apoptosis in C6 glioma cells suggests that C6 glioma cells,

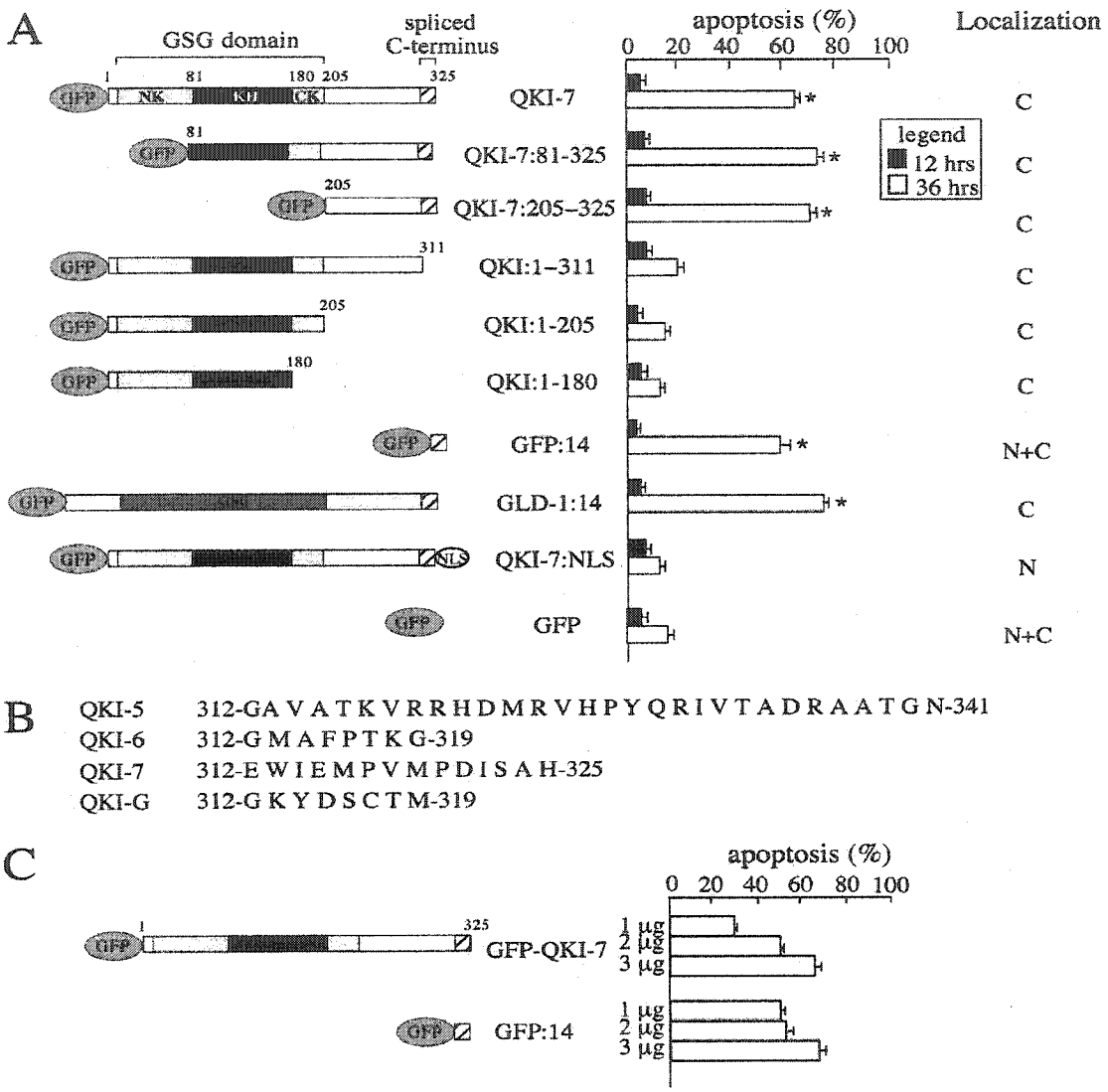

Figure 2-2. The C-terminal 14 amino acids of QKI-7 induce apoptosis. (*A*) A schematic diagram of GFP fusion proteins is shown at *left*. The GSG, KH, NK, and CK regions are indicated. The striped box at the C terminus denotes the unique sequences of the QKI alternatively spliced isoforms. NLS represents the SV40 large T antigen nuclear localization signal. Expression plasmids encoding these GFP fusion proteins were transfected in NIH 3T3 cells. After 12 hours (solid bars) and 36 hours (open bars) the cells were fixed and stained with DAPI to visualize the apoptotic nuclei and expressed as a percentage of green cells. The localization of each protein at 12 hours is indicated at *right*. (C) A predominant cytoplasmic localization; (N) a predominant nuclear localization. Each bar represents the mean + S.E. of 3 experiments ($n > 450$). (*) Values that differ significantly from GFP at 36 hours (*, $P < 0.01$). (*B*) The amino acids of the unique sequences of the different QKI isoforms used. (*C*) C6 glioma cells were transfected with the indicated amount of the expression plasmids encoding GFP-QKI-7 or GFP:14. After 12 and 36 hours the cells were fixed and stained with DAPI to visualize the apoptotic nuclei and expressed as a percentage of green cells. Each bar represents the mean + S.E. of three experiments ($n > 300$).

unlike NIH 3T3 cells where QKI-7 and GFP:14 were equipotent, are able to neutralize the ability of QKI-7 to induce apoptosis.

2.5.3 The QKI isoforms.

To evaluate the ability of the different QKI isoforms to induce apoptosis, we generated cDNAs for isoforms QKI-5, QKI-6, and QKI-G. The integrity of the QKI isoforms was verified by immunoblotting HeLa cell lysates transfected with myc epitope-tagged QKI proteins by using anti-myc, anti-'pan' QKI antibodies, anti-QKI-5-, anti-QKI-6-, or anti-QKI-7-specific antibodies. The anti-'pan' QKI antibody recognizes the KH domain of the QKI isoforms (Chen and Richard 1998) and the anti-QKI-5, -QKI-6, and -QKI-7 antibodies recognize the C-terminal specific sequence of each isoform (Hardy et al. 1996). The QKI isoforms migrated on SDS-polyacrylamide gels with molecular masses ranging from 40 to 47 kD and were all recognized with anti-myc and anti-'pan' QKI antibodies (Fig. 2-3A). The proteins encoded by the QKI-5, QKI-6, and QKI-7 cDNAs were also each recognized with anti-QKI-5, -QKI-6, and -QKI-7 antibodies, respectively (Fig. 2-3B). The QKI proteins were fused to GFP, transfected in NIH 3T3 cells, and analyzed by immunoblotting with the anti-'pan' QKI antibody. The GFP-QKI fusion proteins migrated with mobilities of ~70 kD (Fig. 2-3C). Doublets were observed that most likely represent a second start site in the GFP coding region. Endogenous proteins with approximate molecular masses of 36 kD were observed by immunoblotting NIH 3T3 cells with anti-'pan' Qk1 or HeLa cells with anti-QKI-6 antibodies (Fig. 2-3B,C). The identity of these protein is unknown. In summary, these immunoblotting experiments demonstrate that the cDNAs encode the proper QKI isoforms.

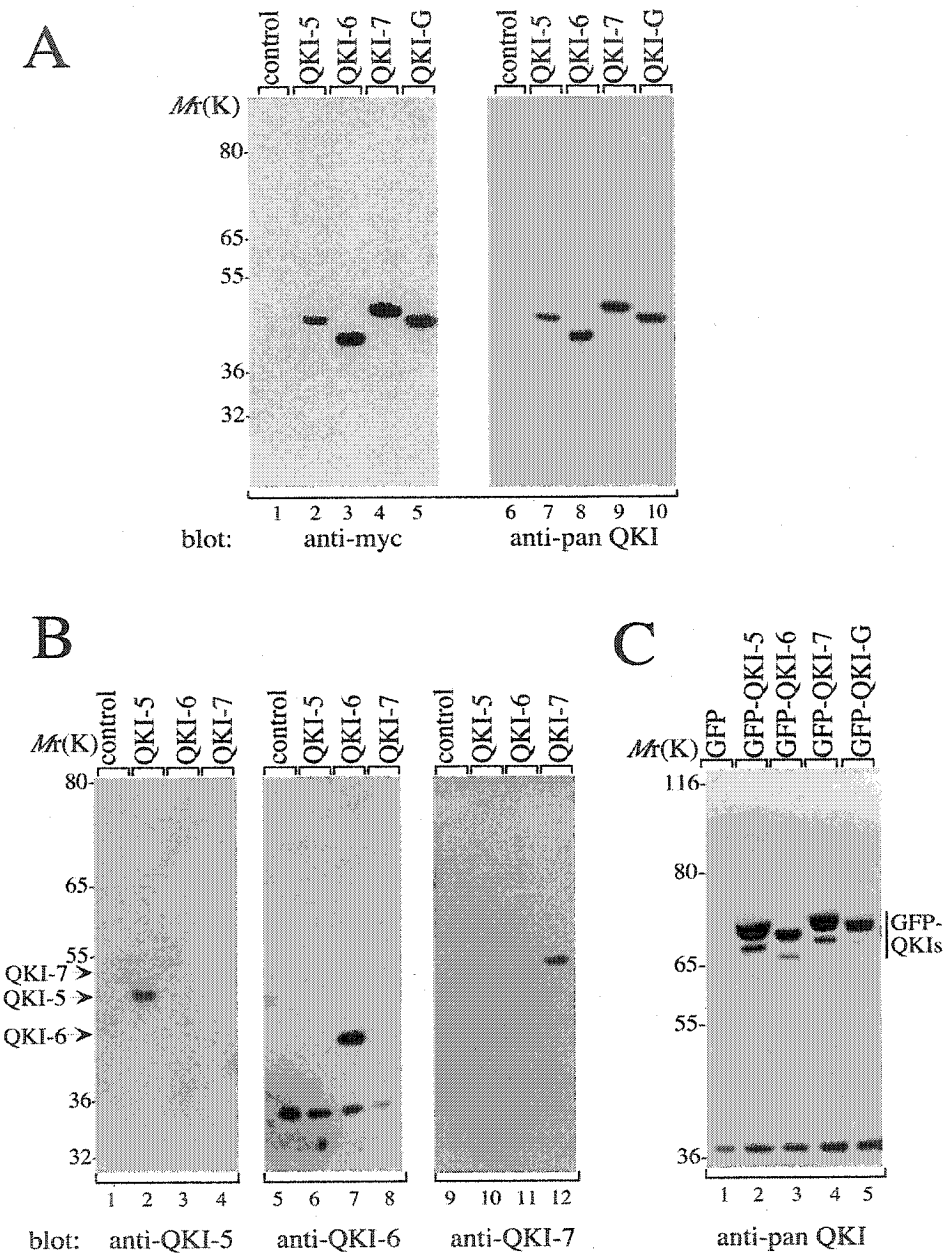

Figure 2-3. Expression of the QKI isoforms. (A) Untransfected (control) or myc-QKI-5, myc-QKI-6, myc-QKI-7, and myc-QKI-G were expressed in HeLa cells. The cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-myc (lanes 1-5) or anti-pan QKI antibodies (lanes 6-10). The molecular mass markers are shown at left in kD. (B) HeLa cells were transfected with the indicated myc-QKI as in A and analyzed by immunoblotting with anti-QKI-5 (lanes 1-4), anti-QKI-6 (lanes 5-8), and anti-QKI-7 (lanes 9-12) antibodies. The migration of QKI-5, QKI-6, and QKI-7 is indicated. (C) NIH 3T3 cells were transfected with expression plasmids expressing the indicated GFP fusion protein. The cells were lysed and analyzed by immunoblotting with anti-pan QKI antibodies.

2.5.4 Localization of the QKI isoforms.

The cellular localization of GFP-QKI-5, GFP-QKI-6, GFP-QKI-7, and GFP-QKI-G was determined. HeLa cells were transfected with expression vectors encoding the QKI isoforms for 12 hours and visualized by fluorescence microscopy. GFP-QKI-5 was predominantly nuclear (Fig. 2-4B), consistent with the presence of a nuclear localization signal at its C terminus (Wu et al. 1999). GFP-QKI-6- and GFP-QKI-G-expressing cells contained the GFP fusion protein in the nucleus and the cytoplasm (Fig. 2-4C,E). QKI-7 was predominantly cytoplasmic (Fig. 2-4D). Some cytoplasmic punctate staining was observed with QKI-6, QKI-7, and QKI-G that may represent focal adhesion structures. In summary, QKI-7 is predominantly cytoplasmic, QKI-5 is predominantly nuclear, and the QKI isoforms QKI-6 and QKI-G are localized in both the cytoplasm and the nucleus.

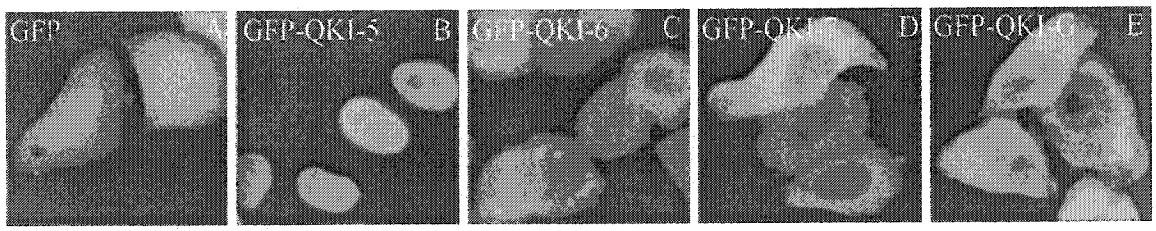

Figure 2-4. The localization of the transfected QKI isoforms in HeLa cells. Expression plasmids expressing GFP (*A*), GFP-QKI-5 (*B*), GFP-QKI-6 (*C*), GFP-QKI-7 (*D*), or GFP-QKI-G (*E*) were transfected in HeLa cells. After 12 hours the cells were fixed and visualized using fluorescence microscopy.

2.5.5 The different QKI isoforms and apoptosis.

The ability of the different QKI isoforms to induce apoptosis was examined by transfecting NIH 3T3 cells with expression vectors encoding the GFP-QKI isoforms and

analyzing the green cells for the presence of apoptosis by fluorescence microscopy at 12 and 36 hours after transfection. GFP-QKI-7 was the only wild-type isoform able to induce apoptosis at 36 hours (Fig. 2-5A). Approximately 60% of the GFP-QKI-7 transfected cells were apoptotic 36 hours after DNA transfection. In contrast, background levels of apoptosis (~15%) were observed at 36 hours with cells transfected with GFP-QKI-5, GFP-QKI-6, GFP-QKI-G, or GFP alone (Fig. 2-5A). These findings demonstrate that the QKI isoforms do not have a general toxic effect but that QKI-7 has the unique ability to signal to the cell death machinery.The ethylnitrosourea-induced mutation qk^{kt4} altering glutamic acid 48 to glycine (E48G) (Justice and Bode 1986; Ebersole et al. 1996) was introduced in the different isoforms and the resulting proteins were examined for their ability to induce apoptosis in NIH 3T3 cells. Surprisingly, all QKI isoforms including QKI-5, QKI-6, QKI-7, and QKI-G containing the E48G substitution were now able to induce apoptosis when expressed in NIH 3T3 cells (Fig. 2-5A; data not shown). The number of green cells remaining 36 hours after DNA transfection was also reduced, consistent with cell death. These data suggest that the apoptosis observed with the substitution of QKI glutamic acid 48 to glycine may represent a gain-of- function that is independent of the C-terminal unique sequences.

2.5.6 Suppression of apoptosis by coexpressing QKI-5 and QKI-6 .

The observation that elevated levels of QKI-7 were required to induce apoptosis in C6 glioma cells and the fact that the E48G substitution induces apoptosis implied that dimerization plays a role in the regulation of apoptosis. To determine the role of dimerization in QKI-mediated apoptosis, GFP-QKI-7 was cotransfected with either GFP

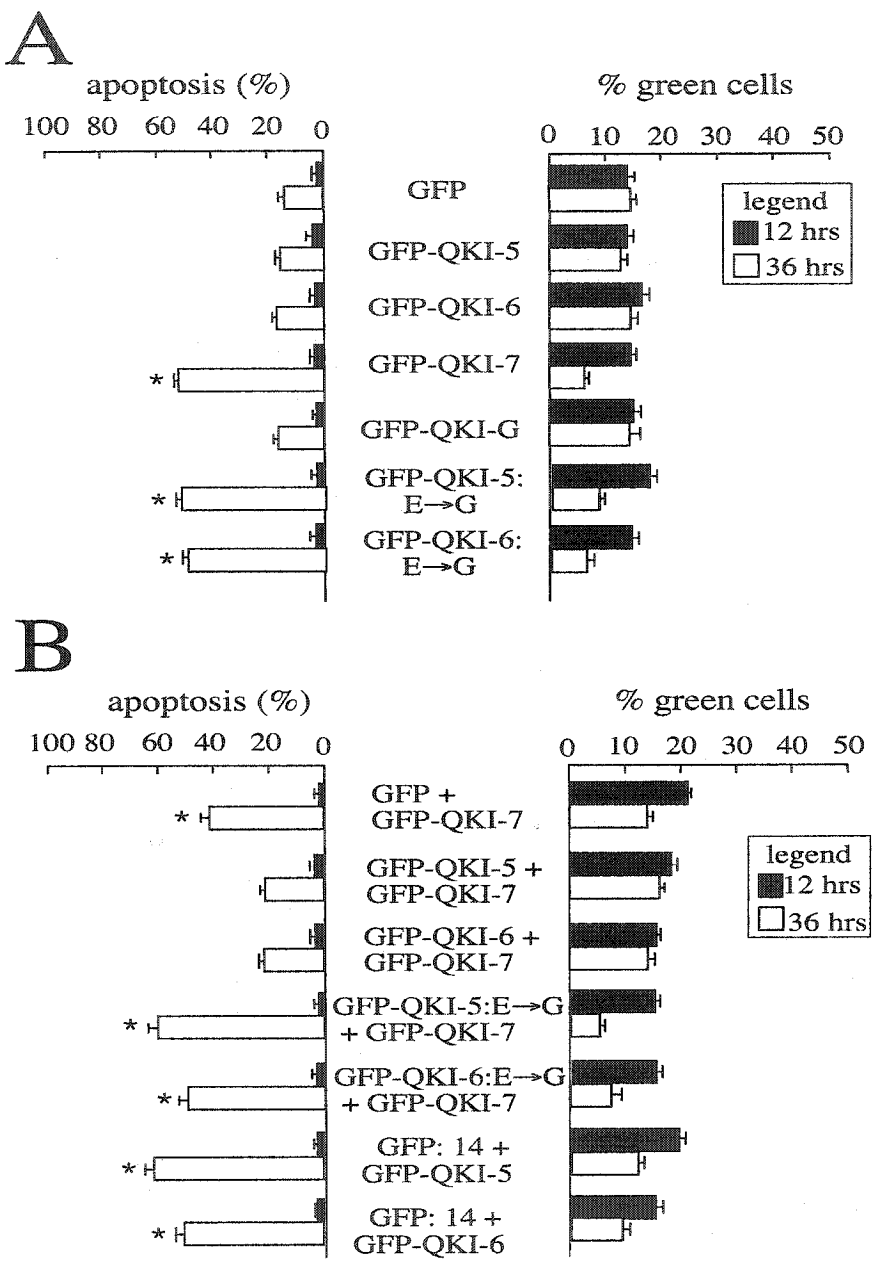

Figure 2-5. Characterization of the apoptosis induced by the QKI isoforms. (*A*) QKI-7 is the only QKI apoptotic inducer. NIH 3T3 cells were transfected with expression vectors encoding the indicated GFP fusion protein. After 12 hours (solid bars) and 36 hours (open bars) the cells were fixed and stained with DAPI. The % green cells represents the number of transfected cells as a percentage of the total number of cells as visualized by DAPI. Each bar represents the mean + S.E. of 3 experiments ($n > 450$). (*) Values that differ significantly from GFP at 36 hours ($P < 0.01$). (*B*) Dimerization is required for QKI-5 or QKI-6 to suppress the apoptosis induced by QKI-7. NIH 3T3 cells were cotransfected with expression plasmids encoding the GFP proteins indicated and the data were expressed as in *A*.

alone, GFP-QKI-5, or GFP-QKI-6 and assessed for apoptotic cell death by using the nuclear stain DAPI. The expression of GFP-QKI-5 or GFP-QKI-6 with GFP-QKI-7 suppressed the ability of GFP-QKI-7 to induce apoptosis (Fig. 2-5B). This suppressive effect was not observed when GFP alone was transfected with GFP-QKI-7. We next examined the ability of GFP-QKI-5:EG or GFP-QKI-6:EG to suppress the apoptosis mediated by GFP-QKI-7 (Fig. 2-5B). GFP-QKI-5:EG and GFP-QKI-6:EG failed to suppress the apoptosis induced by GFP-QKI-7 demonstrating that dimerization is essential for preventing cell death. To further establish that dimerization is essential for the suppression of apoptosis, we investigated the ability of QKI-5 and QKI-6 to suppress the apoptosis induced by GFP:14, a protein that cannot dimerize. Thirty-six hours after transfection, ~60% of the cells expressing GFP:14 were apoptotic regardless of whether GFP-QKI-5 or GFP-QKI-6 was expressed (Fig. 2-5B). These data suggest that QKI-5 and QKI-6 suppress apoptosis by forming heterodimers with QKI-7.

Our major concern with the cotransfection studies in Figure 2-5B was the difficulty in assessing whether the green cells expressed both isoforms. To identify the cells that were transfected with two isoforms GFP alone, GFP-QKI:1-311, or GFP-QKI-5 were cotransfected with myc-QKI-7 in NIH 3T3. The expression of myc-QKI-7 was visualized by indirect immunofluorescence by using anti-myc antibodies followed by a rhodamine-conjugated secondary antibody. The cells that were both green and red were visualized for apoptotic cell death by using the nuclear stain DAPI. Representative fields are shown (Fig. 2-6A) and the data were quantitated and expressed as percentage apoptosis (Fig. 2-6B). The cells expressing myc-QKI-7 alone, myc-QKI-7/GFP, or myc-QKI-7/GFP-QKI:1-311 exhibited characteristics of apoptosis including cell shrinkage,

cytoplasm condensation (Fig. 2-6A, panels A, D, and G, respectively), and irregular, condensed, and fragmented nuclei (Fig. 2-6A, panels B, E, and H, respectively). The myc-QKI-7-expressing cells displayed ~50% apoptosis and ~5% of the transfected cells remained attached to the dish 36 hours after transfection (Fig. 2-6B). The cells that were cotransfected with GFP-QKI-5 and myc-QKI-7 displayed a healthy and normal appearance 36 hours post-transfection (Fig. 2-6A, panels I-K). These cells had background apoptosis at 36 hours (~10%, Fig. 2-6B, left) and most of the cells transfected survived or remained on the dish (Fig. 2-6B, right). We also noted that the majority of cells that expressed one isoform also expressed the other (Fig. 2-6A). These findings suggested that our cotransfection procedure resulted in a high percentage of cells that expressed both isoforms, implying that the majority of the green cells in Figure 3-5B contained both GFP fusion proteins. The experiments demonstrate the ability of isoforms QKI-5 and QKI-6 (data not shown), but not QKI:1-311, to suppress the apoptosis induced by QKI-7. QKI:1-311 is expressed exclusively in the cytoplasm (Fig. 2-2A) and was unable to rescue the apoptosis induced by QKI-7 (Fig. 2-6). These findings suggest that the expression of a QKI isoform or protein fragment able to heterodimerize with QKI-7 is not sufficient to suppress the apoptosis, but that the heterodimerizing QKI isoform or protein fragment must have access to the nucleus.

2.5.7 Heterodimerization of the QKI isoforms in mammalian cells.

We noticed that QKI-7 entered the nucleus in the presence of QKI-5 (Fig. 2-6A, panel J). These findings suggested that heterodimerization was regulating the localization of QKI-7. To confirm that the QKI isoforms were forming heterodimers in mammalian

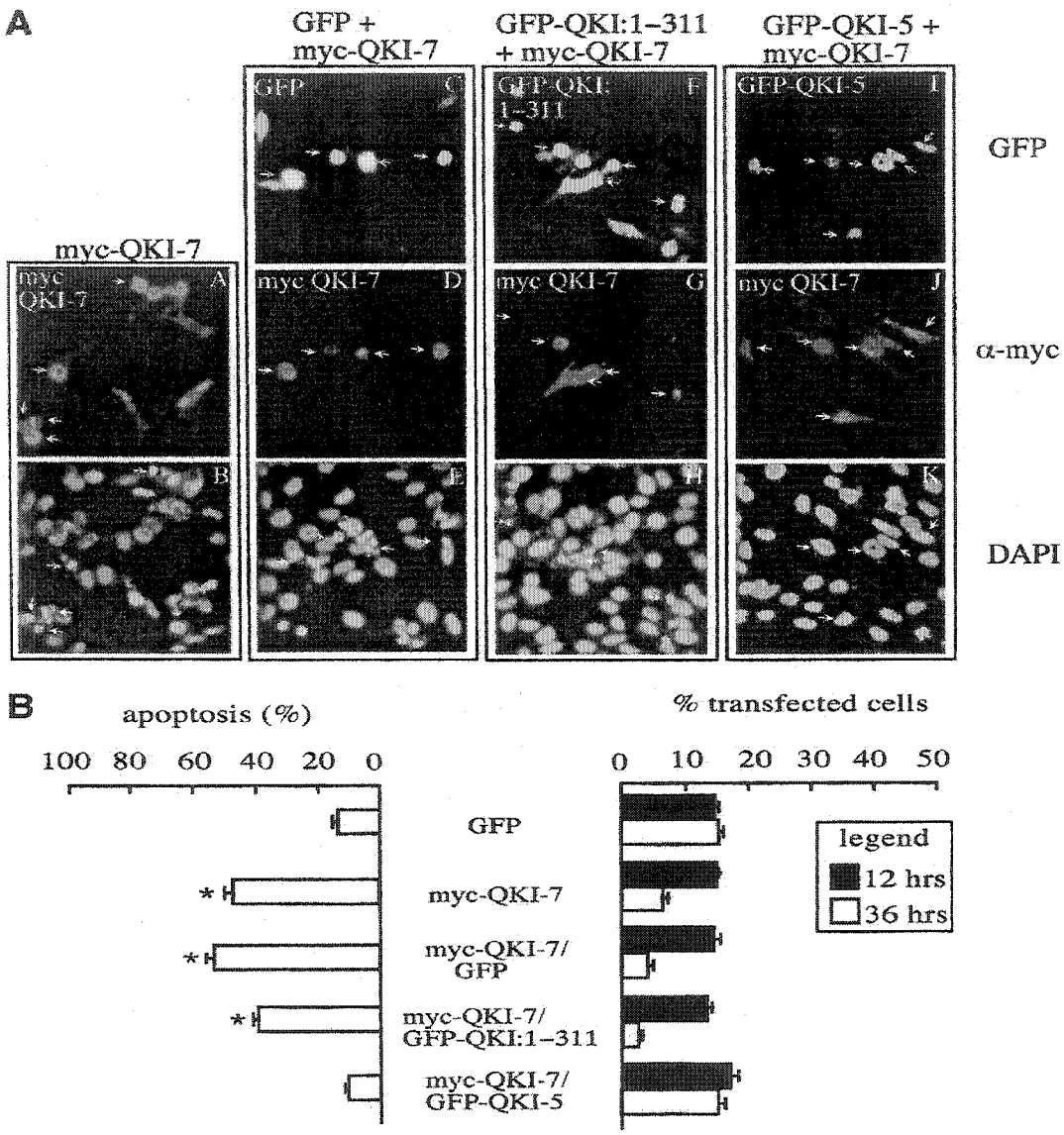

Figure 2-6. QKI-7-induced apoptosis is suppressed by QKI-5. (*A*) NIH 3T3 cells were transfected with an expression plasmid that encodes myc-QKI-7 alone (*A,B*) or with expression vectors for GFP (*C-E*), GFP-QKI:1-311 (*F-H*), or GFP-QKI-5 (*I-K*). After 36 hours the cells were fixed and visualized by indirect immunofluorescence by using the anti-myc antibody followed by rhodamine-conjugated secondary antibody. DAPI was used to visualize the nuclei. Each column represents the same field of cells as visualized under the green (*top*), red(*middle*), and blue (*bottom*) filters. The arrows are used to align the panels. (*B*) Quantitation of the apoptosis suppressed by QKI-5. NIH 3T3 cells were transfected as in *A*. For the cells expressing both a GFP fusion protein and a myc epitope-tagged protein, the apoptotic cells were expressed as a percentage of the cells that were both green and red. The panel at *right* indicates the percentage of cells that were transfected from the total number of cells. Each bar represents the mean + S.E. of 3 experiments ($n > 450$). (*) Values that differ significantly from GFP at 36 hours ($P < 0.01$).

cells, we cotransfected the different isoforms in HeLa cells and performed coimmunoprecipitation studies. Hemagglutin (HA)-tagged QKI-5 was transfected alone or cotransfected with myc-QKI-5, myc-QKI-6, or myc-QKI-7. The cells were lysed, the lysates were divided equally, and the proteins immunoprecipitated with control immunoglobulin G (IgG) and anti-myc antibodies. The proteins were separated by SDS-PAGE and analyzed by immunoblotting with anti-HA antibodies. HA-QKI-5 coimmunoprecipitated with myc-QKI-5, myc-QKI-6, and myc-QKI-7 (Fig. 2-7A, lanes 1-12), demonstrating that heterodimerization occurs in vivo. Similar findings were obtained with HA-QKI-6 (Fig. 2-7A, lanes 13-21). These observations show that heterodimerization occurs between QKI isoforms that display predominant cytoplasmic and nuclear localizations such as QKI-7 and QKI-5. We next examined whether the E48G substitution could prevent heterodimerization. HA-tagged QKI-5:EG or QKI-6:EG were cotransfected with myc-QKI-5, myc-QKI-6, or myc-QKI-7 and analyzed as described above. HA-QKI-5:EG and HA-QKI-6:EG did not coimmunoprecipitate with any of the wild-type isoforms (Fig. 2-7B, lanes 1-21). These results suggest that the heterodimerization domain is similar to the homodimerization domain located in the NK region of the GSG domain.

2.5.8 Heterodimerization controls QKI-7 localization.

The relocalization of the QKI isoforms was further investigated in HeLa cells, a cell type where it could be easily observed. Myc-QKI-7 was transfected or cotransfected with GFP-QKI-5 or GFP-QKI-6 in HeLa cells and visualized by using fluorescence microscopy 12 hours post-transfection. The 12-hour time point was chosen because no

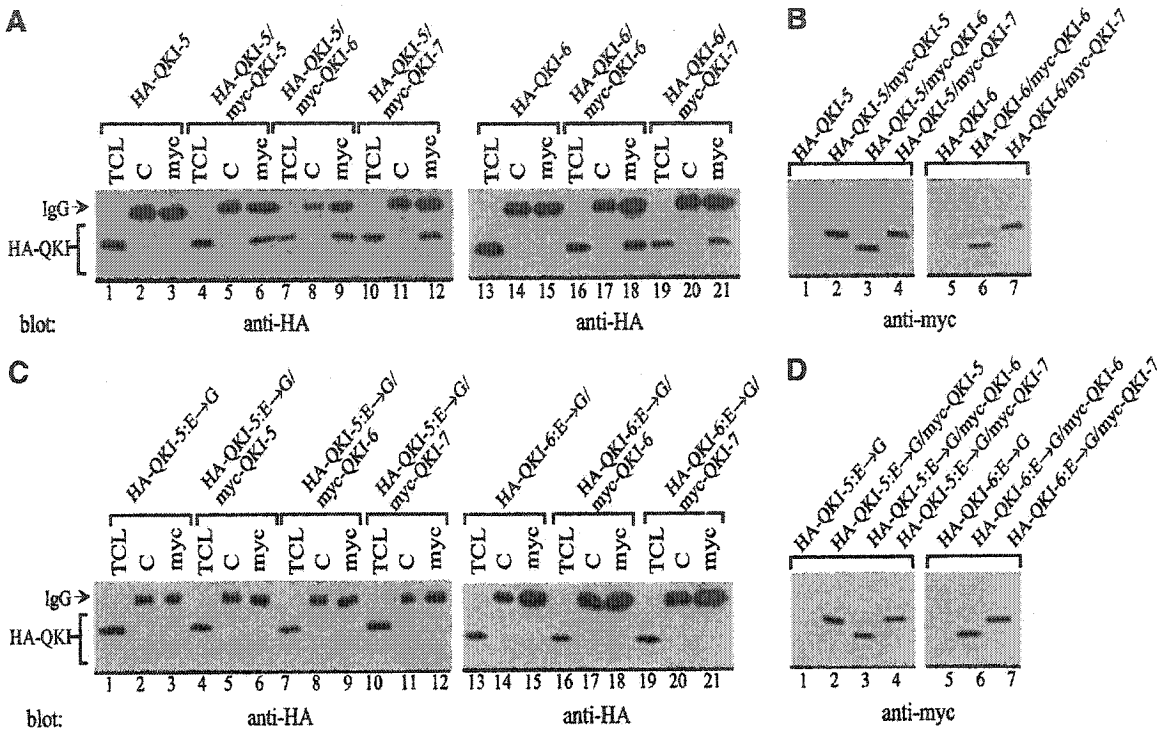

Figure 2-7. Heterodimerization of the QKI isoforms in fibroblasts is abolished by the EG amino acid substitution. (*A,C*) HeLa cells were transfected with myc-QKI and HA-QKI isoforms as indicated. The cells were lysed and immunoprecipitated with IgG as a control (C) or anti-myc antibodies (myc). The bound proteins as well as an aliquot of total cellular lysate (TCL) were separated by SDS-PAGE and analyzed by immunoblotting with anti-HA antibodies. The migration of the heavy chain of IgG and the HA-QKI isoforms is shown. (*B,D*) The total cellular lysate of panels *A* and *C* were confirmed for the equivalent expression of the myc epitope-tagged QKI proteins.

apoptosis was observed at this time. Myc-QKI-7 localized exclusively to the cytoplasm of HeLa cells (Fig. 2-8A,B). However, myc-QKI-7 staining could be observed throughout the cell, with prominent nuclear localization, when coexpressed with GFP-QKI-5 (Fig. 2-8C-E). Interestingly, the localization of QKI-5 did not relocalize to the cytoplasm with the coexpression of QKI-7 (Fig. 2-8, C-E). The coexpression of GFP-QKI-6 also relocalized myc-QKI-7 to the nucleus (Fig. 2-8F-H), but less nuclear staining was observed as compared with GFP-QKI-5 (Fig. 2-8, cf. D and G). Although significant

cytoplasmic staining of QKI-7 was observed with QKI-6 (Fig. 2-8G), QKI-6 was able to suppress the apoptosis by QKI-7 (Fig. 2-5B). These findings suggest that in addition to relocalizing QKI-7 to the nucleus, QKI-6 may also inactivate cytoplasmic QKI-7 by forming heterodimers. If dimerization is required for relocalization, then the E48G substitution should impair the relocalization. HeLa cells were cotransfected with myc-QKI-7 and GFP-QKI-5:EG or GFP-QKI-6:EG and visualized by fluorescence microscopy. Myc-QKI-7 failed to relocalize to the nucleus with the coexpression of GFP-QKI-5:EG or GFP-QKI-6:EG (Fig. 2-8I-N). These results suggest that the nuclear localization of QKI-7 is controlled by heterodimerization.

2.5.9 QKI-7 adenovirus induces apoptosis in primary rat OLs.

The dysmyelination phenotype observed in the qk^{v} mice suggests that the QKI isoforms are involved in the normal physiology of the OLs (Hogan and Greenfield 1984). We wanted to examine whether QKI-7 was a potent apoptotic inducer in a cell type where it is known to have a physiological role. We constructed adenoviruses that express tetracycline-inducible QKI-5, QKI-6, and QKI-7. The QKI adenoviruses also express GFP constitutively and it is a marker of infection. Initially, we performed experiments with HeLa cells to verify that the adenoviruses were expressing the QKI isoforms in an inducible manner. HeLa cells were coinfected with two adenoviruses: one supplying constitutive levels of the tetracycline-regulated transactivator (tTA) and the other expressing the desired QKI isoform under a tetracycline-inducible promoter. Induced and noninduced infected HeLa cells were lysed, and the proteins were separated by SDS-PAGE and immunoblotted with anti-myc antibodies. QKI-5, QKI-6, and QKI-7 were

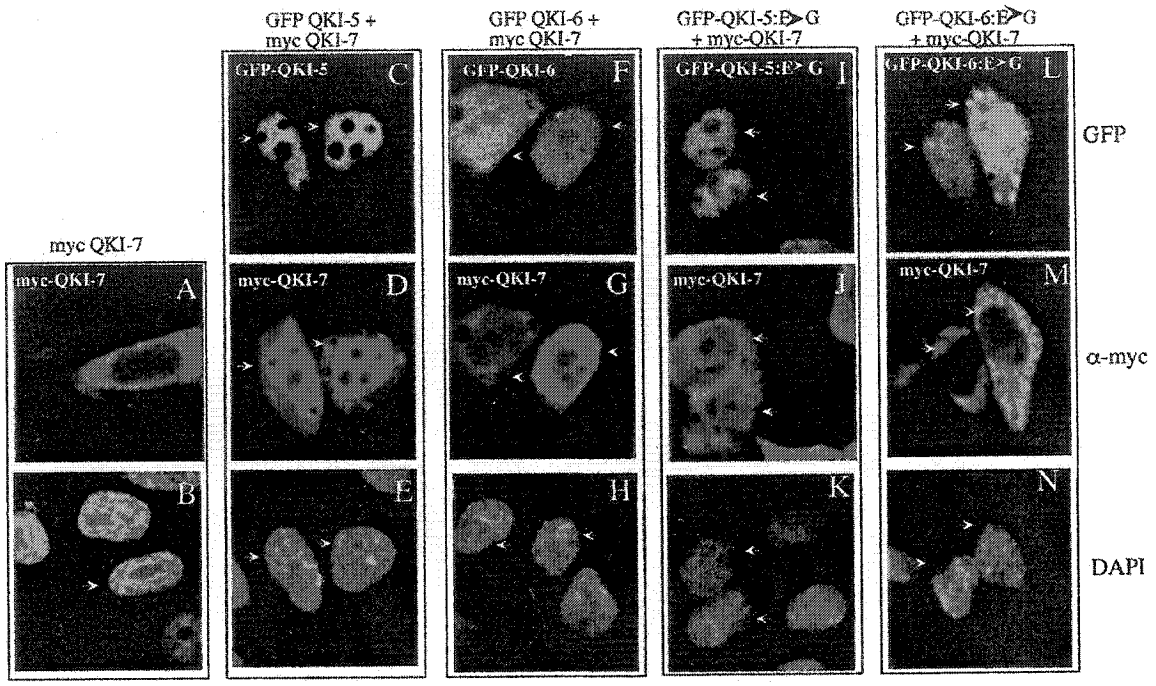

Figure 2-8. Nuclear translocation of QKI-7 with QKI-5 or QKI-6 but not with QKI-5:EG or QKI-6:EG. Myc-QKI-7 was either transfected alone (*A,B*) or cotransfected with GFP-QKI-5 (*C-E*), GFP-QKI-6 (*F-H*), GFP-QKI-5:EG (*I-K*), or GFP-QKI-6:EG (*L-N*) into HeLa cells. After 12 hours the cells were fixed, immunostained with an anti-myc antibody followed by a rhodamine-conjugated secondary antibody, and mounted onto a glass slide in the presence of the nuclear stain DAPI. The cells were visualized by fluorescence microscopy. Each column represent the same field of cells as visualized under the green (*top*), red (*middle*), and blue (*bottom*) filters. The arrows are used to align the panels.

expressed in a tetracycline-inducible manner (Fig. 2-9A). The expression of the QKI isoforms was tightly regulated, as little or no expression was observed in the noninduced cultures. Primary rat OLs were isolated from newborn rats as described (Lubetzki et al. 1991). After 2 days of maturation in the presence of growth factors, the cells displayed an OL appearance with little or no contaminating astrocytes (Fig. 2-9B, left). Approximately 43% of the cells stained with anti-galactocerebroside antibody conjugated to FITC, a well-known marker for mature OLs (Fig. 2-9B, right). Thus, our cultures contained ~40%

mature OLs and ~60% immature OLs. These cells were coinfected as described above with a tTA-expressing adenovirus and the QKI-inducible adenoviruses and could be visualized by indirect immunofluorescence by using the anti-myc antibody. The myc epitope-tagged QKI isoforms were expressed in their respective compartments in the OLs. QKI-5 was nuclear, QKI-6 localized throughout the cell, and QKI-7 was cytoplasmic (Fig. 2-9C). We next examined whether the overexpression of the QKI isoforms resulted in cell death. The induced and noninduced infected cells were stained live with annexin V-phycoerythrin to detect early signs of apoptosis. The stained cells were analyzed by flow cytometry for the expression of GFP and annexin V-phycoerythrin. Approximately ~65% of the cells infected with the QKI-7 adenovirus in the ON state stained positive for annexin V-phycoerythrin (Fig. 2-9D, bottom right). The infection with QKI-5 or QKI-6 induced and noninduced had no significant increase in staining with annexin V (Fig. 2-9D, top right). All OLs cultures were infected at equivalent MOIs as observed with the expression of the GFP marker (Fig. 2-9D, left). These data suggest that QKI-7 is an apoptotic inducer in mature and immature primary rat OLs.

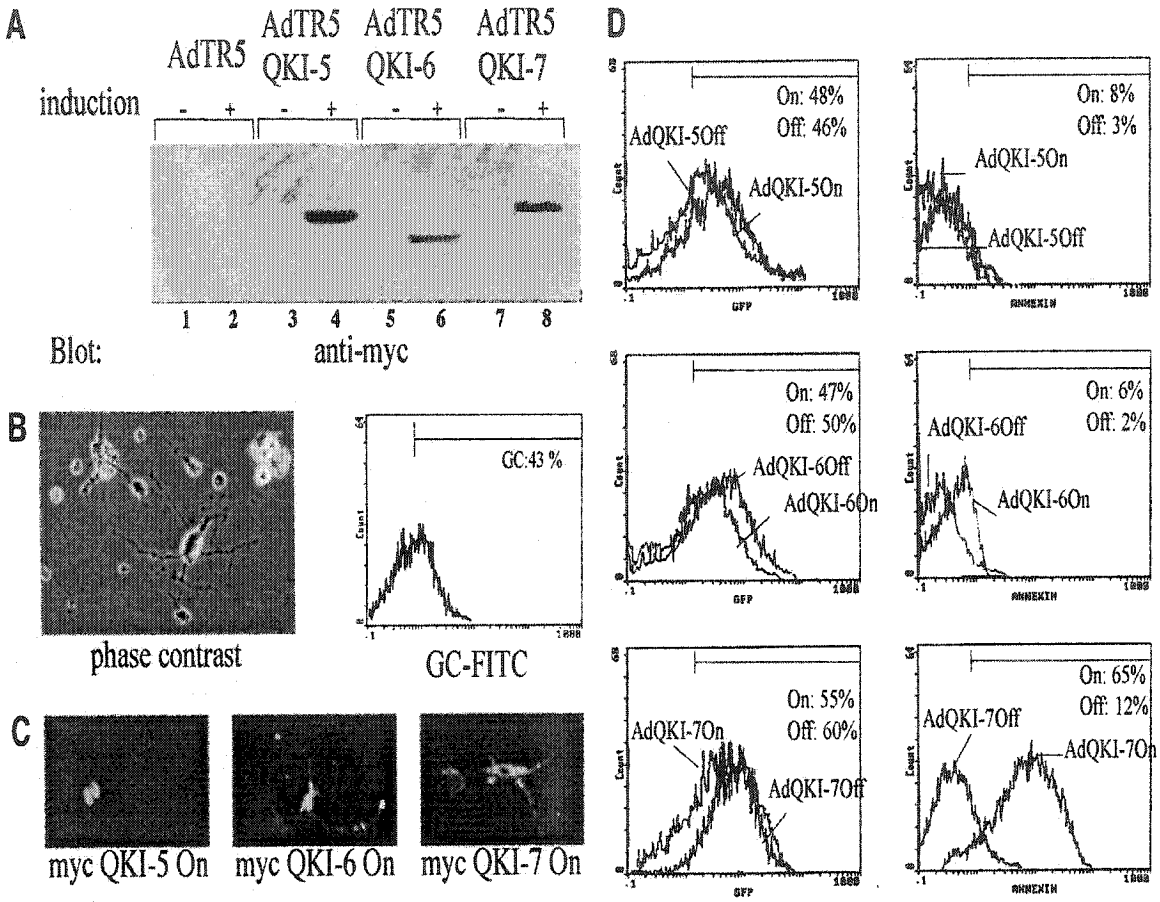

Figure 2-9. Adenoviruses expressing QKI-7 induce apoptosis in primary rat OLs. (*A*) The generation of QKI-inducible adenoviruses. HeLa cells were coinfected at an M.O.I. of 10 with an adenovirus that constitutively expresses the tTA and a tetracycline-inducible adenovirus expressing myc-QKI-5, myc-QKI-6, or myc-QKI-7. (+) Absence of doxycyclin; (-) addition of 1 μg/mL of doxycyclin. Essentially 100% of the cells were green 36 hours after infection (not shown). The cells were harvested, lysed in sample buffer, separated by SDS-PAGE, and immunoblotted with anti-myc antibodies. pAdTR5 denotes an adenovirus generated with vector alone. pAdTR5-QKI-5, pAdTR5-QKI-6, and pAdTR5-QKI-7 represent the adenoviruses expressing QKI-5, QKI-6, and QKI-7, respectively. (*B*) Phase contrast photograph of a typical primary rat OLs preparation 2 days after maturation in growth factors (*left* panel). Uninfected OLs were stained live with anti-galactocerebroside FITC-conjugated antibodies and analyzed by flow cytometry. (*C*) Primary rat OLs were coinfected as in *A*, fixed, permeabilized, and stained with anti-myc antibodies followed by a rhodamine-conjugated secondary antibody. The cells were visualized by fluorescence microscopy. (*D*) Primary rat OLs were coinfected as in *A*, stained live with annexin V-phycoerythrin, and analyzed by flow cytometry. The percentage of GFP and annexin V positive cells is indicated for each condition. The experiment shown is representative of three experiments.

2.6 Discussion

In this study, we show that a balance of the QKI isoforms regulates the activity of the QKI-7 apoptotic inducer. The expression of the different QKI isoforms including QKI-5, QKI-6, and QKI-G in fibroblasts or primary rat OLs did not induce apoptosis. The coexpression of either QKI-5 or QKI-6 with QKI-7 caused the nuclear translocation of QKI-7 and suppressed the apoptosis normally observed with the expression of QKI-7. These data suggest that heterodimerization causes the nuclear translocation of QKI-7 and may be the major mechanism responsible for its inactivation.

The region necessary for the induction of apoptosis was mapped to the unique C-terminal 14 amino acids of QKI-7. This represents a new functional domain in the QKI proteins. Other functional domains characterized previously in QKI include the GSG domain required for dimerization and RNA binding (Chen and Richard 1998) and an NLS in the unique sequences of QKI-5 (Wu et al. 1999). The finding that the C-terminal sequences of QKI-7 are sufficient to confer the ability to induce apoptosis to GFP and GLD-1 suggests that the 14 amino acids of QKI-7 signal to the apoptotic machinery independent of other functional domains. Thus, the RNA-binding activity of QKI-7 is not required for induction of cell death. Mutations or deletions within the QKI-7 GSG domain did not suppress the apoptosis (data not shown). Because the 14 amino acids of QKI-7 confer the ability to induce apoptosis to heterologous proteins, we propose the name 'killer sequence' for this sequence. Database searches using the 14-amino-acid killer sequence EWIEMPVMPDISAH did not reveal a novel protein module. However, a core motif (underlined) was found for *Drosophila, C. elegans*, and mammalian proteins in GenBank. The proteins that contained this motif included *C. elegans* CED-9 (the

histidine is replaced with a lysine) and hypothetical protein C09H10.9 (accession no. T19165), human and murine Hect2/rjs protein (accession no. AAC31433), *Drosophila* CG11958 and CG9906 gene products (accession nos. AAF57631 and AAF48618). The mechanism by which the killer sequence QKI-7 communicates to the apoptotic machinery is unknown.

The suppression of apoptosis by the coexpression of QKI-5 or QKI-6, but not QKI:1-311 suggests that heterodimerization with QKI-7 is not sufficient for the suppression. It has been shown that QKI-5 is capable of nucleoplasmic shuttling (Wu et al. 1999). Thus, QKI-5 and other QKI isoforms such as QKI-6 and QKI-G may shuttle into the nucleus as a heterodimer with QKI-7. Because QKI:1-311 has an exclusive cytoplasmic localization, QKI-7/QKI:1-311 heterodimers would remain cytoplasmic resulting in apoptosis. Our data suggest that cytoplasmic but not nuclear QKI-7 signals to the apoptotic machinery.

The qk^v mice have been shown to contain an enhancer/promoter deletion in the *qk* gene (Ebersole et al. 1996). It has been shown that the qk^v OLs express QKI-5, but not QKI-6 and QKI-7 (Hardy et al. 1996). How an enhancer/promoter deletion prevents the production of the QKI-6 and QKI-7 isoforms in OLs is unknown. It is thought that the absence of QKI-6 and QKI-7 prevents the proper maturation of the OLs and/or the process of myelination (Hardy et al. 1996). Our findings suggest that the balance in the QKI isoforms is critical for the normal function of the QKI proteins and cell viability. Based on our findings, the loss of QKI-6 and QKI-7 should not affect the viability of the OLs in the qk^v mice and indeed normal quantities and hyperplasia of OLs have been reported (Friedrich 1975). We believe that the loss of QKI-6 and QKI-7 would provide an

imbalance in the QKI isoforms that would impair the differentiation and maturation of the OLs resulting in the dysmyelination phenotype observed in the qk^{v} mice. A balance between QKI isoforms (HOW; held-out-wings) has been reported for the export of the stripe mRNA in *Drosophila melanogaster* (Nabel-Rosen et al. 1999). This supports the hypothesis that the balance in the QKI isoforms regulates their function.

The ethylnitrosourea-induced point mutation altering glutamic acid 48 to glycine prevents homo- and heterodimerization of all QKI isoforms (Fig 2-7; Chen and Richard 1998; Wu et al. 1999). Moreover, we showed that all QKI isoforms containing the E48G amino acid substitution are able to induce apoptosis, regardless of whether they contained the killer sequence. These data suggest that the QKI:E48G represents a gain-of-function amino acid substitution that is separate from the QKI-7 killer sequence. Thus, the QKI proteins have multiple regions that can induce apoptosis: QKI-7 has the killer sequence and all isoforms have an NK region that can be altered to induce cell death. These findings further support our hypothesis that the QKI isoforms are critical proteins for maintenance of cell viability and cell death. It is possible that QKI isoforms other than QKI-7 are apoptosis inhibitors or growth inducers themselves. The fact that QKI-5:E48G is a potent inducer of apoptosis suggests that the embryonic lethality observed in embryos containing the qk^{kt4} allele (Justice and Bode 1986; Ebersole et al. 1996) is caused by apoptotic cell death.

There are several known genes that give rise to alternatively spliced transcripts encoding proteins with opposing cell death functions. The genes for *bcl-x* (Boise et al. 1993) and *C. elegans ced-4* (Shaham and Horvitz 1996) each encode a long and a short transcript. The longer transcripts encode proteins that protect against cell death and the

shorter transcripts encode proteins that promote cell death. Two genes encoding caspases including Ich-1 (Wang et al. 1994) and interleukin-1-converting enzyme (Alnemri et al. 1995) have been shown to produce alternatively spliced transcripts that have positive and negative influences on programmed cell death. In the examples mentioned above, it has been proposed by the authors that it is the balance between the activator and repressor of programmed cell death, controlled by factors that influence splicing, which determines whether a cell will live or die. It has been shown that the two splicing factors SC35 and hnRNP A1 have opposing roles in the alternative splicing of the Ich-1 or caspase 2 gene (Jiang et al. 1998).

In summary, we characterized four different QKI isoforms and their ability to induce apoptosis. The expression of the cytoplasmic QKI-7 isoform is sufficient to induce apoptosis in the absence of other signals. The expression of the other alternatively spliced QKI isoforms induced cell survival by protecting the cell against the QKI-7 apoptotic inducer. The balance between QKI-7 and the QKI isoforms was required for cell survival. If QKI-7 is the major isoform or if it is overexpressed, QKI-7 localizes to the cytoplasm and induces cell death. If the QKI isoforms are expressed with QKI-7 such that an appropriate balance is achieved, the proteins heterodimerize and cause the nuclear translocation of QKI-7 resulting in cell survival. In addition, our studies identify a short peptide sequence that could be used to tag proteins and create chimeric proteins able to induce cell death. Our findings support the hypothesis that factors that influence splicing can regulate cell death and survival.

2.7 Acknowledgments

We thank Karen Artzt for providing the isoform-specific anti-QKI antibodies, Bernard Massie for providing pAdTR5-K7-GFPq and the adenovirus expressing the tTA, and Walter Nishioka for the Bcl-2 expression vector. We are grateful to Taiping Chen for performing some of the initial C-terminal QKI deletions. This work was supported by a grant from the Multiple Sclerosis Society of Canada. J.P. is a recipient of the Research Studentship from the Multiple Sclerosis Society of Canada and S.R is a Scholar of the Canadian Institutes of Health Research of Canada.

2.8 References

Adams, J.M. and Cory, S. 1998. The Bcl-2 protein family: Arbiters of cell survival. Science 281: 1322-1326.

Alnemri, E.S., Fernandes-Alnemri, T., and Litwack, G. 1995. Cloning and expression of four novel isoforms of human interleukin-1 converting enzyme with different apoptotic activities. J. Biol. Chem. 270: 4312-4317.

Ashkenazi, A. and Dixit, V.M. 1998. Death receptors: Signaling and modulation. Science 281: 1305-1308.

Baehrecke, E.H. 1997. who encodes a KH RNA binding protein that functions in muscle development. Development 124: 1323-1332.

Boise, L.H., Gonzales-Garcia, M., Postema, C.E., Ding, L., Lindsten, T., Turka, L.A., Mao, X., Nunez, G., and Thompson, C.B. 1993. *bcl-x*, a *bcl-2*-related gene that functions as a dominant regulator of apoptotic cell death. Cell 74: 597-608.

Chen, T. and Richard, S. 1998. Structure-function analysis of QKI: A lethal point mutation in mouse *quaking* prevents homodimerization. Mol. Cell. Biol. 18: 4863-4871.

Chen, T., Damaj, B., Herrerra, C., Lasko, P., and Richard, S. 1997. Self-association of the single-KH domain family members Sam68, GRP33, GLD-1 and Qk1: Role of the KH domain. Mol. Cell. Biol. 17:5707-5718.

Cox, R.D., Hugill, A., Shedlovsky, A., Noveroske, J.K., Best, S., Justice, M.J., Lehrach, H., and Dove, W.F. 1999. Contrasting effects of ENU induced embryonic lethal mutations of the *quaking* gene. Genomics 57: 333-34.

Di Fruscio, M., Chen, T., Bonyadi, S., Lasko, P., and Richard, S. 1998. The identification of two *Drosophila* KH domain proteins: KEP1 and SAM are members of the Sam68 family of GSG domain proteins. J. Biol. Chem. 273: 30122-30130.

Ebersole, T.A., Chen, Q., Justice, M.A., and Artzt, K. 1996. The *quaking* gene unites signal transduction and RNA binding in the developing nervous system. Nat. Genet. 12: 260-265.

Evan, G. and Littlewood, T. 1998. A matter of life and cell death. Science 281:1317-1321.

Francis, R., Maine, E., and Schedl, T. 1995. Gld-1: A cell-type specific tumor suppressor gene in *C. elegans*. Genetics 139: 607-630.

Friedrich, V.L.J. 1975. Hyperplasia of oligodendrocytes in *quaking* mice. Anat. Embryol. 147:259-271.

Gibson, T.J., Thompson, J.D., and Heringa, J. 1993. The KH domain occurs in a diverse set of RNA-binding proteins that include the antiterminator NusA and is probably involved in binding to nucleic acid. FEBS Letts. 324: 361-366.

Gross, A., McDonnell, J.M., and Korsmeyer, S.J. 1999. BCL-2 family members and the mitochondria in apoptosis.Genes & Dev. 13: 1899-1911.

Hardy, R.J., Loushin, C.L., Friedrich, V.L., Jr., Chen, Q., Ebersole, T.A., Lazzarini, R.A., and Artzt, K. 1996. Neural cell type-specific expression of QKI proteins is altered in the *quaking* viable mutant mice. J. Neurosci. 16: 7941-7949.

Hogan, E.L. and Greenfield, S. 1984. Animal models of genetic disorders of myelin. In Myelin (ed. P. Morell), pp. 489-534. Plenum, New York.

Jan, E., Motzny, C.K., Graves, L.E., and Goodwin, E.B. 1999. The STAR protein, GLD-1, is a translational regulator of sexual identity in *Caenorhabditis elegans*. EMBO J. 18: 258-269

Jiang, Z.-H., Zhang, W.-J., Rao, Y., and Wu, J.Y. 1998. Regulation of Ich-1 pre-mRNA alternative splicing and apoptosis by mammalian splicing factors. Proc. Natl. Acad. Sci. 95: 9155-9160.

Jones, A.R. and Schedl, T. 1995. Mutations in GLD-1, a female germ cell-specific tumor suppressor gene in *C. elegans*, affect a conserved domain also found in Sam68. Genes & Dev. 9: 1491-1504.

Justice, M. and Bode, V. 1986. Induction of new mutations in a mouse t-haplotype using ethylnitrosourea mutagenesis. Genet. Res. 47: 187-192.

Justice, M.J. and Bode, V.C. 1988. Three ENU-induced alleles of the murine *quaking* locus are recessive embryonic lethal mutations. Genet. Res. 51: 95-102.

Ko, L.J. and Prives, C. 1996. p53: Puzzle and paradigm. Genes & Dev. 10: 1054-1072.

Kondo, T., Furuta, T., Mitsunaga, K., Ebersole, T.A., Shichiri, M., Wu, J., Artzt, K., Yamamura, K., and Abe, K. 1999. Genomic organization and expression analysis of the mouse *qkI* locus. Mamm. Genome 10:662-669.

Livingstone, L.R., White, A., Sprouse, J., Livanos, E., Jacks, T., and Tisty, T.D. 1992. Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. Cell 70: 923-935.

Lubetzki, C., Goujet-Zalc, C., Gansmuller, A., Monge, M., Brillat, A., and Zalc, B. 1991. Morphological, biochemical, and function characterization of bulk isolated glial progenitor cells. J. Neurochem. 56:671-680.

Massie, B., Couture, F., Lamoureux, L., Mosser, D.D., Guilbault, C., Jolicoeur, P., Belanger, F., and Langelier, Y. 1998. Inducible overexpression of a toxic protein by an adenovirus vector with a tetracycline-regulatable expression cassette. J. Virol. 72: 2289-2296.

Massie, B., Mosser, D.D., Koutroumanis, M., Vitté-Mony, I., Lamoureux, L., Couture, F., Paquet, L., Guilbault, C., Dionne, J., Chahla, D. et al. 1999. New adenovirus vectors for protein production and gene transfer. Cytotechnology 28: 1-12.

Mezquita, J., Pau, M., and Mezquita, C. 1998. Four isoforms of the signal-transduction and RNA-binding protein Qk1 expressed during chicken spermatogenesis. Mol. Reprod. Devel. 50: 70-78.

Nabel-Rosen, H., Dorevitch, N., Reuveny, A., and Volk, T. 1999. The balance between two isoforms of the *Drosophila* RNA-binding protein How controls tendon cell differentiation. Mol. Cell 4: 573-584.

Nicholson, D.W., Ali, A., Thornberry, N.A., Vaillancourt, J.P., Ding, C.K., Gallant, M., Gareau, Y., Griffin, P.R., Labelle, M., and Lazebnik, Y.A. 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 376: 37-43.

Porter, A.G. 1999. Protein translocation in apoptosis. Trends Cell Biol. 9: 394-401.

Reed, J.C. 1998. Bcl-2 family proteins. Oncogene 17: 3225-3236.

Saccomanno, L., Loushin, C., Jan, E., Punkay, E., Artzt, K., and Goodwin, E.B. 1999. The STAR protein Qk1-6 is a translational repressor. Proc. Natl. Acad. Sci. 96: 12605-12610.

Shaham, S. and Horvitz, H.R. 1996. An alternatively spliced *C. elegans ced-4* RNA encodes a novel cell death inhibitor. Cell 86: 201-208.

Shedlovsky, A., King, T.R., and Dove, W.F. 1988. Saturation germ line mutagenesis of the murine t region including a lethal allele of the *quaking* locus. Proc. Natl. Acad. Sci. 85: 180-184.

Siomi, H., Siomi, M.C., Nussbaum, R.L., and Dreyfuss, G. 1993. The protein product of the fragile X gene, FMR1 has characteristics of an RNA binding protein. Cell 74: 291-298.

Tanaka, H., Abe, K., and Kim, C.H. 1997. Cloning and expression of the *quaking* gene in the Zebrafish embryo. Mech. Develop. 69: 209-213.

Tartaglia, L.A. and Goeddel, D.V. 1992. Two TNF receptors. Immunol. Today 13: 151-153.

Vernet, C. and Artzt, K. 1997. STAR, a gene family involved in signal transduction and activation of RNA. Trends Genet. 13: 479-484.

Wan, L., Dockendorff, T.C., Jongens, T.A., and Dreyfuss, G. 2000. Characterization of dFMR1, a *Drosophila* melanogaster homolog of the fragile X mental retardation protein. Mol. Cell. Biol. 20:8536-8547.

Wang, L., Miura, M., Bergeron, L., Zhu, H., and Yuan, J. 1994. Ich-1, an Ice/ced-3-related gene encodes both positive and negative regulators of programmed cell death. Cell 78: 739-750.

Wu, J., Zhou, L., Tonissen, K., Tee, R., and Artzt, K. 1999. The *quaking* I-5 (QKI-5) has a novel nuclear localization signal and shuttles between the nucleus and the cytoplasm. J. Biol. Chem. 274:29202-29210.

Zaffran, S., Astier, M., Gratecos, D., and Semeriva, M. 1997. The held out wings (how) *Drosophila* gene encodes a putative RNA binding protein involved in the control of muscular and cardiac activity. Development 124: 2087-2098.

Zhu, J. and Chen, X. 2000. MCG10, a novel p53 target gene that encodes a KH domain RNA-binding protein, is capable of inducing apoptosis and cell cycle arrest in G2-M. Mol. Cell. Biol. 20:5602-5618.

Zorn, A.M. and Krieg, P.A. 1997. The KH domain protein encoded by *quaking* functions as a dimer and is essential for notochord development in *Xenopus* embryos. Genes & Dev. 11:2176-2190.

Zorn, A.M., Grow, M., Patterson, K.D., Ebersole, T.A., Chen, Q., Artzt, K., and Krieg, P.A. 1997. Remarkable sequence conservation of transcripts encoding amphibian and mammalian homologues of *quaking* a KH domain RNA-binding protein. Gene 188: 199-206.

Chapter 3

NUCLEAR RETENTION OF MBP mRNAS IN THE *QUAKING VIABLE* MICE

3.1 Preface

Many myelin proteins are affected by the *quaking* viable mouse mutation. Since *quaking* is an RNA-binding protein and is mostly expressed in myelinating glial cells such as oligodendrocytes, it suggests an involvement of this protein in the regulation of mRNAs encoding myelin proteins. We were interested in finding a RNA target for the QUAKING proteins, therefore we looked at myelin proteins altered in the *quaking* viable mouse and identified MBP as a potential target. We were also interested in understanding the reason for the myelination defects in the *quaking viable* mouse, which will be the themes covered in the following study.

3.2 Summary

Quaking viable (qk^v) mice fail to properly compact myelin in their central nervous systems. Although the defect in the qk^v mice involves a mutation affecting the expression of the alternatively spliced qk gene products, their roles in myelination are unknown. We show that the QKI RNA binding proteins regulate the nuclear export of MBP mRNAs. Disruption of the QKI nucleocytoplasmic equilibrium in oligodendrocytes results in nuclear and perikaryal retention of the MBP mRNAs and lack of export to cytoplasmic processes, as it occurs in qk^v mice. MBP mRNA export defect leads to a reduction in the

MBP levels and their improper cellular targeting to the periphery. Our findings suggest that QKI participates in myelination by regulating the mRNA export of key protein components.

3.3 Introduction

Quaking viable (qk^v) mice contain a spontaneous mutation resulting in hypomyelination of the central and peripheral nervous systems (Sidman et al., 1964). These animals have been studied for over thirty years and represent an animal model for dysmyelination (reviewed in Hogan and Greenfield, 1984). These mutant mice develop normally until postnatal day 10 when they display rapid tremors or "quaking" that is especially pronounced in the hindlimbs and experience convulsive tonic-clonic seizures as they mature (reviewed in Hogan and Greenfield, 1984). Studies using the qk^v mice have identified that oligodendrocyte (OL) function was impaired and that the intracellular transport of myelin components might be the underlying defect (reviewed in Hogan and Greenfield, 1984). The mutation in qk^v mice has been genetically identified and does not involve a component of myelin. The qk^v mutation consists of a one megabase deletion that includes the promoter and enhancer regions of the *qk* gene, which encodes a family of alternatively spliced RNA binding proteins (Ebersole et al., 1996). Oligodendrocytes (OLs) of normal mice express three major *qk* mRNAs of 5, 6, and 7 kb encoding QKI-5, QKI-6, and QKI-7, respectively (Ebersole et al., 1996). The promoter deletion observed in qk^v mice prevents the expression of alternatively spliced QKI-6 and QKI-7 isoforms in OLs, as analyzed by immunocytochemistry (Hardy et al., 1996b). These observations suggest that the balance between the different QKI RNA binding isoforms may control myelination. The other class of recessive *qk* mutations are ethylnitrosourea-induced recessive mutations, which cause embryonic lethality (Bode, 1984; Justice and Bode, 1988; Shedlovsky et al., 1988). One such allele, qk^{ki4}, was found to alter QKI glutamic acid 48 to glycine (Ebersole et al., 1996). This amino acid substitution abrogates QKI

dimerization mediated by a coiled-coil region in the N-terminal region of QKI and may be the molecular defect of qk^{kt4} (Chen and Richard, 1998). Another allele, qk^{k2}, altering QKI valine 157 to a glutamic acid, remains uncharacterized (Cox et al., 1999).

The dysmyelination phenotype of the qk^{v} mice implicates QKI proteins in the process of myelination. Defects in mRNA processing, mRNA localization, and protein expression of the major protein components of myelin have been observed in the qk^{v} mice (Vernet and Artzt, 1997; Hardy, 1998). The expression and the incorporation of the MBPs in myelin are decreased in the brain of qk^{v} mice (Brostoff et al., 1977; Delassalle et al., 1981; Carnow et al., 1984; Sorg et al., 1986). OLs from qk^{v} mice in culture and in vivo are unable to specifically translocate MBP mRNAs to the periphery (Campagnoni et al., 1990; Barbarese, 1991), where they are locally translated by polyribosomes (Colman et al., 1982; Verity and Campagnoni, 1988). The rate of synthesis of MBPs is unaffected (Brostoff et al., 1977), but the levels of MBP mRNAs in qk^{v} mice are reduced (Li et al., 2000). The level of the alternatively spliced mRNAs and proteins for the small and large isoforms of myelin-associated glycoprotein are also altered in qk^{v} mice (Frail and Braun, 1985; Braun et al., 1990; Fujita et al., 1990; Bartoszewicz et al., 1995; Trapp et al., 1984; Bo et al., 1995). The levels of proteolipid protein mRNA have also been shown to be reduced in qk^{v} (Sorg et al., 1986, 1987). These observations raise the possibility that the QKI proteins are directly involved in the regulation of RNA metabolism of myelin components.

The QKI proteins contain a hnRNP *K Homology* (KH) domain embedded in a larger domain called the GSG (*G*RP33, *S*am68, *G*LD-1) domain (Jones and Schedl, 1995). These proteins are often referred to as STAR (*S*ignal *T*ransduction *A*ctivators of

*R*NA) proteins because of their potential link to signal transduction pathways (Vernet and Artzt, 1997). The QKI GSG domain has been shown to be necessary and sufficient for RNA binding and dimerization (Chen and Richard, 1998). The C-terminal QKI sequences that vary from 8 to 30 amino acids due to alternative splicing are involved in targeting the QKI proteins to different cellular compartments (Hardy et al., 1996b). The unique C-terminal sequences of QKI-5 harbor a nuclear localization signal and target QKI-5 to the nucleus (Wu et al., 1999). QKI-6 and QKI-7 are predominantly cytoplasmic. The QKI isoforms are thought to shuttle between the nucleus and the cytoplasm as homo- and heterodimers (Wu et al., 1999; Pilotte et al., 2001). QKI orthologs have been identified from *Xenopus*, *Drosophila*, chicken, and zebrafish, suggesting that the *qk* gene may have a fundamental cellular function (Baehrecke, 1997; Tanaka et al., 1997; Zaffran et al., 1997; Zorn et al., 1997; Mezquita et al., 1998). Recent evidence has indicated the involvement of several QKI orthologs in RNA metabolism. For instance, *Drosophila* *H*eld-*O*ut-*W*ings (How) has been shown to function in the mRNA export of the transcription factor Stripe during tendon development (Nabel-Rosen et al., 1999). Although the QKI proteins have been shown to control cell survival and cell death (Pilotte et al., 2001), their molecular function in RNA metabolism and their physiological mRNA targets remain unknown.

We now show that the QKI RNA binding proteins bind a short element in the MBP 3' untranslated region. The balance between the nuclear and cytoplasmic isoforms of QKI, which are derived from alternative splicing, controls the nuclear export of MBP mRNAs and the cellular localization of exon II MBP isoform 17 and 21.5. By using OL

cultures overexpressing QKI-5, we have re-created the MBP defects observed in qk^{v} mice. Our data provide evidence that the QKI proteins are involved in myelination.

3.4 Materials and methods

3.4.1 Primary rat OL cultures and adenovirus infections.

Cultures of OL progenitors were generated as described (Almazan et al., 1993). OL progenitors, also termed O-2 A progenitors for their ability to generate OLs and type 2 astrocytes in vitro, were plated on 6-well dishes at a density of 15 x 10^3 cells/cm^2. In order to expand their numbers and prevent differentiation, the cultures were grown in media containing 2.5 ng/ml basic fibroblast growth factor (Peprotec) and platelet-derived growth factor AA for 4 days. The cultures were infected with the indicated adenovirus co-expressing QKI-5 from the TR5 promoter and GFP from the CMV promoter (AdTR5-QKI-5; Massie et al., 1998b; Pilotte et al., 2001) or control adenovirus (AdTR5) expressing GFP from the TR5 promoter and an adenovirus AdCMV-tTA that expresses the tTA (Massie et al., 1998a). A multiplicity of infection (MOI) of 100 was sufficient to infect about 80% of the cells as judged by GFP-positive cells. After infection, the cells were grown in differentiation media containing bFGF, PDGF-AA, and T3 at 40 ng/ml. Morphological examination established that the progenitor cultures were essentially homogeneous bipolar cells, and acquired ramified processes as they differentiated into mature OLs in vitro.

For stereotactic adenovirus injection, 1 μl of 1 x 10^7 pfu/μl adenovirus was injected using a 31-gauge needle in the corpus callosum of postnatal day 1 inbred C57BL/6 mice at the following localization: bregma -1 mm; lateral 0.5 mm; depth of 1 mm. The survival rate was >95% with over 20 pups. The pups were returned to the mother and monitored daily. At postnatal day 10, the pups were sacrificed and their brains processed for GFP and MBP protein levels by immunofluorescence.

3.4.2 Tissue processing and immunolabeling of brain cross-sections.

For immunofluorescence studies, 30-day-old mice were treated intraperitoneal with sodium pentobarbital before intracardial perfusion at 40 ml with 0.1 M cold PBS and then 40 ml of ice-cold 4% paraformaldehyde in PBS, pH 7.4. The entire brain was removed and post-fixed in 4% paraformaldehyde at 4°C overnight, and then immersed in 30% sucrose for 2 days. Tissue blocks were embedded in OCT compound and frozen on dry ice. Serial coronal sections at a thickness of 15 μm were cut in a cryostat, collected on +/+ glass slides (Fisher) pretreated with gelatin-chromium sulfate, and stored at -70°C. Sections were pre-incubated for 30 minutes in 5% calf serum-1% Triton X-100 in PBS. This was followed by a 24 hr incubation in PBS-0.1% Triton with a mix of two monoclonal MBP antibodies. The monoclonal anti-MBP antibody (1:2500, Sternberger LTD) and mAb 387 (1:1000, Chemicon International) were used for staining the MBP isoforms in the mouse brain cross-sections. Affinity-purified anti-MBPexII was used as described previously (Pedraza et al., 1997). Species-specific Alexa 546 (Molecular Probes) secondary antibodies diluted 1:400 in PBS:0.1%Triton were used for detection.

3.4.3 DNA constructs.

The constructs encoding HA-QKI-7, myc-QKI-5, myc-QKI-6, myc-QKI-7, myc-QKI-7:E48G, glutathione S-transferase (GST)-QKI-7, and GST-QKI-7:E48G were described previously (Chen and Richard, 1998; Pilotte et al., 2001). Full-length MBP cDNAs for mouse 14, 17, 18.5, and 21.5 kDa were obtained from the Laboratory for

Genome Exploration Research Group RIKEN Genomic Sciences Center (GSC), RIKEN Yokohama Institute Japan. Myc-QKI-7:V157E was constructed by inverse PCR with myc-QKI-7 and MBP3'UTR and MBP full-length were generated by PCR.

3.4.4 Preparation of mouse OLs.

OLs progenitor cells were purified from postnatal day 11 mouse brain. At that age, the *qk*^{v} phenotype is clearly visible by a typical tremor in the hindlimbs. Five wild-type C57BL/6 females and five *qk*^{v} females were used for the mouse OL culture experiments. Purification and culture of OL progenitor cells were performed according to the percoll gradient procedure (Lubetzki et al., 1991). These OL progenitor cells were plated on poly-D-lysine-coated glass coverslips. Cultures were incubated for 10 days in OL differentiation media containing T3 hormone as described (Almazan et al., 1993).

For the endogenous MBPexII proteins, analysis from brain crude extract, entire brains were lysed by sonication in 1x Laemmli buffer containing 8 M urea without bromphenol blue. The quantity of total protein in each sample was estimated by the Bradford assay (Bio-Rad, Hercules, California).

3.4.5 Protein analysis.

Myc-QKI-7 and HA-QKI-7 were transfected in HeLa cells, lysed, immunoprecipitated, and analyzed as previously described (Chen and Richard, 1998). Samples were immunoblotted with anti-myc (9E10), anti-HA (12CA5), anti-MBP (Dako Diagnostics), or anti-actin (Chemicon) antibodies followed by a goat anti-mouse antibody

conjugated to horse radish peroxidase (ICN). Chemiluminescence was used for protein detection (Dupont).

3.4.6 In vitro transcription.

^{32}P-labeled MBP RNA fragments were generated by in vitro transcription using the T7 RNA polymerase following the protocols recommended by the manufacturer (Promega). The DNA templates used for in vitro transcription were PCR fragments of different regions of the MBP cDNA with an engineered 5' T7 promoter. The oligonucleotide sequences used to generate the MBP RNAs will be given upon request.

3.4.7 RNA binding assays.

RNA binding of immunoprecipitated QKI was performed as previously described (Chen and Richard, 1998). For EMSA, ^{32}P-labeled RNA (10^5 cpm) was incubated at RT for 30 minutes with GST or GST-QKI fusion proteins (0.5–2.0 μg) in PBS supplemented with 1% Triton X-100, 1 mg/ml of heparin, and 250 μg/ml of tRNA. The samples were electrophoresed on a 4% native polyacrylamide gel (acrylamide:bis, 60:1) in 0.5 x TBE and visualized by autoradiography. For the analysis of QKI-MBP mRNA association in vivo, astrocytes and OLs (OLs) isolated from newborn rats were lysed with lysis buffer supplemented with the RNase inhibitor RNAguard (Pharmacia), and the cell lysates were immunoprecipitated with anti-QKI antibody (Chen and Richard, 1998) or normal rabbit serum (NRS). Associating MBP mRNAs were amplified by RT-PCR and Southern blot

analysis. For UV crosslinking, a synthetic ^{32}P-labeled RNA corresponding to nucleotides 626–885 of the MBP mRNA was prepared by in vitro transcription using ^{32}P-CTP.

3.4.8 In situ hybridization and fluorescence staining.

COS cells were plated on glass coverslips in 6-well dishes and transfected by using Lipofectamine Plus (Canadian Life). Twelve hours after transfection, the cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 1% Triton X-100 in PBS. If the cells were transfected with GFP constructs, the coverslips were mounted onto glass slides with Immuno-Mount (Shandon Inc.) containing DAPI or To-Pro3 (Molecular Probes) to stain the nuclei. For Myc and MBP immunostaining, the permeabilized cells were blocked with 10% calf serum in PBS for 30 minutes, incubated with 9E10 (1:1000) or the monoclonal anti-MBP antibody (1:2500, Sternberger LTD) in PBS containing 3% BSA for 1 hour, and then incubated with rhodamine- (1:200) or FITC-conjugated (1:400) goat anti-mouse secondary antibodies (Jackson Laboratories) for 20 minutes. The cells were visualized with a Leitz (Wetzlar, Germany) Aristoplan fluorescence microscope or by confocal microscopy using Zeiss LSM-510 system.

A DNA fragment encompassing nucleotides 35–190 of the 14 kDa MBP isoform was amplified by PCR and in situ hybridization was carried out as described (Lawrence and Singer, 1986). Digoxigenin-labeled RNA was detected with the anti-digoxigenin antibody conjugated to rhodamine in COS cells, whereas the fluorescent antibody enhancement set for DIG detection (Roche) was used on OL slides and visualized as describe above.

3.5 Results

3.5.1 Identification of a 110 Nucleotide QKI recognition element in the 3'-UTR of the MBP mRNAs.

The myelination defect observed in the qk^v mice prompted us to investigate whether the QKI RNA binding proteins bound the mRNAs of the major protein components of myelin. One such component is the myelin basic protein (MBP). Mice contain four alternatively spliced isoforms of MBP (14, 17, 18.5, and 21.5 kDa) that differ in their coding sequence, but not in the untranslated regions of their mRNAs (de Ferra et al., 1985). To investigate whether the QKI proteins bound the mRNAs encoding the MBPs, synthetic RNA transcripts corresponding to portions of the longest mouse MBP mRNA (21.5 kDa, Figure 3-1A) were ^{32}P-labeled and examined for their ability to associate with the QKI proteins. Transfected HeLa cells expressing myc-epitope-tagged QKI-7 were immunoprecipitated with control (IgG) or anti-myc antibodies followed by protein A-Sepharose beads. The control and myc-QKI-7 bound beads were incubated with equal quantities of ^{32}P-labeled MBP transcripts, washed, and the bound RNAs quantitated by scintillation counting. The 3'-UTR of the MBP mRNAs bound immunoprecipitated myc-QKI-7 approximately 30-fold over control immunoprecipitations (Figure 3-1A, compare bars representing IgG with myc for 626-2111). RNA transcripts corresponding to the 5'-UTR (1-49) and the coding region (1-625) of the 21.5 kDa MBP did not associate with QKI-7 (Figure 3-1A). Shorter RNA segments of the 3'-UTR of the MBPs were examined for QKI binding in order to map a short recognition element. The majority of QKI binding resided from nucleotides 626 to 885 in the 3'-UTR of MBP, and a minor binding site was also observed from nucleotides

1441 to 1770 (Figure 3-1A). QKI-5 and QKI-6 also bound the MBP 3'-UTR spanning nucleotides 626 to 790, demonstrating that MBP mRNA binding was not QKI isoform specific (Figure 3-1A). These findings suggest that the mRNAs encoding the MBPs contain one major *Q*KI *R*ecognition *E*lement (QRE) residing from nucleotides 626 to 790.

To confirm that the QKI-7 binding to the MBP mRNAs was a direct interaction, an ultraviolet light (UV)-crosslinking assay was performed. Myc-QKI-7 transfected or untransfected HeLa cells were lysed, and cell lysates were incubated with a ^{32}P-labeled synthetic RNA transcript encompassing nucleotides 626 to 885 and irradiated with UV light. The cell lysates were RNase treated to remove unprotected RNA, immunoprecipitated using control (IgG) or anti-myc antibodies, and the bound proteins separated on SDS 10%-polyacrylamide gels and visualized by autoradiography. A ^{32}P-labeled protein of ~45 kDa corresponding to the size of QKI-7 was present in Myc immunoprecipitates from cells transfected with QKI-7, but not untransfected cells (Figure 3-1B). This experiment provides strong evidence that the interaction between the QKI proteins and the MBP QRE is direct. To further confirm a direct interaction, electrophoretic mobility shift assays (EMSA) were performed using recombinant QKI proteins and MBP ^{32}P-labeled RNA transcripts. Recombinant glutathione-S-transferase (GST) QKI-7 fusion protein and the control GST fusion partner were incubated with a ^{32}P-labeled MBP synthetic RNA corresponding to nucleotides 626 to 885. The QKI-RNA complexes and unbound RNAs were separated on native 4% polyacrylamide gels and visualized by autoradiography. A slow migrating complex that corresponded to a QKI-RNA complex was observed with GST-QKI-7, but not with GST or phosphate-buffered

saline (PBS) alone (Figure 3-1C). QKI bound to a shorter RNA transcript encompassing nucleotides 626 to 790 but not nucleotides 626 to 710 (Figure 3-1C, lanes 6 and 9). Two 5'-deletions were performed and tested for QKI binding. The removal of nucleotides 626 to 679 did not affect QKI binding (lanes 10–15), but the deletion of nucleotides 626 to 719 abrogated QKI binding (lanes 16–21). Thus, the shortest region identified resides from nucleotides 680 to 790 in the mouse MBP 3'-UTR (Figure 3-1C, lanes 13–15) and this area was designated the minimal QRE. The interaction between endogenous QKI proteins and MBP mRNAs was verified in primary rat OLs. The OLs were lysed and immunoprecipitated with control normal rabbit serum (NRS) or anti-QKI antibodies, known to immunoprecipitate the QKI proteins (Chen and Richard, 1998). The presence of coimmunoprecipitating MBP mRNAs was verified by using reverse transcription (RT)-PCR analysis with MBP-specific primers. A DNA fragment of the correct size was observed in the anti-QKI antibody immunoprecipitates only in the presence of the reverse transcriptase, suggesting that the DNA fragment was amplified from mRNA and not genomic DNA (Figure 3-1D, lanes 1–5). To confirm that the DNA fragment was indeed from the MBP mRNAs, a southern blot was performed using an MBP-specific DNA ^{32}P-labeled probe. The DNA fragment amplified from the anti-QKI antibody immunoprecipitation hybridized with the MBP-specific probe (Figure 3-1D, lanes 6–10). These findings demonstrate that the QKI proteins associate with the MBP mRNAs in vivo.

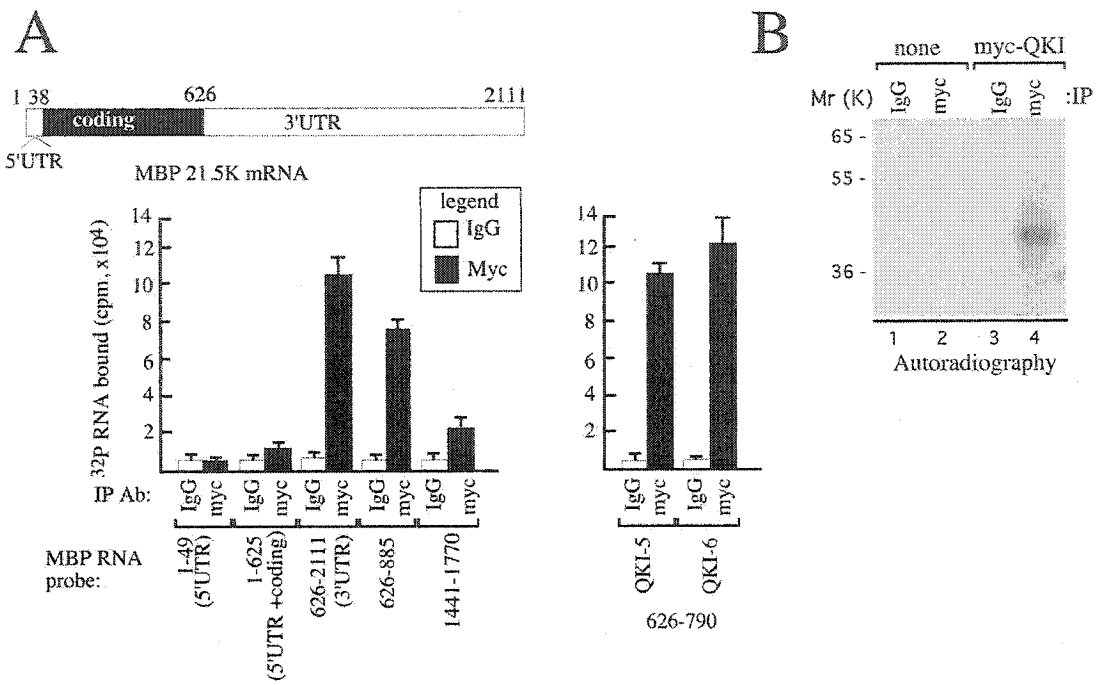

Figure 3-1. QKI proteins bind an RNA element in the MBP 3'-UTR. (A) A schematic diagram representing different regions of the 21.5 K MBP mRNA is shown. QKI-5, -6, or -7 were expressed in HeLa cells and immunoprecipitated with control or myc antibodies. The immunoprecipitates were incubated with ^{32}P-labeled RNA MBP fragments as indicated and quantitated. Each bar represents the mean ± standard deviation of data from n > 6. (B) QKI binds the 3'-UTR of MBP directly. HeLa (none) or QKI-7 transfected HeLa cells were lysed and incubated with a ^{32}P-labeled MBP RNA 626–885 and UV crosslinked. The unbound RNAs were digested and the QKI protein immunoprecipitated with control (IgG) or anti-myc antibodies. The bound proteins were separated by SDS-PAGE and visualized by autoradiography.

3.5.2 QKI embryonic lethal point mutations disrupt RNA binding activity.

The identification of a physiological RNA target (MBP) for QKI permitted us to examine whether the two embryonic lethal point mutations identified in the QKI GSG domain altered specific RNA binding. Recombinant GST-QKI-7 bound the MBP QRE in a dose- dependent manner, as visualized by electrophoretic mobility shift assays (Figure

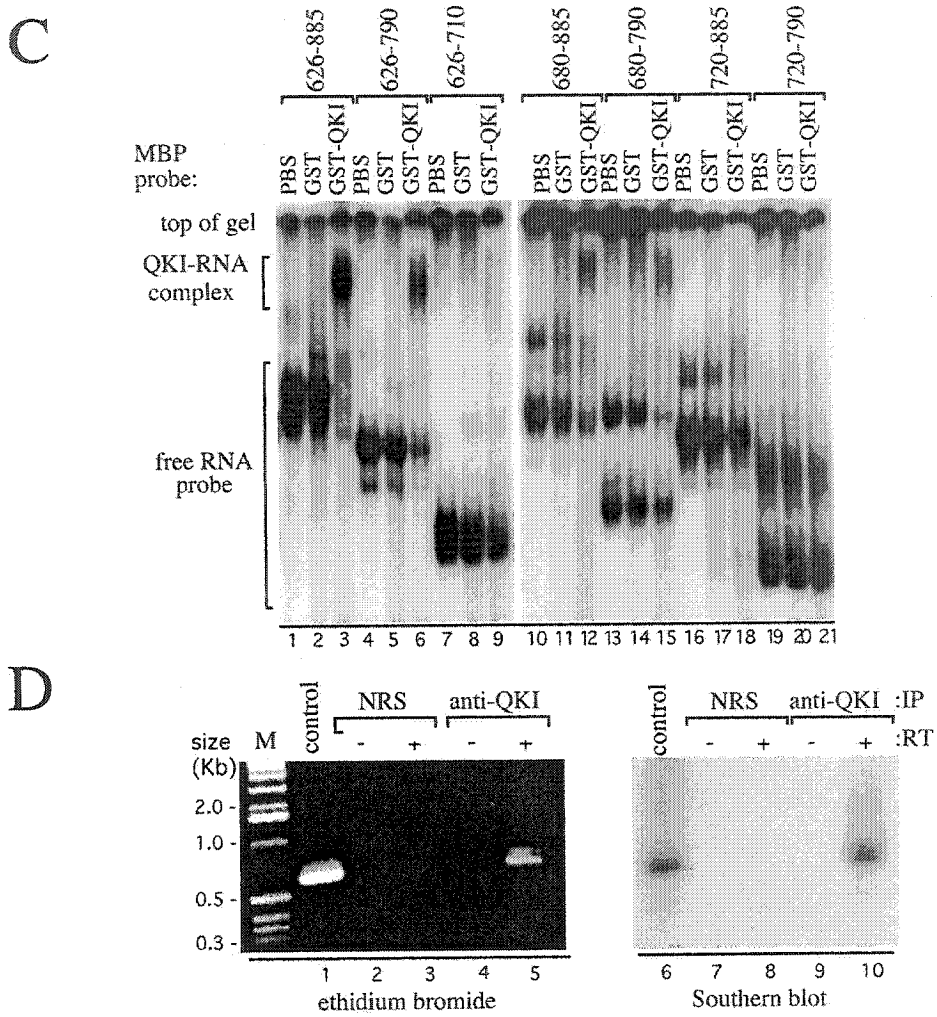

Figure 3-1 (2). QKI Proteins bind an RNA element in the MBP 3'-UTR. (C) The QKI recognition element (QRE) is located within nucleotides 680–790 of MBP mRNA. ^{32}P-labeled RNA probes corresponding to different portions of the MBP mRNA were incubated with PBS, GST (1.0 μg), or GST-QKI-7 (1.0 μg), and the reactions were analyzed with native acrylamide gel electrophoresis and autoradiography. The migration of QKI-RNA complexes and free RNA probes is indicated. (D) Mixed rat astrocytes and OLs were homogenized in lysis buffer and immunoprecipitated with normal rabbit serum (NRS) or anti-QKI antibodies. RNAs were extracted from the immunoprecipitates and the presence of MBP mRNA was detected by RT-PCR. For positive control, MBP cDNA was used as a template (control) and for negative control, reverse transcriptase (RT) was omitted. The PCR products were first visualized by staining and verified by Southern blot analysis with an MBP-specific probe.

3-2A, lanes 5–7). The substitution of valine 157 to glutamic acid (V157E) or the substitution ofglutamic acid 48 to glycine (E48G) in QKI abolished the ability of the recombinant proteins to bind the QRE (Figure 3-2A, lanes 8–13). We have shown in earlier studies that the substitution of QKI E48G prevents homodimerization by disrupting predicted coiled-coil interactions (Chen and Richard, 1998). Therefore, we wanted to examine whether the inability of the V157E amino acid substitution was a failure to dimerize. HeLa cells were transfected with myc- and HA-epitope-tagged QKI proteins containing the amino acid substitutions. The cells were lysed and immunoprecipitated with control (IgG) or anti-HA antibodies, the bound proteins were separated by SDS-PAGE and immunoblotted with anti-myc antibodies. HA immunoprecipitates of wild-type QKI coimmunoprecipitated myc-epitope-tagged QKI and QKI:V157E, but not QKI:E48G (Figure 3-2B, lanes 1–9). These findings suggest that the V157E mutation prevents RNA binding and that the E48G substitution prevents dimerization. Since the substitution of E48G is unable to bind RNA, this suggests that dimer formation may be essential for MBP RNA binding.

3.5.3 Nuclear export of the MBP mRNAs is controlled by the balance of the QKI isoforms.

The relationship between the localization of the MBP mRNAs and the QKI proteins was examined in COS cells. Since COS cells do not endogenously express the QKI isoforms (Chen and Richard, 1998), the contribution of the different QKI isoforms in MBP mRNA localization can be assessed separately or collectively using transfected genes. COS cells were transfected with an expression vector containing a full-length 14

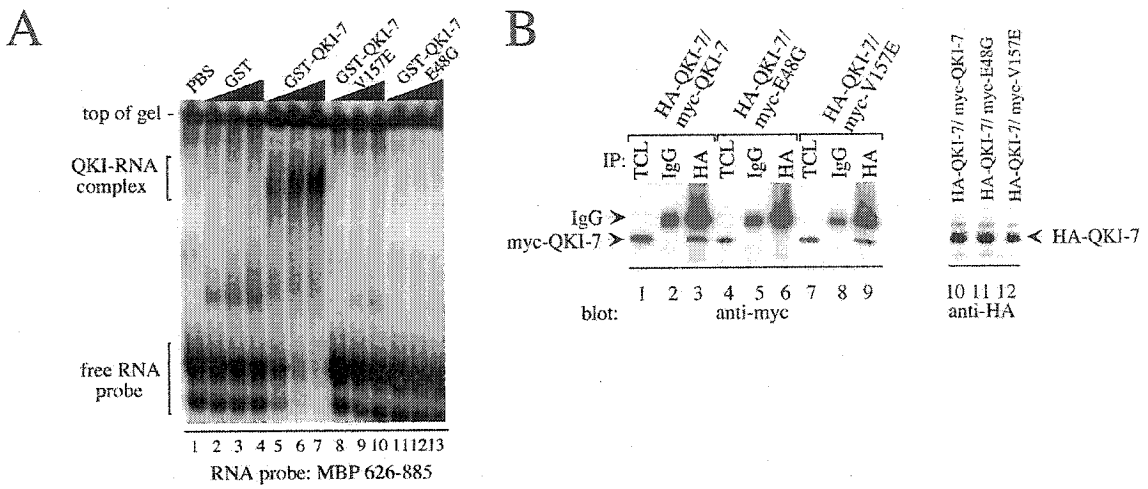

Figure 3-2. The *qk* lethal point mutations abrogate RNA binding. (A) V157E or E48G mutations abolish MBP binding. PBS or an increasing amount (0.5, 1.0, and 2.0 μg) of GST, GST-QKI-7, GST-QKI-7:V157E, and GST-QKI-7:E48G were incubated with a ^{32}P-labeled MBP RNA fragment containing the QRE. The reactions were analyzed with native PAGE and autoradiography. (B) V157E substitution has no effect on QKI dimerization. HA-tagged QKI-7 was cotransfected in HeLa cells with myc-tagged QKI-7, QKI-7:E48G, or QKI-7:V157E as indicated. Total cell lysates (TCL), IgG, and anti-HA immunoprecipitates were analyzed by immunoblotting with anti-myc antibodies (lanes 1–9). TCL were also immunoblotted with anti-HA antibodies to verify the expression (lanes 10–12).

kDa mouse MBP cDNA. The cells were fixed and the MBP mRNA was detected by using in situ hybridization. The MBP mRNA was localized predominantly in the cytoplasm of COS cells, as expected for mRNA (Figure 3-3A, panel A). The co-transfection of myc-epitope-tagged QKI-5 with an MBP expression vector containing the untranslated regions caused the nuclear retention of the MBP mRNA (Figure 3-3A, panelD). MBP mRNA without the 3'-UTR (MBP3'UTR) exited the nucleus and failed to be retained by QKI-5 (Figure 3-3A, panel G). Similar results were observed with the MBP 21 kDa isoform (data not shown). These findings suggest that the 3'-UTR of the MBP mRNA is required for QKI-5-mediated nuclear retention.

The heterodimerization of the QKI isoforms (Chen and Richard, 1998) and their nucleocytoplasmic shuttling ability (Wu et al., 1999) suggest that QKI dimers may function in the nuclear export of the MBP mRNAs. If this is indeed the case, the presence of the cytoplasmic QKI-6 and QKI-7 isoforms might relieve the QKI-5-mediated MBP mRNA nuclear retention. COS cells were co-transfected with expression vectors encoding the 14 kDa MBP and various combinations of QKI isoforms. The presence of QKI-6 or QKI-7 relieved part of the QKI-5-mediated nuclear retention of the MBP mRNA. However, a significant portion of MBP mRNA was unable to exit the nucleus (Figure 3-3A, panels J and M). The presence of all three QKI isoforms greatly restored the ability of the MBP mRNA to fully exit the nucleus (Figure 3-3A, panel P). Reverse transcription (RT)-PCR was performed on RNA isolated from cells transfected with or without QKI-5. The presence of QKI-5 did not affect the overall levels of MBP mRNA (Figure 3-3B, compare lanes 3–5 to 7–9 and lanes 11–13 to 15–17). Immunoblotting confirmed that the expression of QKI-5 remained equivalent with the expression of QKI-6 and/or QKI-7 isoforms (Figure 3-3C). These findings demonstrate that the balance of the QKI isoforms regulates the retention and release of the MBP mRNAs from the nucleus.

3.5.4 QKI-5 retains MBP mRNAs in the nucleus of OLs.

The ability of QKI proteins to control the nuclear export of MBP mRNAs was examined in OLs, a cell type where QKI has a physiological role. OLs of qk^{v} mice only express the QKI-5 isoform (Hardy et al., 1996b), implying that the dysmyelination could be the result of mRNA nuclear export defects of key myelin components. To test this

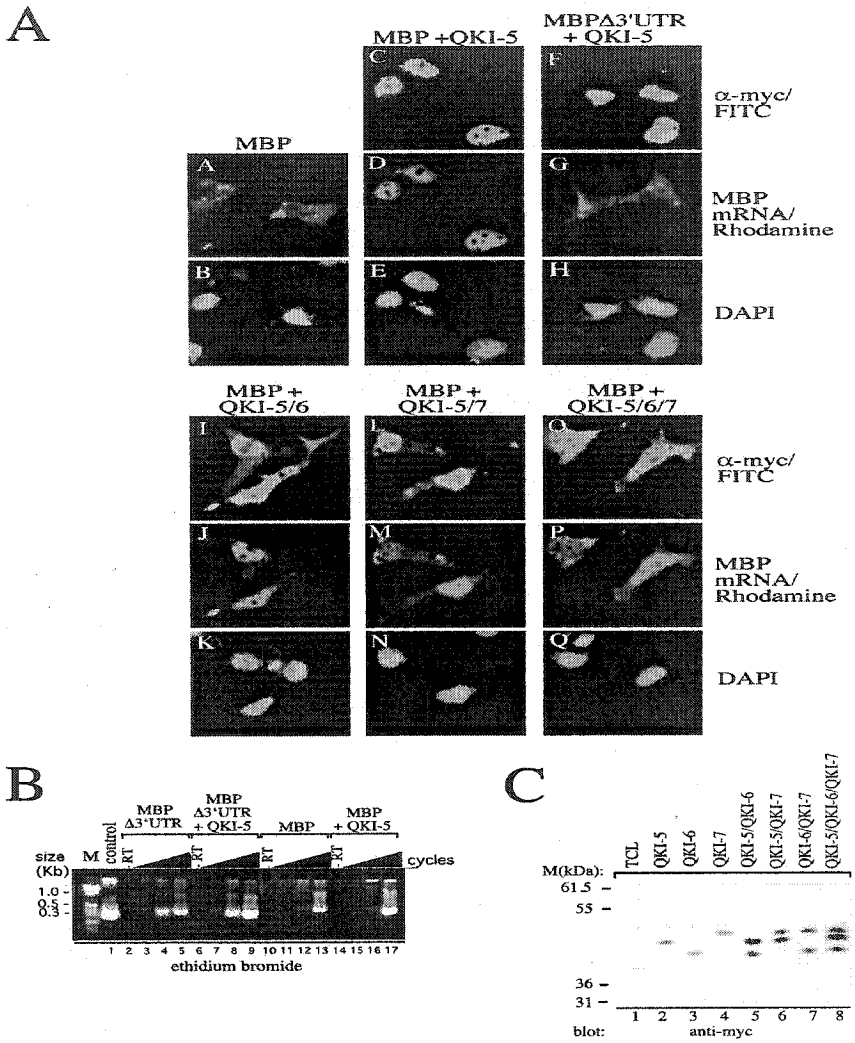

Figure 3-3. The QKI isoforms control the nuclear export of the MBP mRNAs. (A) COS cells were transfected with the plasmids encoding the 14 K MBP or MBP 3'-UTR in combination with the myc-QKI isoforms. After 12 hour, the cells were fixed, permeabilized, and in situ hybridization and immunofluorescence were performed. A digoxigenin-labeled RNA probe was used followed by an anti-digoxigenin rhodamine-conjugated antibody, whereas an anti-myc antibody followed by a FITC-conjugated secondary antibody was used to detect the myc-QKI. The cells were mounted onto a glass slide in the presence of the nuclear stain DAPI and visualized by fluorescence microscopy. (B) The stability of MBP mRNA is unaffected by QKI-5 expression. The 14 K MBP or MBP 3'-UTR were transfected alone or in combination with QKI-5 in COS cells. The RNA was isolated with Trizol, treated with DNase, and subjected to RT-PCR with MBP-specific primers. DNA fragments were separated on agarose gels and visualized by staining. The MBP cDNA was used as a positive control, whereas RT was omitted (-) during reverse transcription as a negative control. (C) QKI-5 expression is unaltered by the presence of the other QKI isoforms. COS cells were transfected with either myc-tagged QKI-5, -6, or -7 alone and in different combinations. The cell lysates were analyzed by immunoblotting with anti-myc antibodies.

possibility and to mimic the situation in qk^{v} mice, QKI-5 was overexpressed in OL cultures. OLs were co-infected with adenoviruses expressing QKI-5 (AdTR5-QKI-5) and the tetracycline transactivator (tTA) at MOIs sufficient to transduce about 80% of the cells as shown by the expression of the green fluorescent protein (GFP) which is constitutively coexpressed from the QKI-5 virus. The presence of the tTA is necessary to drive the expression of the inducible QKI-5. The distribution of the QKI-5 protein was predominantly nuclear as viewed by indirect immunofluorescence with an anti-myc epitope antibody 3 days post-infection (Pilotte et al., 2001). OLs infected with AdTR5-QKI-5 were fixed, permeabilized, and MBP mRNAs detected by using in situ hybridization. The "green" QKI-5 overexpressing OLs displayed mainly a nuclear accumulation of MBP mRNAs with only some staining in the perikaryon, as visualized by confocal microscopy (Figure 3-4, panel E). Longer exposure of the MBP in situ hybridization demonstrates that little to no MBP mRNAs are in the OL processes and the periphery (Figure 3-4, panel F). In contrast, OLs infected with a control adenovirus (AdTR5) expressing only GFP localized the MBP mRNAs in the perikaryon and processes with little to no staining in the nucleus (Figure 3-4, panel B). Longer exposure clearly demonstrates that the MBP mRNAs extend into the processes and reach the periphery (Figure 3-4, panel C). Our findings suggest that a balance toward elevated QKI-5 causes nuclear export defects of MBP mRNAs in OLs.

The interaction of QKI with the 3'-UTR of the MBPs could have several functional consequences ultimately affecting the protein levels of the MBPs. The effects of retaining the MBP mRNAs in the nucleus and the perikaryon of OLs was verified at

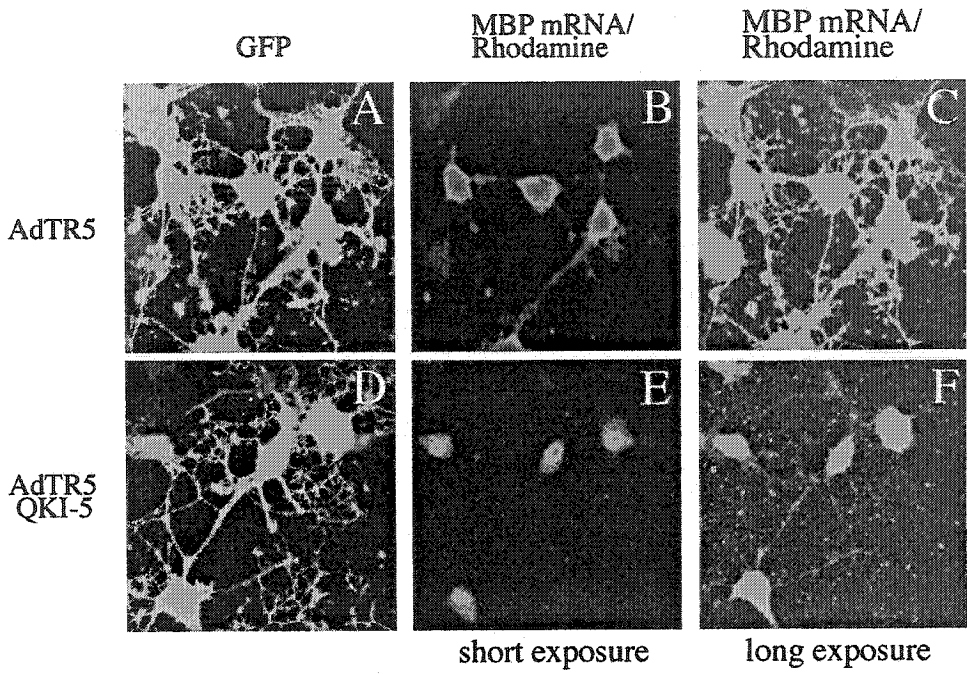

Figure 3-4. Nuclear retention of MBP mRNAs in QKI-5 overexpressing OLs. Primary rat OLs were co-infected with adenoviruses that constitutively expresses the tTA and a tetracycline-inducible adenovirus expressing GFP alone (A, B, and C) or GFP with myc-QKI-5 (D, E, and F). After 3 days, the cells were fixed, and permeabilized to perform in situ hybridization with a digoxigenin-labeled RNA probe. The cells were visualized by confocal microscopy.

the MBP protein level. QKI-5-infected OLs were immunolabeled with a monoclonal anti-MBP antibody and visualized by immunofluorescence. QKI-5-infected OLs had a visibly weaker staining for the MBPs (Figure 3-5A, compare panels b and d) and this was confirmed by Western blot analysis. Thus the five MBP isoforms normally expressed in rat OLs were proportionally reduced (Figure 3-5B). Another observation was that in theQKI-5-infected OLs, MBPs were mostly localized in the perikarya. The failure of MBPs to migrate to the distal branching sites was visualized by immunofluorescence using a conventional (Figure 3-5A, panel d) or a confocal microscope (Figure 3-5C, compare panels b and d). In control cultures infected with the GFP construct alone, the MBPs were localized at the periphery (Figures 5-5A and 5-5C, panels b), demonstrating

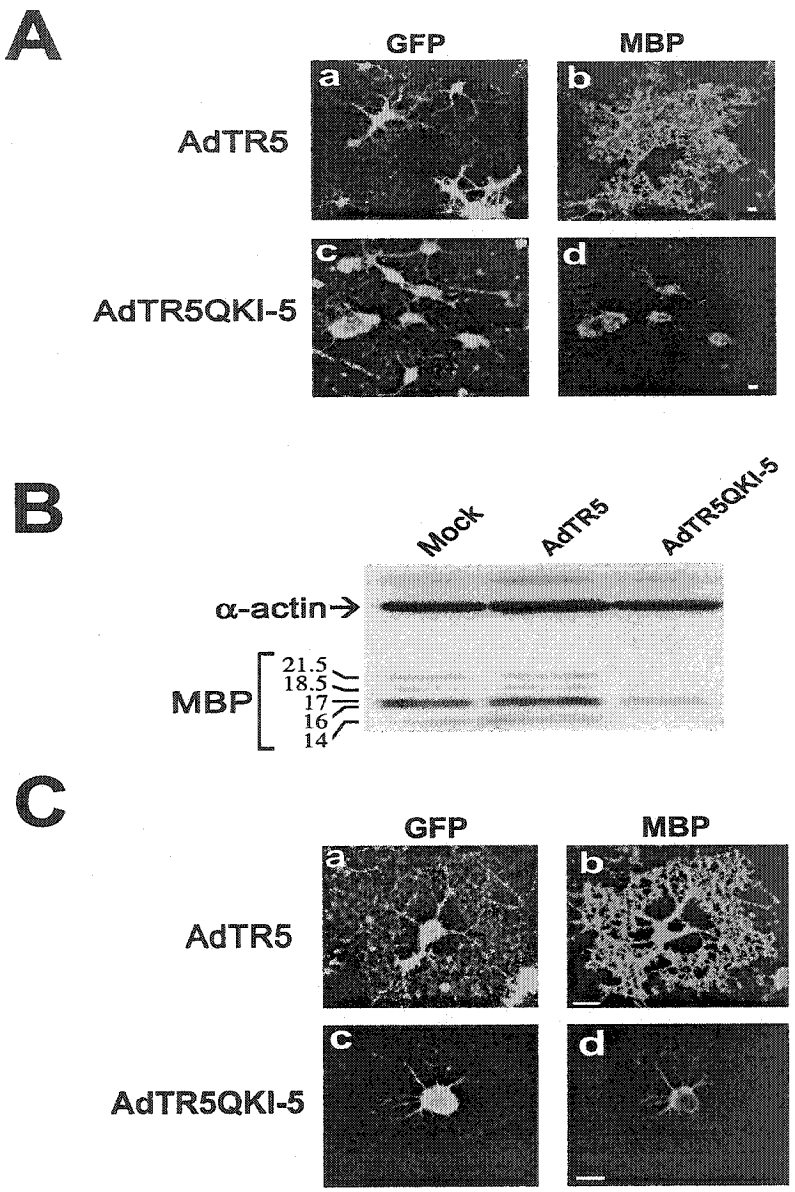

Figure 3-5. The overexpression of QKI-5 suppresses the expression of MBPs and alters their proper localization in OLs. (A) QKI-5 protein expression causes retention of the MBPs in the perikarya of OLs. OLs progenitors were infected with adenoviruses coding for QKI-5 (panels c and d) and GFP only expressing control AdTR5 (panels a and b) and allowed to differentiate. On day 3 after QKI-5 expression, the MBPs were immunostained with anti-MBP antibodies (red) and visualized by fluorescence microscopy (panels b and d). GFP (green) positive cells denote the infected cells in this experiment. Bar scale represents 10 μm. (B) Expression of the MBPs decreases in the presence of QKI-5. OL progenitors infected as in (A) were lysed, separated by SDS-PAGE, and immunoblotted with anti-MBP and -actin antibodies, n = 3. (C) The localization of MBPs was examined by confocal microscopy. Cells infected as in (A) were immunostained with anti-MBP antibodies (red, panels b and d). The GFP (green) is a marker of infection. Bar scale represents 10 μm.

that adenovirus infection per se did not alter the normal localization of the MBPs in OLs. These data suggest that the nuclear export defect induced by the overexpression of QKI-5 ultimately translates into lower levels of expression of the myelin basic proteins as well as their mislocalization.

3.5.5 Nuclear retention of MBP mRNAs in *qk*^{v} mice.

MBP mRNAs are concentrated in the cell body of *qk*^{v} OLs, as assessed in 1991 by in situ hybridization using a ^{35}S-labeled MBP probe (Barbarese, 1991). The resolution of this assay did not permit distinction between the cell body, the perinuclear compartments, or the nucleus of the OLs. To better define the compartment in which MBP mRNAs are located in *qk*^{v} mice, we performed in situ hybridization using a digoxigenin-labeled probe and confocal microscopy. Brain slices from the cortex of normal mice showed that MBP mRNAs were located along axons and this was not observed in *qk*^{v} mice (Figure 3-6A). Primary OL cultures from postnatal day 11 wild-type and *qk*^{v} mice were generated and the localization of the MBP mRNAs was visualized. The mRNAs for the MBPs were observed to be located at the periphery of OLs from wild-type mice (Figure 3-6B, panel B). The mRNAs for the MBPs were localized in the nuclei and the perinuclear regions of OLs from *qk*^{v} mice, as visualized by confocal microscopy (Figure 3-6B, panel F). The OLs from *qk*^{v} mice appeared immature and bipolar compared to the OLs from wild-type mice (Figure 3-6B, panels C and G). The bright foci observed with the Hoechst dye do not represent cell death and are characteristic of mice cells (Figure 3-6B, panels A and E). These findings suggest that OLs from *qk*^{v} mice are defective in the nuclear export of the MBP mRNAs.

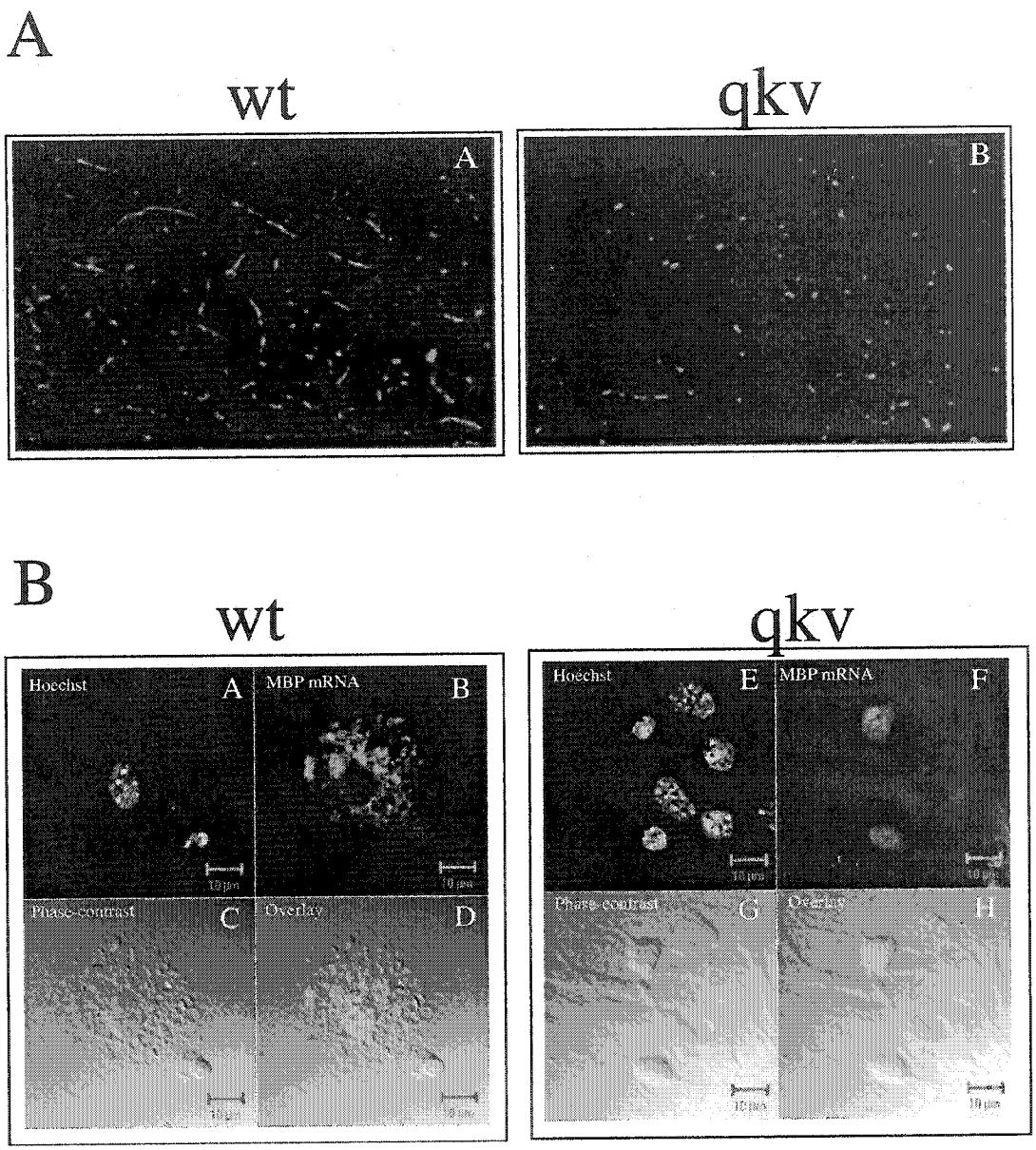

Figure 3-6. mRNAs encoding the MBPs are nuclear in OLs from qk^v mice. (A) Fifteen micrometer slices from 30-day-old C57BL/6 or qk^v mouse brains were fixed, permeabilized, and hybridized with a DIG-labeled MBP RNA probe. The RNA was detected with an anti-DIG antibody conjugated to rhodamine, and the brain slices were visualized by fluorescence microscopy. The panels shown represent the cortex region of the mouse brain. (B) MBP mRNAs are retained in the nuclei of OLs in qk^v mice. Primary mouse OLs were isolated from postnatal day 11 wild-type (wt) or qk^v mice. The cells were fixed, permeabilized, and subjected to in situ hybridization with a DIG-labeled MBP RNA probe. The cells were visualized by using a Zeiss confocal microscope. Each panel represents the same field of cells as visualized under blue (Hoechst) and red (rhodamine) and phase contrast filters. The overlay represents the merge of the three fields.

3.5.6 The localization of MBPexII isoforms in *quaking* viable mice.

The exon II containing MBP isoforms 17 and 21.5 are nuclear in young OLs, and their presence is thought to regulate mRNA movement to the periphery during myelination (Allinquant et al., 1991; Pedraza et al., 1997). The defect in MBP mRNA export might be explained by the abundance of MBPexII in the OL nuclei in qk^v mice. Although we did not see nuclear retention of MBP in OL culture with QKI-5 (Figure 3-5C, panel d), we wanted to confirm if MBPexII localization was affected in qk^v mutants. Brain slices containing the corpus callosum, a region rich in myelinating OLs, from qk^v mutant and wild-type mice were examined for the MBPexII distribution by using an anti-MBPexII-specific antibody (Pedraza et al., 1997). A cell body staining was observed from the corpus callosum in the wild-type brain sections (Figure 3-7A). In qk^v mice, MBPexII isoforms were clearly localized in the cytoplasm and primary branches of OLs, consistent with the presence of young OLs (Figure 3-7A, panels b and d). The presence of bipolar cells suggests that a maturation defect or delay is observed in qk^v OLs. OL cultures from wild-type and qk^v mice that were matured for 10 days in vitro localized MBPexII proteins in the perinuclear and cytoplasmic regions (Figure 3-7A, panels e and f). Moreover, the ratio of MBP isoforms 17 and 21.5 was not altered between wild-type and qk^v mice, suggesting that the QKI proteins may not regulate inclusion/exclusion of MBP exon II (Figure 3-7B), as recently suggested (Wu et al., 2002). These observations suggest that the MBP mRNA nuclear retention in qk^v mice is not caused by an excess of MBPexII isoforms in the nucleus, but that qk^v OLs clearly have maturation defects.

To examine the role of the QKI proteins on the cellular localization of exon II containing MBP isoforms, primary rat OLs were infected with QKI-5, -6, and -7

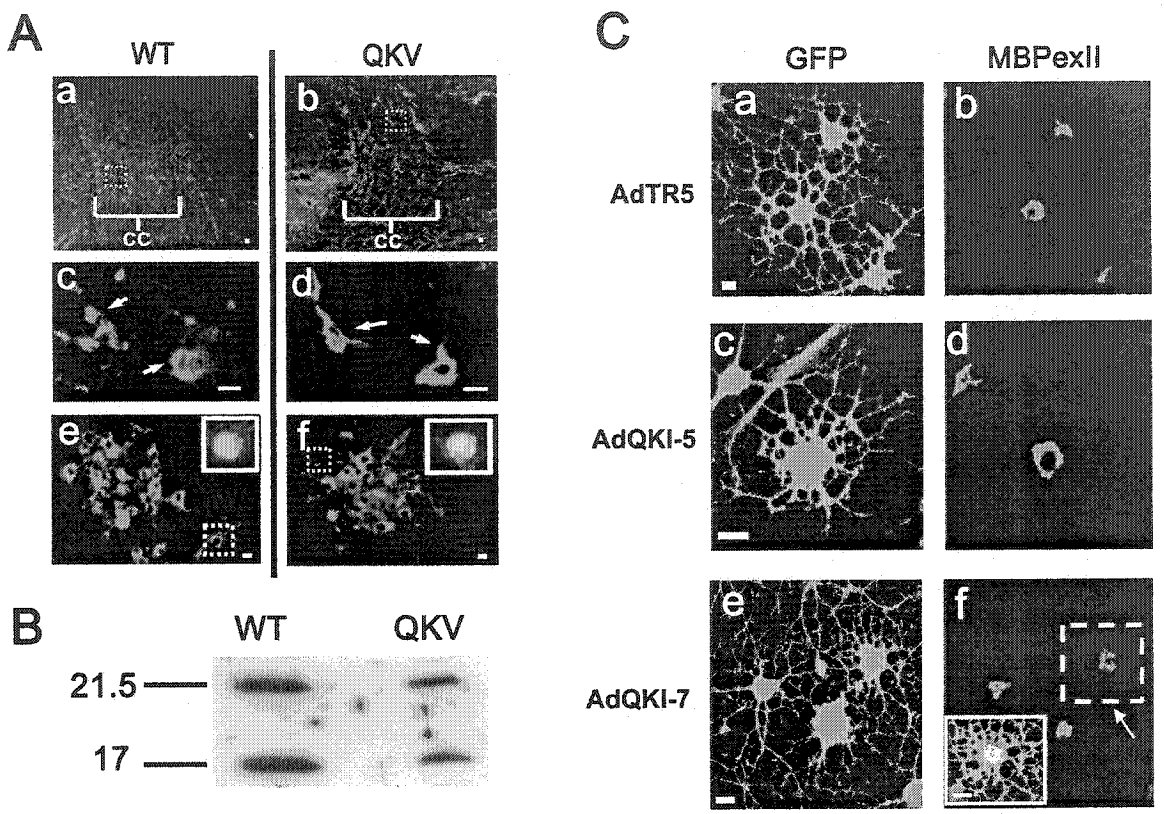

Figure 3-7. Abnormal distribution of MBP exon II isoforms in OLs from qk^v. (A) Coronal sections through the corpus callosum of postnatal day 30 wild-type (wt) and qk^v mice were immunostained with anti-MBPexII antibodies. A secondary antibody conjugated to rhodamine was used and the staining was visualized by confocal microscopy. Panels c and d represent amplifications of the regions that are boxed in panels a and b. The bar scale represents 10 μm. Panels e and f, OL culture from wild-type and qk^v mice, respectively. The inset is an image from the merge of MBPexII and DAPI staining. (B) Protein extracts from 30-day-old wild-type and qk^v mice were separated by SDS-PAGE and immunoblotted with the anti-MBPexII antibody. The positions of mouse MBP isoforms 17 and 21.5 are indicated. (C) The overexpression of QKI-7 causes abnormal distribution of MBP exon II isoforms in OLs. Rat OL progenitors were infected with adenoviruses coding for QKI-5 (panels c and d), QKI-7 (panels e and f), and control AdTR5 (panels a and b) and allowed to differentiate. On day 3, after infection, the cells were immunostained with anti-MBP exon II antibodies (red) and visualized by fluorescence microscopy. The green cells denote the infected cells. An example of nuclear staining is shown in the boxed region of panel f. The inset is an image from a merge of To-Pro3+GFP+MBPexII staining. The white staining denotes the nucleus. The bar scale represents 10 μm.

adenoviruses. Control infected OLs localized MBPexII to the cytoplasm and the perinuclear compartment as detected by immunofluorescence (Figure 3-7C, panels a and b). The presence of QKI-5 (panels c and d), or QKI-6 (data not shown) did not affect MBPexII localization. The overexpression of QKI-7 exclusively localized MBPexII isoforms to the nuclei of OLs (Figure 3-7C, panels e and f). Although we have shown that QKI-7 overexpression causes cell death in OLs (Pilotte et al., 2001), MBPexII localization was assessed at a pre-apoptotic stage. Although wild-type OLs (Figure 3-7C, panel b) contain QKI-7, it is the imbalance in the QKI isoforms that causes the MBPexII retention (Figure 3-7C, panel f). These findings suggest that the absence of QKI-7 in qk^{v} mice prevents the nuclear localization of MBP exon II isoforms.

3.5.7 QKI-5 downregulates the expression of the MBPs in vivo.

To confirm that QKI-5 downregulates the expression of the MBPs in vivo, the corpus callosum of postnatal day 1 mice was injected with a control or QKI-5-expressing adenovirus. The corpus callosum was chosen as the site of injection because it is myelin rich and adenovirus injections are known to spread along the white matter tracts (Kuo et al., 1995). The body weight of QKI-5-injected mice was consistently less than those of control-injected mice at postnatal day 10. The QKI-5 adenovirus-injected mice displayed a reduced weight (3.88 g ± 0.12, n = 6) compared to the control-injected adenovirus (4.30 g ± 0.14, n = 6), and the reason for this is unknown. Brain sections were prepared and stained with anti-MBP antibodies (red, Figures 3-8C to 3-8F) and visualized by confocal microscopy. A single injection was sufficient to spread the GFP-positive adenovirus throughout the corpus callosum, as well as the lateral ventricle (Figure 3-8B). Sections

from two independent injections with the control adenovirus AdTR5 demonstrated that the tracts in the corpus callosum stained positive for both GFP and MBP (Figures 3-8C and 3-8E). In panel C, a single OL can be observed by the arrow where its cell body is green (GFP) and its primary processes are red (MBP), indicating that adenovirus-infected cells are able to produce the MBPs. In contrast, brain sections from two separate mice demonstrated that the tracts in the corpus callosum that were GFP positive (i.e., expressing QKI-5) were MBP negative (Figures 3-8D and 3-8F). The brain section in panel F is quite striking and in addition depicts an area of degeneration. These results provide strong evidence that disturbing the balance of the QKI equilibrium toward nuclear QKI isoform suppresses the expression of the myelin basic proteins.

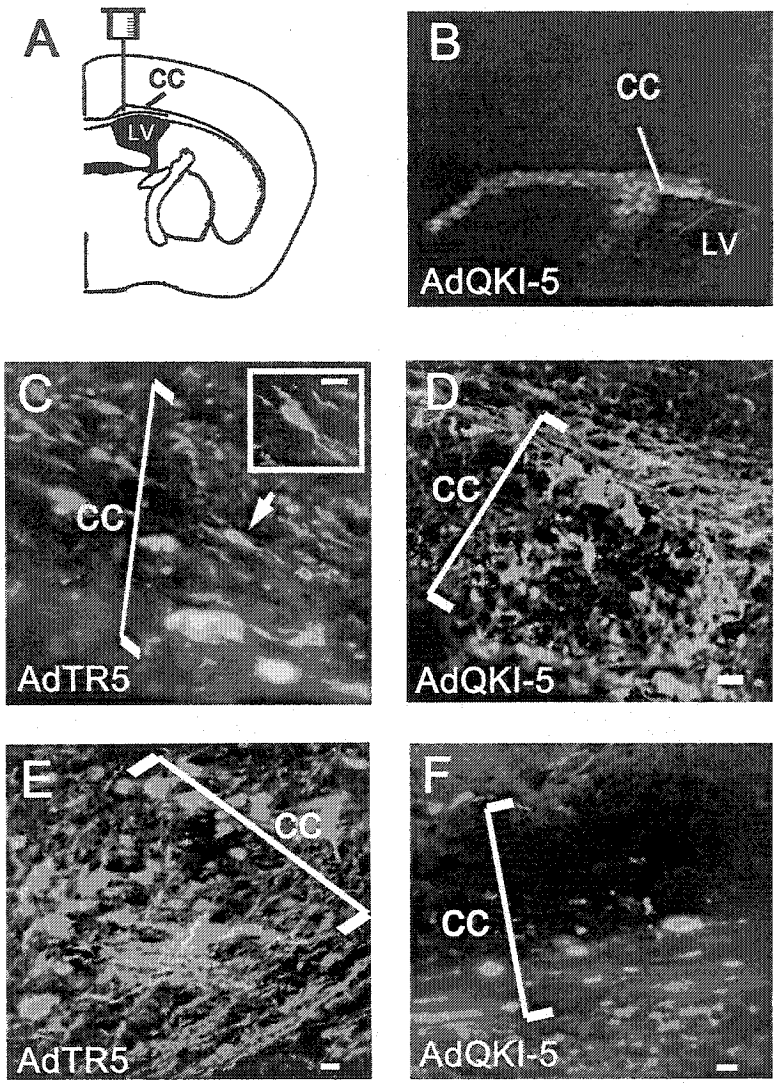

Figure 3-8. QKI-5 causes loss of MBP staining in vivo in the corpus callosum. (A) A schematic diagram of the site of injection is shown. (B) A coronal section of a QKI-5-injected mouse brain. The GFP was visualized by confocal microscopy. The corpus callosum of new born mice were injected with control AdTR5 adenovirus (C and E) or AdTR5 expressing QKI-5 (D and F). Ten days later, the mice were sacrificed and brain sections were stained with anti-MBP antibodies. The visualization of GFP (green) and MBP (red) or both (yellow) was observed by confocal microscopy. The corpus callosum (cc) is denoted by the white brackets. LV denotes the lateral ventricle. The bar scale represents 10 μm. The arrow in (C) points to an infected MBP-positive OL that has been enlarged in the inset.

3.6 Discussion

In this study, we show that MBP mRNA is a target of the QKI isoforms, specifically through a recognition element of ~100 nucleotides situated at position 680–790 of the mouse 3'-untranslated region. We have named this region the QKI recognition element or QRE. The QKI proteins bind the QRE as dimers since an amino acid substitution that prevents dimerization abrogates the ability to bind the QRE. The overexpression of QKI-5 in OLs disrupts the QKI nucleocytoplasmic equilibrium and causes the retention of the MBP mRNAs in the nucleus and perikaryon. The nuclear accumulation of MBP mRNA was further confirmed in OLs of qk^{v} mice. The injection of QKI-5-expressing adenovirus in new born C57BL/6 mice caused a reduction in MBP staining. Our data implicate the QKI RNA binding proteins as regulators of nuclear export of the MBP mRNAs in OLs and provide evidence that the QKI proteins are implicated in myelination.

QKI has been shown to associate with the *tra2* and *gli* element (TGE) bound by another STAR protein *C. elegans* GLD-1 (Jones and Schedl, 1995; Jan et al., 1999; Saccomanno et al., 1999). Although QKI associates with the TGE, the physiological relevance of this interaction is unknown. Schedl and coworkers identified several mRNA targets for GLD-1 and were unable to identify a consensus RNA binding site (Lee and Schedl, 2001). Inspection of the QRE and computer searches have also failed to identify similar sequences in other mRNAs. QKI and GLD-1 may bind a short sequence within a given secondary structure making computer searches erroneous. The QRE, however, is predicted to form several short stem-loop structures (data not shown). During the course of our studies, a QKI RNA binding site was identified in the 3'-UTR of MBP by Feng

and coworkers (Zhang and Feng, 2001). The identification of the nucleotides and RNA secondary structures bound by QKI will require further mapping with RNA footprinting techniques as recently performed for the product of the fragile X syndrome protein (Darnell et al., 2001). The major domain required to bind RNA is the QKI GSG domain (Chen and Richard, 1998), and this is indeed supported by the fact that the V157E amino acid substitution in the KH domain abrogates RNA binding.

The identification of genetic point mutations in the *qk* gene has helped understand the properties of the QKI proteins. We showed previously that the qk^{kt4} allele altering QKI glutamic acid 48 to glycine prevents dimerization by disrupting a predicted coiled-coil (Chen and Richard, 1998). However, we were unable to see a reduction in binding to total cellular mRNAs (Chen and Richard, 1998). Here we extend our studies to show that QKI E48G amino acid substitution cannot bind the QRE in the MBP mRNA. These findings suggest that the QKI proteins bind as dimers to specific mRNA targets. The other amino acid substitution known to cause embryonic lethality in mice is QKI valine 157 to glutamic acid (Cox et al., 1999). This point mutation localizes in the KH domain that resides ~100 amino acids C-terminal to the coiled-coil region and is not predicted to influence dimerization. We observed that QKI V157E abolishes RNA binding, but not dimerization. Thus the molecular defect of the qk^{k2} allele may be the inability of QKI-5 to retain certain mRNAs during development.

QKI-5 is the only isoform expressed during mouse embryogenesis (Ebersole et al., 1996). Expression of QKI-6 and QKI-7 peaks at postnatal day 16 coinciding with the onset of myelination (Ebersole et al., 1996). The mechanism that triggers the expression of QKI-6 and QKI-7 is unknown. Our data suggest that the expression of QKI-6 and

QKI-7 is essential for the "release" or the derepression of the MBP mRNAs imposed by the nuclear QKI-5. The fact that the QKI proteins dimerize (Chen and Richard, 1998) and shuttle between the nucleus and the cytoplasm (Wu et al., 1999) suggest that they function as heterodimers to export the MBP mRNAs. QKI proteins may be directly involved in nuclear export mechanics or may be required to "coat" the MBP mRNAs into mature messenger ribonucleoprotein particles (mRNPs) to be recognized by the export machinery. QKI-5 has been shown to shuttle in fibroblasts, as assessed by heterokaryon assays (Wu et al., 1999). We confirmed that QKI-5 indeed has the ability to shuttle but to a lesser extent than hnRNPK, a well-known shuttling protein (Michael et al., 1997; data not shown). This leads to the question of how an RNA binding protein with shuttling abilities can retain mRNAs in the nucleus. Our data clearly demonstrate that MBP mRNAs are retained in nuclei of OLs and COS cells by QKI-5 and that only in the presence of QKI-6 and QKI-7 are they released in the cytoplasm. This leads us to propose that MBP mRNAs may prevent QKI-5 shuttling, or alternatively the model we favor, that QKI-6 and QKI-7 are involved in the release of mRNAs from QKI-5 by competition for the QRE.

The presence of mRNAs encoding the MBPs in myelin (Colman et al., 1982) and of soluble polyribosomes in OLs (Verity and Campagnoni, 1988) led to the proposal that MBP mRNAs are transported along processes to the periphery, where they are locally translated and incorporated into myelin. The proteins and RNA elements responsible for this journey are beginning to be identified (Kiebler and DesGroseillers, 2000). Microinjection of labeled MBP mRNAs in OLs and the formation of mRNP granules that are transported down the processes has led to the identification of the RNA trafficking

signal (RTS) (Ainger et al., 1993, 1997) . Other RTS found in mRNAs transported along processes include those for myelin OL basic protein (MOBP), -CAMKII, tau, and amyloid precursor protein (APP) (Barbarese et al., 2000; Carson et al., 2001). The RTS is localized from nucleotides 794 to 814 of rat MBP mRNAs or nucleotides 999 to 1019 of mouse MBP (Ainger et al., 1997). Thus the RTS is localized ~200 nucleotides downstream of the QRE in the MBP mRNA. The RTS is sufficient to permit transport of the MBP mRNAs to the processes and is recognized by hnRNP A2 (Ainger et al., 1997; Hoek et al., 1998). Since hnRNP A2 is predominantly nuclear, it is thought that hnRNP A2 binds the RTS of the MBP mRNAs and permits their nuclear export and is implicated in the mRNA transport along the cytoskeletal network in the OL processes (Carson et al., 2001). Our data demonstrate that the QKI proteins are necessary to allow the MBP mRNAs out of the nucleus. Since the QKI and hnRNP A2 bind different elements, they may colocalize in mRNPs and function cooperatively in the nuclear export of the MBP mRNAs. The QRE is also distinct from other RNA elements in the untranslated region of MBP including the Y element (Han et al., 1995; Wu and Hecht, 2000) and the RNA localization region (Ainger et al., 1997). The QKI-6 and QKI-7 isoforms have been shown to localize in the processes of OLs (Hardy et al., 1996b; Wu et al., 2001), but their function in these processes remains unknown. It is tempting to speculate that QKI-6 and QKI-7 may be involved in the translational suppression of the MBPs during the journey along the processes especially since the QKI proteins have been shown to functionally substitute for GLD-1, a translational suppressor (Saccomanno et al., 1999).

OLs from *qk*^{v} have been proposed to have maturation defects (reviewed in Hogan and Greenfield, 1984). An elevated presence of bipolar OLs that stained with MBPexII

antibodies was observed in the corpus callosum of qk^v mice. Moreover, much fewer primary OLs were obtained from qk^v mice and the cells generally exhibited a bipolar morphology (see Figure 3-6B, panel G and Figure 3-7A, panel d). Thus, OLs of qk^v mice have a delay in OL maturation compared with wild-type OLs at the same developmental stage. It is currently unknown whether the absence of QKI-6 and QKI-7 is the cause or the effect of the maturation defects. The presence of the cytoplasmic QKI-7 permits cultured rat OLs to localize MBPexII within the nucleus. These studies suggest that cytoplasmic QKI isoforms may allow MBPexII nuclear import that will properly initiate the maturation process of the OLs. Thus the QKI isoforms regulate the early events of myelination. It is unknown whether QKI-7 is directly involved in the mechanism by which MBPexII are transported to the nucleus. For example, QKI-7 may remove an exon II NLS masking protein. Alternatively, QKI-7 may have a global effect on the cell that triggers it to myelinate. It is unclear whether this effect is separate from its ability to induce cell death. The brain injection of the QKI-5 isoform in new born inbred C57BL/6 mice resulted in disruption of the MBP-positive tracts in the corpus callosum. These experiments further demonstrate that the disrupted balance toward the nuclear isoform, QKI-5, is sufficient to induce MBP defects in vivo. Thus it may be possible to induce re-myelination by displacing the equilibrium toward the cytoplasmic QKI isoforms.

Drosophila tendon cell differentiation has been shown to involve the opposing activities of two QKI homologs, How(L) and How(S) (Baehrecke, 1997; Zaffran et al., 1997). These How isoforms have been shown to regulate the mRNA export and the degradation rate of the Stripe mRNA (Nabel-Rosen et al., 1999, 2002) . We have also shown that a similar balance between the mammalian QKI isoforms controls cell survival and cell

death (Pilotte et al., 2001). Although the mRNA retention of MBP by QKI-5 parallels the observations of Stripe and How(L) (Nabel-Rosen et al., 1999), QKI-5 is not involved in mRNA stability, unlike How(L) (Figure 3-3B and data not shown). It has been postulated that QKI-5 may have such activity toward the mRNA of Krox-20 (Nabel-Rosen et al., 2002), but QKI-5 was expressed in insect cells and it is unclear whether QKI-5 regulates protein translation, mRNA stability, or mRNA export in that system.

While this paper was under revision, QKI-5 was shown to regulate alternative splicing (Wu et al., 2002). This is most likely an embryonic function because the other QKI isoforms are absent during embryogenesis. Our working model is that during myelination, the elevated presence of QKI-6 and QKI-7 (Ebersole et al., 1996) most likely suppresses the nuclear function of QKI-5 (i.e., alternative splicing) and permits the participation of QKI-5 in the mRNA export with QKI-6 and QKI-7. Thus the presence of splicing defects in qk^{v} mice is most likely caused by the QKI-5 embryonic function that cannot be suppressed by QKI-6 and QKI-7 because of their absence in OLs from qk^{v} mice. It will be important to identify the developmental genes regulated by QKI-5 during embryogenesis and to investigate the alternative splicing function of QKI-5 in the presence of QKI-6 and QKI-7 isoforms.

In conclusion, we have shown that the MBP mRNAs are physiological targets of the QKI family of proteins and that the QKI proteins function in the regulation of mRNA export out of the nucleus. The elucidation of the role of the QKI proteins during myelination will only be achieved by studying the isoforms together in OLs. The present manuscript introduces the various QKI isoforms in OLs and recreates a molecular defect observed in qk^{v} mice. The qk^{v} phenotype is complicated because some areas of the brain

maintain QKI-5 expression and others are completely devoid of QKI proteins (Hardy et al., 1996a). Our studies show that QKI-5 overexpression, which is predicted to disrupt the nuclear to cytoplasmic ratio of QKI proteins (Pilotte et al., 2001), reproduces in OLs the nuclear retention of the MBP mRNAs. These findings imply that the absence of QKI-6 and QKI-7 in OLs of the qk^{v} mice is sufficient to cause myelination defects.

3.7 Acknowledgement

We would like to thank Samuel David and Nahum Sonenberg for advice, useful discussions, and for critically reading the manuscript. We would like to thank Yue Feng and David Colman for critically reading the manuscript and helpful comments and discussion. We thank Ben Barres for providing advice with the OL cultures and staining. We are grateful to Kostas Pantopoulos for his help with RNA gel shifts. We thank Maxime Hallé at McGill Cancer Center for excellent assistance with the confocal microscopy and François Bouthillier from the BRI for the production of adenovirus. This work was supported by a grant from the Multiple Sclerosis Society of Canada to S.R.; D.L. and J.P. are recipients of studentships from the K.M. Hunter/CIHR Foundation and the CIHR, respectively. P.L. is a CIHR Investigator. S.R. is a chercheur-boursier from the Fonds de la Recherche en Santé du Québec and a CIHR Investigator. S.R. is a recipient of the Terry Fox Young Investigator Award from National Cancer Institute of Canada.

3.8 References

Ainger K., Avossa D., Morgan F., Hill S.J., Barry C., Barbarese E. and Carson J.H. 1993. Transport and localization of exogenous myelin basic protein mRNA microinjected into oligodendrocytes. J. Cell Biol., 123:431-441.

Ainger K., Avossa D., Diana A.S., Barry C., Barbarese E. and Carson J.H. 1997. Transport and localization elements in myelin basic protein mRNA. J. Cell Biol., 138:1077-1087.

Allinquant B., Staugaitis S.M., D'Urso D. and Colman D.R. 1991. The ectopic expression of MBP isoforms in shiverer oligodendrocytes: implications for myelinogenesis. J. Cell Biol., 113:393-403.

Almazan G., Afar D.E.H. and Bell J.C. 1993. Phosphorylation and disruption of intermediate filament protein in oligodendrocyte precursor cultures treated with calyculin A. J. Neurosci. Res., 36:163-172.

Baehrecke E.H. 1997. who encodes a KH RNA binding protein that functions in muscle development. Development, 124:1323-1332.

Barbarese E. 1991. Spatial distribution of myelin basic protein mRNA and polypeptide in Quaking oligodendrocytes in culture. J. Neurosci. Res., 29:271-281.

Barbarese E., Brumwell C., Kwon S., Cui H. and Carson J.H. 2000. RNA on the road to myelin. J. Neurocytol., 28:263-270.

Bartoszewicz Z.P., Noronha A.B., Fujita N., Sato S., Bo L., Trapp B.D. and Quarles R.H. 1995. Abnormal expression and glycosylation of the large and small isoforms of myelin-associated glycoprotein in dysmelinating quaking mutants. J. Neurochem. Res., 41:27-38.

Bo L., Quarles R.H., Fujita N., Bartoszewiez Z., Sato S. and Trapp B.D. 1995. Endocytic depletion of L-MAG from CNS myelin in quaking mice. J. Cell Biol., 131:1811-1820.

Bode V.C. 1984. Ethylsnitrosourea mutagenesis and the isolation of mutant alleles for specific genes located in the t region of the mouse chromosome 17. Genetics, 108:457-470.

Braun P.E., Horvath E. and Edwards A.M. 1990. Two isoforms of myelin associated glycoprotein accumulate in quaking mice: only the large polypeptide is phosphorylated. Dev. Neurosci., 12:286-292.

Brostoff S.W., Greenfield S. and Hogan E.L. 1977. The differentiation of synthesis form incorporation of basic protein in quaking mutant mouse myelin. Brain Res., 120:517-520.

Campagnoni A.T., Verdi J.M., Verity A.N. and Amur-Umarjee S. 1990. Posttranscriptional events in the expression of myelin protein genes. Ann. N Y Acad. Sci., 605:270-279.

Carnow T.B., Carson J.H., Brostoff S.W. and Hogan E.L. 1984. Myelin basic protein gene expression in quaking, jimpy and myelin synthesis-deficient mice. Dev. Biol., 106:38-44.

Carson J.H., Cui H. and Barbarese E. 2001. The balance of power in RNA trafficking. Curr. Opin. Neurobiol., 11:558-563.

Chen T. and Richard S. 1998. Structure-function analysis of QKI: A lethal point mutation in mouse quaking prevents homodimerization. Mol. Cell. Biol., 18:4863-4871.

Colman D.R., Kreibich G., Frey A.B. and Sabatini D.D. 1982. Synthesis and incorporation of myelin polypeptides into CNS myelin. J. Cell Biol., 95:598-608.

Cox R.D., Hugill A., Shedlovsky A., Noveroske J.K., Best S., Justice M.J., Lehrach H. and Dove W.F. 1999. Contrasting effects of ENU induced embryonic lethal mutations of the quaking gene. Genomics, 57:333-341.

Darnell J.C., Jensen K.B., Jin P., Brown V., Warren S.T. and Darnell R.B. 2001. Fragile X mental retardation protein targets G quartet mRNAs important for neuronal function. Cell, 107:489-499.

de Ferra F., Engh H., Hudson L., Kamholz J., Puckett C., Molineaux S. and Lazzarini R.A. 1985. Alternative splicing accounts for the four forms of myelin basic protein. Cell, 43:721-727.

Delassalle A., Zalc B., Lachapelle F., Raoul M., Collier P. and Jacque C. 1981. Regional distribution of myelin basic protein in the CNS of quaking, jimpy and normal mice during development and aging. J. Neurosci. Res., 6:303-313.

Ebersole T.A., Chen Q., Justice M.J. and Artzt K. 1996. The *quaking* gene unites signal transduction and RNA binding in the developing nervous system. Nat. Genet., 12:260-265.

Frail D.E. and Braun P.E. 1985. Abnormal expression of the myelin-associated glycoprotein in the central nervous system of dysmyelinating mutant mice. J. Neurochem., 45:1071-1075.

Fujita N., Sato S., Ishiguro H., Inuzuka T., Baba H., Kurihara T., Takahashi Y. and Miyatake T. 1990. The large isoform of the myelin-associated glycoporotein is scarcely expressed in the quaking mouse brain. J. Neurochem., 55:1056-1059.

Han J.R., Yiu G.K. and Hecht N.B. 1995. Testis/Brain RNA-binding protein attaches translationally repressed and transported mRNAs to microtubules. Proc. Natl. Acad. Sci. USA, 92:9550-9554.

Hardy R.J. 1998. Molecular defects of the dysmyelinating mutant quaking. J. Neurosci. Res., 51:417-422.

Hardy R.J., Lazzarini R.A., Colman D.R. and Friedrich V.L. Jr. 1996. Cytoplasmic and nuclear localization of myelin basic proteins reveals heterogeneity among oligodendrocytes. J. Neurosci. Res., 46:246-257.

Hardy R.J., Loushin C.L., Friedrich V.L. Jr., Chen Q., Ebersole T.A., Lazzarini R.A. and Artzt K. 1996. Neural cell type-specific expression of QKI proteins is altered in the *quaking* viable mutant mice. J. Neurosci., 16:7941-7949.

Hoek K.S., Kidd G.J., Carson J.H. and Smith R. 1998. hnRNP A2 selectively binds the cytoplasmic transport sequence of myelin basic protein mRNA. Biochemistry, 37:7021-7029.

Hogan, E.L., and Greenfield, S. 1984.. Animal models of genetic disorders of myelin. Myelin 489–534.

Jan E., Motzny C.K., Graves L.E. and Goodwin E.B. 1999. The STAR protein, GLD-1, is a translational regulator of sexual identity in Caenorhabditis elegans. EMBO J., 18:258-269.

Jones A.R. and Schedl T. 1995. Mutations in GLD-1, a female germ cell-specific tumor suppressor gene in *C.elegans*, affect a conserved domain also found in Sam68. Genes Dev., 9:1491-1504.

Justice M.J. and Bode V.C. 1988. Three ENU-induced alleles of the murine quaking locus are recessive embryonic lethal mutations. Genet. Res., 51:95-102.

Kiebler M.A. and DesGroseillers L. 2000. Molecular insights into mRNA transport and local translation in the mammalian nervous system. Neuron, 25:19-28.

Kuo H., Ingram D.K., Crystal R.G. and Mastrangeli A. 1995. Retrograde transfer of replication deficient recombinant adenovirus vector in the central nervous system for tracing studies. Brain Res., 705:31-38.

Lawrence J.B. and Singer R.H. 1986. Intracellular localization of messenger RNAs for cytoskeletal proteins. Cell, 45:407-415.

Lee M.-H. and Schedl T. 2001. Identification of in vivo mRNA targets of GLD-1, a maxi-KH motif containing protein required for C. elegans germ cell development. Genes Dev., 15:2408-2420.

Li Z., Zhang Y., Li D. and Feng Y. 2000. Destabilization and mislocalization of the myelin basic protein mRNAs in quaking dysmyelination lacking the Qk1 RNA-binding proteins. J. Neurosci., 20:4944-4953.

Lubetzki C., Goujet-Zalc C., Gansmuller A., Monge M., Brillat A. and Zalc B. 1991. Morphological, biochemical, and function characterization of bulk isolated glial progenitor cells. J. Neurochem., 56:671-680.

Massie B., Couture F., Lamoureux L., Mosser D.D., Guilbault C., Jolicoeur P., Belanger F. and Langelier Y. 1998. Inducible overexpression of a toxic protein by an adenovirus vector with a tetracycline-regulatable expression cassette. J. Virol., 72:2289-2296.

Massie B., Mosser D.D., Koutroumanis M., Vitte-Mony I., Lamoureux L., Couture F., Paquet L., Guilbault C., Dionne J. and Chahla D. et al. 1998. New adenovirus vectors for protein production and gene transfer. Cytotechnology, 28:53-64.

Mezquita J., Pau M. and Mezquita C. 1998. Four isoforms of the signal-transduction and RNA-binding protein Qk1 expressed during chicken spermatogenesis. Mol. Reprod. Dev., 50:70-78.

Michael W.M., Eder P.S. and Dreyfuss G. 1997. The K nuclear shuttling domain: a novel signal for nuclear import and nuclear export in the hnRNP K protein. EMBO J., 16:3587-3598.

Nabel-Rosen H., Dorevitch N., Reuveny A. and Volk T. 1999. The balance between two isoforms of the Drosophila RNA-binding protein How controls tendon cell differentiation. Mol. Cell, 4:573-584.

Nabel-Rosen H., Volohonsky G., Reuveny A., Zaidel-Bar R. and Volk T. 2002. Two isoforms of the Drosophila RNA binding protein, How, act in opposing directions to regulate tendon cell differentiation. Dev. Cell, 2:183-193.

Pedraza L., Fidler L., Staugaitis S.M. and Colman D.R. 1997. The active transport of myelin basic protein into the nucleus suggests a regulatory role in myelination. Neuron, 18:579-589.

Pilotte J., Larocque D. and Richard S. 2001. Nuclear translocation controlled by alternatively spliced isoforms inactivates the QUAKING apoptotic inducer. Genes Dev., 15:845-858.

Saccomanno L., Loushin C., Jan E., Punkay E., Artzt K. and Goodwin E.B. 1999. The STAR protein Qk1–6 is a translational repressor. Proc. Natl. Acad. Sci. USA, 96:12605-12610.

Shedlovsky A., King T.R. and Dove W.F. 1988. Saturation germ line mutagenesis of the murine t region including a lethal allele of the quaking locus. Proc. Natl. Acad. Sci. USA, 85:180-184.

Sidman R.L., Dickie M.M. and Appel S.H. 1964. Mutant mice (quaking and jimpy) with deficient myelination in the central nervous system. Science, 144:309-311.

Sorg B.J., Agrawal D., Agrawal H.C. and Campagnoni A.T. 1986. Expression of myelin proteolipid protein and basic protein in normal and dysmyelinating mutant mice. J. Neurochem., 46:379-387.

Sorg B.A., Smith M.M. and Campagnoni A.T. 1987. Developmental expression of the myelin proteolipid protein and basic protein mRNAs in normal and dysmyelinating mutant mice. J. Neurochem., 49:1146-1154.

Tanaka H., Abe K. and Kim C.H. 1997. Cloning and expression of the quaking gene in the Zebrafish embryo. Mech. Dev., 69:209-213.

Trapp B.D., Quarles R.H. and Suzuki K. 1984. Immunocytochemical studies of quaking mice support a role of the myelin-associated glycoprotein in forming and maintaining the periaxonal space and periaxonal cytoplasmic collar of myelinating schwann cells. J. Cell Biol., 99:594-606.

Verity A.N. and Campagnoni A.T. 1988. Regional expression of myelin protein genes in the developing mouse brain: In situ hybridization studies. J. Neurosci. Res., 21:238-248.

Vernet C. and Artzt K. 1997. STAR, a gene family involved in signal transduction and activation of RNA. Trends Genet., 13:479-484.

Wu J., Zhou L., Tonissen K., Tee R. and Artzt K. 1999. The quaking I-5 (QKI-5) has a novel nuclear localization signal and shuttles between the nucleus and the cytoplasm. J. Biol. Chem., 274:29202-29210.

Wu X.-Q. and Hecht N.B. 2000. Mouse testis brain RNA binding protein/Translin colocalizes with microtubules and is immunoprecipitated with mRNAs encoding MBP, alpha CaMKII and protamines 1 and 2. Biol. Reprod., 62:720-725.

Wu H.Y., Dawson M.R.L., Reynolds R. and Hardy R.J. 2001. Expression of QKI proteins and MAP1B identifies actively myelinating oligodendrocytes in adult rat brain. Mol. Cell. Neurosci., 17:292-302.

Wu J.I., Reed R.B., Grabowski P.J. and Artzt K. 2002. Function of quaking in myelination: regulation of alternative splicing. Proc. Natl. Acad. Sci. USA, 99:4233-4238.

Zaffran S., Astier M., Gratecos D. and Semeriva M. 1997. The held out wings (how) Drosophila gene encodes a putative RNA binding protein involved in the control of muscular and cardiac activity. Development, 124:2087-2098.

Zhang Y. and Feng Y. 2001. Distinct molecular mechanisms lead to diminished myelin basic protein and 2',3'-cyclic nucleotide 3'-phosphodiesterase in qk(v) dysmyelination. J. Neurochem., 77:165-172.

Zorn A.M., Grow M., Patterson K.D., Ebersole T.A., Chen Q., Artzt K. and Krieg P.A. 1997. Remarkable sequence conservation of transcripts encoding amphibian and mammalian homologues of *quaking*, a KH domain RNA-binding protein. Gene, 188:199-206.

Chapter 4

General discussion

The *quaking* viable mouse was discovered almost 40 years ago. However, characterization of the *quaking* gene itself was accomplished only in the last decade. *Quaking* viable mice generally have a myelination defect and a better understanding of the myelination process will improve our knowledge on the molecular pathology of the *quaking* mice as well as other myelination-related diseases like multiple sclerosis and secondary-injury-induced demyelination. The identification of the RNA-binding GSG domain in the various QKI isoforms suggests a link between RNA metabolism and myelination. The main focus of my project was to characterize the QKI-7 protein. We showed that QKI-7 is involved in apoptosis and is dependent on the other QKI isoforms to keep cells from dying. We also worked on finding physiological myelin mRNA targets in order to better understand the role of this protein in myelination and RNA metabolism. This led to the discovery that QKI proteins are involved in the timely nuclear export of the MBP mRNA via interaction with its 3'UTR.

4.1 The QKI isoforms control cell survival

We discovered throughout my studies that the balance of the QKI proteins isoforms is responsible for cell survival. The QKI-7 isoform had previously been shown in our laboratory to induce apoptosis in mouse fibroblast cells (Chen and Richard, 1998), and we confirmed these results in oligodendrocytes. The QKI-7 isoform possesses a domain which we named the 'KILLER' sequence, consisting of the 14 amino acid

produced by alternative splicing of its C-terminus. The 14 amino acid sequence did not uncover other identical motifs by BLAST searches, but did have some similarity to a series of unknown proteins as well as the *C.elegans* CED-9 protein, the homologue of mammalian Bcl-2. The other major QKI isoforms, QKI-5 and QKI-6, do not contain this 'KILLER' sequence and therefore do not have any effect on the induction of apoptosis. The QKI-7 cell death induction seems to be dependent on its localization to the cytoplasm. QKI-6 is found in the cytoplasm and the nucleus and QKI-5 is localized primarily to the nucleus (Hardy et al., 1996). The QKI-7 protein has been shown to homodimerize through the NK domain, and cross-linking assays have suggested that the QKI proteins heterodimerize (Chen and Richard, 1998). We have confirmed that all three QKI isoform are able to heterodimerize, which in turn causes the relocalization of the proteins from one compartment to another. This was especially striking for the QKI-7 protein, which relocalizes mostly to the nucleus when co-expressed with QKI-5. Results are similar with QKI-6, and as a result of this relocalization, QKI-7 induced apoptosis is repressed. Placing a nuclear localization signal (NLS) at the end of QKI-7 also inhibited apoptosis, showing once again the importance of its location in the cytoplasm in order to communicate with the apoptotic machinery. There is always the possibility that the dimerization or addition of the NLS causes the masking of a domain necessary for apoptosis in the C-terminus of QKI-7. Stochiometric amount of the QKI proteins was responsible for quenching the apoptotic effect of QKI-7, as increasing the amount of either QKI-5 or QKI-6 increased cell survival. Therefore the balance of these isoforms in the cell is crucial for keeping cells alive. As already mentioned in the discussion of Chapter 2, alternative splicing of proteins with opposite effects on regulating cell survival

has already been shown. Splicing of the apoptotic proteins Bcl-x, ced-4 in *C.elegans*, Ich-1 and interleukin-1-converting enzyme all lead to an isoform able to act contrary to the original function of the protein (Alnemri et al., 1995; Boise et al., 1993; Shaham and Horvitz, 1996; Wang et al., 1994).

Another interesting outcome of the apoptotic study was that the E48G mutation led to cell death in all the QKI isoforms. This mutation was first identified in the ENU-induced mutated *quaking* mouse qk^{ki4}, which is recessive lethal (Justice and Bode, 1988). The E48G mutation is located in the NK region of the GSG, previously demonstrated to abolish dimerization (Chen and Richard, 1998). This induction of apoptosis is different from the 'KILLER' sequence of the QKI-7 protein, which does not require this domain for cell death. Therefore we speculate that this event may occur as a result of a change in the 2^0 structure of the protein caused by the position of the mutation in the predicted coil-coiled domain. This in turn might expose a site responsible for inducing cell death providing binding to death inducing proteins. It is possible as well that there is an inability of these monomers to associate with survival proteins such as Bcl-2, or that the dimererization is needed for this interaction. This once again implicates the importance of KH proteins in cell survival.

QUAKING is not the only RNA binding protein known to induce apoptosis. As mentioned in section 1.9.1, 15 out of 21 RNA binding proteins were recognized as potential gene targets activated by Fas-induced apoptosis (Thiede et al., 2001). The MCG-10 protein and the *Drosophila* dFMR1, KEP1 and Sam50 are all KH-containing proteins involved in cell death regulation (Di Fruscio et al., 1998; Wan et al., 2000; Zhu and Chen, 2000). Apoptosis induced by QKI-7 over-expression in cultured 1^0 rat

oligodendrocytes suggests that QKI could take part of programmed cell death in vivo. It has been documented that extra premyelinating oligodendrocytes are eliminated by programmed cell death (Barres et al., 1992; Barres and Raff, 1994; Trapp et al., 1997). More than 50% of these newly differentiated oligodendrocytes do not survive, therefore there must be specific signals instructing these extra cells to self-destruct. It is hypothesized that axonal contact with the oligodendrocyte generates cell survival signals (Hardy and Reynolds, 1993). After the peak of myelination, QKI-5 is greatly reduced, while only QKI-6 is left to quench QKI-7's ability to induce cell death. This also coincides with the time that extra non-myelinating oligodendrocytes are eliminated by the process of apoptosis. Thus it would be interesting to see whether the balance between QKI-6 and QKI-7 is altered in these cells programmed to die. This could be achieved by looking at expression levels of the QKI isoforms in cells marked for programmed cell death. I hypothesize that the signals involved in the elimination of non-myelinating oligodendrocytes or the absence of cell survival factors might be implicated in this event. There is also the possibility that QKI-7 can somehow detect cells that are not properly myelinating axons, targeting the cell for destruction. It will be interesting to study whether QKI-7 participates in this process, as well as elucidate the pathway utilized and identify the proteins targeted.

Another interesting point strengthening the role of the QKIs in cell survival and cell death was brought up by a Japanese group who demonstrated that the QKI isoforms are altered in certain gliomas (Li et al., 2002). A number of gliomas (~30%) had reduced levels of human QKI, particularly the apoptotic QKI-7. This is specific to this type of tumor, as schawnnomas or meningiomas were not affected. These numbers might be even

higher, as these studies were done by PCR detection, which does not distinguish point mutations from wild-type. It is also interesting to note that insertion of a normal chromosome 6 fragment containing the hQKI gene into a tumor cell line with the 6q deletion resulted in suppression of tumorigenecity (Trent et al., 1990). These observations support our findings that deregulation in the balance of the QKI isoforms probably leads to cell cycle alteration and that loss of the apoptotic inducer QKI-7 would lead to tumorigenecity. In fact, there is some evidence of hyperplasia in optic nerve oligodendrocytes of the *quaking* viable mouse, where QKI-7 is not expressed (Friedrich, 1975). It would be interesting to see if these mice have a higher propensity of forming gliomas and how these mice react to cancer inducing agents. This would also support other GSG proteins believed to have roles in tumorigenesis, such as the germ-line tumor suppressor GLD-1, and Sam68, which has been seen in nuclear bodies of certain cancer cell lines (Chen et al., 1999; Jones and Schedl, 1995).

4.2 QKI regulates MBP mRNA

Our second study led to the identification of an mRNA target for QUAKING. We identified the myelin basic protein mRNA as a potential candidate for binding to QKI. All QKI isoforms are able to bind to the MBP mRNA 3'UTR. Binding occurs near the end of the coding sequence, at nucleotides 680-790. We called this sequence QRE, for Quaking Recognition Element. This sequence differs from other RNA signals identified in the 3'UTR such as the RNA transport signal (RTS: nucleotides 999-1019), the RNA localization element (RLR: 1061-1190), and the Y element (Ainger et al., 1997; Ainger et al., 1993; Han et al., 1995; Wu and Hecht, 2000). Binding to the QRE

region requires dimerization of the QUAKING isoforms, as introduction of the dimerization defective mutation E48G (qk^{kt4}) disrupted the RNA binding. Mutation of a conserved residue in the KH domain, V157E (qk^{k2}), also disrupted the RNA binding. These results suggest that lethality in these ENU-induced mutated mice is due to their inability to bind to their RNA targets, most likely needed for proper embryonic development. This also strengthens previous hypotheses that GSG proteins function as dimers and that the KH domain is crucial in RNA binding, as seen from mutations affecting FMR1, GLD-1, KEP-1, How and Sam68 (Coyle et al., 2003; Di Fruscio et al., 2003; Jan et al., 1999; Nabel-Rosen et al., 1999; Siomi et al., 1994).

We also wanted to understand the myelination defect behind the *quaking* viable mouse, therefore we observed the effect of the sole expression of QKI-5 into different systems and its effect on the MBP mRNA localization and expression. Overexpression of QKI-5 in cultured rat oligodendrocytes caused the retention of the MBP mRNA into the nucleus of the cell, where QKI-5 is located. This was confirmed in oligodendrocytes isolated from *quaking* viable mice. We also observed a high number of immature oligodendrocytes, suggesting a slowing or a block in maturation. Trapping of the MBP mRNA in the nucleus led to a reduction of the expression of the MBP protein, as it is unable to exit the nucleus and communicate with the cytoplasmic translation machinery. Co-expression of QKI-6 and QKI-7 in COS cells relieved the MBP mRNA trapped by QKI-5 from the nucleus, presumably by heterodimerization and relocalization of the proteins as seen in Chapter 2. Therefore it seems that overexpression of the QKI-5 isoform in comparison to the other cytoplasmic isoforms leads to a disruption of the nuclear-cytoplasmic equilibrium. We wanted to recreate the effect of the *quaking* viable

mouse in vivo, therefore we injected newborn mice at day one before myelination begun with adenoviral vectors expressing QKI-5. Overexpression of QKI-5 in the brain led to an absence of MBP in the infected area, suggesting that the dysmyelination of the *quaking* viable mouse is indeed caused by the overexpression of the QKI-5 isoform in comparison to the cytoplasmic QKI isoforms.

Another interesting point brought up in Chapter 3 is the localization of the MBP containing exon II (MBPexII). It has been previously reported that the MBPexII isoforms, 21.5K and 17K, are found in the nucleus of young oligodendrocytes contrarily to their presence in the myelin periphery, which led to the hypothesis that their nuclear presence may regulate MBP mRNA transport during myelination (Allinquant et al., 1991; Pedraza et al., 1997). Immunostaining for MBPexII in *quaking* viable oligodendrocytes revealed that these proteins are localized in the cytoplasm and primary branches, consistent with the presence of young oligodendrocytes. A presence of bipolar cells was observed as well, suggesting that a maturation defect or delay is occurring. Moreover, the ratio of MBPexII isoforms was not altered between wild-type and *quaking* viable mice, suggesting that the QKI proteins do not regulate inclusion/exclusion of MBPexII, as recently suggested (Wu et al., 2002). Therefore the MBP mRNA nuclear retention in *quaking* viable mice is not caused by an excess of MBPexII isoforms in the nucleus, but is due to maturation defects. Furthermore, QKI-5 or QKI-6 did not affect MBPexII localization, but the overexpression of QKI-7 exclusively localized MBPexII isoforms to the nuclei of oligodendrocytes. This would suggest that the absence of QKI-7 in *quaking* viable mice prevents the nuclear localization of MBPexII isoforms, which in turn could have deleterious effects on the maturation stage of the oligodendrocyte differentiation.

This perhaps may also explain the low levels of the other MBP mRNA isoforms, which might be upregulated after MBPexII localization to the nucleus. Interestingly, MBPexII isoforms as well as the QKI proteins have been shown to be upregulated in remyelinating cells, questioning their participation in localization and maturation of myelination (Wu et al., 2001).

What are the causes of the dysmyelination in the *quaking* viable mouse? The most obvious possibility is the misexpression of the cytoplasmic isoforms of QUAKING. Without these proteins, the MBP mRNA is trapped in the nucleus, which causes reduced expression and mislocalization of the MBP protein. This leads to a breakdown in the differentiation of the oligodendrocyte and myelination process. Also supporting this theory is our observation suggesting that the absence of QKI-7 in *quaking* viable mice prevents the nuclear localization of MBP exon II isoforms, which is presumed to alter maturation of the oligodendrocyte. Dysmyelination defects in the *quaking* viable mouse seems to improve after three weeks, where MBP levels increase near wild type levels, coinciding with QKI-5 downregulation at the end of myelination (reviewed in Hogan and Greenfield, 1984a). In this case, the MBP mRNA could finally be released into the cytoplasm and be translated. Unfortunately at this point, proper incorporation of MBP into the membrane is compromised and this is not enough to compensate for the lack of myelination thus far, causing thinner myelin membranes (Campagnoni et al., 1987).

The differential expression of the *quaking* gene in the *quaking* viable mouse is still poorly understood. The 1Mb deletion in the promoter/enhancer region lies 913 base pairs away from the transcription site, therefore almost 1 kb of the 5' UTR of the *quaking* transcript is intact (Kondo et al., 1999). It has been observed in one of the *quaking* studies

that all of the *qkI* transcripts are reduced by ~30% in *quaking* viable homozygotes. This also leads to the possibility that the problems with oligodendrocyte maturation and QKI expression are due to lower amounts of QKI-5 (Kondo et al., 1999). The only difference between the QKI isoforms lies in their alternatively spliced C-terminus and 3'UTR, where the latter is identical in the QKI-6 and QKI-7 isoforms. Therefore it is possible that all transcripts are synthesized, and that *cis*-elements are responsible for the cell-specific isoform expression by post-transcriptional/translational events. Stabilizing myelin proteins produced during the process of maturation could prevent transcripts degradation and allow translation of the proteins in a temporal fashion. Just recently, it was confirmed that indeed all of the transcripts are present in the *quaking* viable mouse but in lower amounts. This was specific to myelinating cells, as transcripts are still at normal levels in astrocytes and other tissues such as the heart and testis (Lu et al., 2003). This strengthens the hypothesis that it is a cell specific enhancer element that is deleted in the *quaking* viable mouse. Another possibility is that QKI-6 and QKI-7 are simply not expressed as a result of the disruption of oligodendrocyte maturation, caused by the misexpression of the MBP proteins or other possible myelin components still not identified. This model is not favored, since lower expression of QKI-6 and QKI-7 is not seen in other hypomyelinating mutants such as the *jimpy* mouse. But one interesting study supporting the latter model involves a mouse knock-out of the PNS myelin protein P0, where an absence of QKI-6 and QKI-7 is observed. Interestingly, P0 relatively replaces the role of MBP in the PNS, and the P0 knock-out causes dysmyelination due to problems in Schawnn cell differentiation (Xu et al., 2000). Therefore there could be a signal missing in both of these dysmyelinating mutants that are not affecting other mice mutants such as the *jimpy*

mouse. Answering these questions will lead to a better understanding of the QKI protein function.

4.3 QKI function in embryogenesis

QKI-5 is mostly expressed in embryogenesis, a time where myelination does not yet occur, and embryonic lethality from ENU-induced mutations in *quaking* further confirms its importance at this time. In the $qk^{l\text{-}1}$ mutant, the QKI-5 isoform is the only one eliminated, due to the creation of a differential splice site by the ENU-induced mutation (Cox et al., 1999). When crossed with the *quaking* viable mouse ($qk^{l\text{-}1}/qk^{v}$), the limiting amounts of the three QKI isoforms create a milder phenotype than the qk^{v} homozygote, which disappears with time. But when $qk^{l\text{-}1}$ is homozygous, the complete lack of QKI-5 causes development to stop and the embryo to die at around embryonic day 8-9 (Cox et al., 1999; Shedlovsky et al., 1988). Therefore QKI-5 must have a specific role at this point. Indeed it has been suggested that early expression of QKI is implicated in neuron-glial fates, where only cells expressing QKI differentiate into glial cells (Hardy, 1998). Another group has suggested that QKI is implicated in the development of blood vessels supplying brain and heart development at these times (Noveroske et al., 2002). QKI most likely interacts with RNA targets implicated in the differentiation of the glial cell into an oligodendrocyte. Observations from other QKI homologues such as *Xenopus* Xqua, and zebrafish *zqk* strengthen the possibility QUAKING's involvement in embryogenesis (Tanaka et al., 1997; Zorn et al., 1997; Zorn and Krieg, 1997). Xqua is actively involved in notochord development, and it has been suggested that Xqua is probably involved in the maintenance of the differentiation process rather than determination of notochord cell

fate, by the accumulation of several essential mRNAs (Zorn and Krieg, 1997). The fact that QKI is mostly expressed in cells of neural lineage highly suggests its involvement in the differentiation of glial cells (Hardy, 1998). QUAKING is also expressed in other tissues besides the brain such as the heart, the testis and has even been found in blood leukocytes, including the possibility of other functions besides myelination and differentiation of glial cells (Ebersole et al., 1996; Kondo et al., 1999; Li et al., 2002).

Although *Drosophila* does not create myelin in the nervous system, it does have a mechanism very similar to QKI in muscle differentiation. The QKI-5 nuclear homologue How(L) is expressed early in embryogenesis and the cytoplasmic How(S) is upregulated later. The balance between these two isoforms controls tendon cell differentiation (Nabel-Rosen et al., 1999). Like QKI-5, How(L) is responsible for trapping the Stripe mRNA in the nucleus. Once Vein is expressed, it causes activation of the EGF receptor pathway and the upregulation of the cytoplasmic How(S). How(S) subsequently competes for export of Stripe mRNA into the cytoplasm where it is translated permitting participation in the tendon cell terminal differentiation (Nabel-Rosen et al., 1999; Nabel-Rosen et al., 2002). This upregulation of Stripe increases How(L) in what seems to be an auto-regulatory loop, which suggests that mature MBP might also be regulating the expression of the QKI isoforms (Nabel-Rosen et al., 2002). Krox-2, a PNS homologue of egr2 expressed in Schawnn cells, can be regulated similarly by QUAKING (Nabel-Rosen et al., 2002). QKI-5 led to a reduction of krox-2, whereas QKI-6 and QKI-7 caused an increase of the protein (Nabel-Rosen et al., 2002).

4.4 QKI and RNA processing

QKI-5 presumably has other roles besides retaining RNA into the nucleus, and indeed evidence of splicing is not uncommon for KH-containing proteins, as seen with the GSG protein SF1, as well as Sam68, SLM-2, and KEP-1 (Di Fruscio et al., 2003; Kramer, 1992; Matter et al., 2002; Stoss et al., 2001). It was demonstrated recently in mice spinal cord that QKI-5 might have a role in splicing of another myelin component, MAG. QKI-5 was involved in repressing the inclusion of exon 12 (Wu et al., 2002). Splicing of exon 3b in PLP and exon 6 in MBP have also been reported to be altered due to QKI-5 expression earlier in myelinogenesis, and exon 5 in MBP at later stages (Wu et al., 2002). The fact that QKI-5 is no longer expressed at later stages contradicts later splicing of exon 5 in MBP, and it is interesting to note that QKI-5 can easily be replaced by the PTB splicing protein (Wu et al., 2002). Whether or not QKI-5 acts directly in this event is still unknown, as no direct RNA interaction with these targets has been identified. Additional studies will determine whether QKI-5 is indeed a player in splicing. Other possible roles for the QKI-5 include stabilization of nuclear transcripts, maturation events of the transcripts, and nuclear export. Although we have not detected a link between MBP mRNA decay and QKI in our studies, others have reported these observations. The How protein was shown to prevent decay of the Stripe mRNA, and an accumulation of MBP mRNA was observed with QKI association (Nabel-Rosen et al., 2002; Zhang et al., 2003). QKI-6 and QKI-7 are most likely players in cytoplasmic post-transcriptional/translational events. As mentioned earlier, it is postulated that the QKI isoforms are involved in mRNA transport of the MBP to the cell periphery, and that they may be involved in translational repression. QKI proteins have also been shown to

associate with polyribosomes, confirming a role in translation regulation (Li et al., 2000). Example of a similar scenario involved the FMRP protein, shown to shuttle mRNAs in a translationally inactive form from the nucleus to the postsynaptic sites until synaptic input alters FMRP activity to allow mRNA translation (Darnell et al., 2001). Studies of GLD-1 with the *rme-2* mRNA have shown that binding occurs at the 5' and 3' ends of the mRNA, possibly interfering with the initiation of translation by forming a closed loop between the 5' and 3' ends of the mRNA (Lee and Schedl, 2001).

4.5 QKI and signaling

The QUAKING proteins are part of the family of GSG proteins, also known as STAR for signal transduction and activator of RNA (Hardy et al., 1996). This was inspired by the RNA-binding Sam68, thought to have an effect on signal transduction as it is phosphorylated by Src during mitosis (Taylor and Shalloway, 1994). QUAKING has potential phosphorylation sites in its tyrosine rich C-terminal, and phosphorylation of QKI might lead to changes in RNA binding, like Sam68 (Wang et al., 1995). In fact, a recent study confirmed phosphorylation of QUAKING downstream of Src family protein kinases during CNS phosphorylation, and this event did negatively regulate QKI binding to MBP mRNA (Zhang et al., 2003). Phosphorylation of all of the major QKI isoforms occurred through the tyrosine rich C-terminal, and tyrosine phosphorylation of QKI declined during active myelination, increasing its association with MBP mRNA. Although other Src kinases are present in the myelin producing cells, *fyn* is a likely candidate due to previous reports implicating this kinase in active myelination. *Fyn* is upregulated during oligodendrocyte differentiation, and a mouse knock-out of the kinase causes myelination defects (Umemori et al., 1994). It has been documented that MAG-L

associates with *fyn* during the initial phases of myelination, causing a rapid increase in the specific activity (Osterhout et al., 1999; Umemori et al., 1994). It has also been shown that *fyn* stimulates the transcription of the MBP gene (Umemori et al., 1994; Jaramillo et al., 1994). Since QKI has been implicated in the splicing of MAG and that *fyn* activation leads to oligodendrocyte differentiation, it highly suggests a link between transduction and RNA metabolism. Given that the myelin protein MAG-L is reduced in the *quaking* viable mouse, there is always the possibility that some of the dysmyelination defect is coupled to a decreased *fyn* activation, in turn lowering MBP mRNA levels (Osterhout et al., 1999; Umemori et al., 1999; Umemori et al., 1994; Zhang et al., 2003). It will be interesting to further study the relationship between QKI and *fyn* phosphorylation during myelinogenesis.

4.6 General model of the QKI isoforms

MBP localization in oligodendrocytes is thought to occur in multiple steps: first, there is assembly of the mRNP or granules as a co-transcriptional event with further assembly in the perikaryon, which are then transported out of the cell body along microtubules, where it reaches its destination at the distal end of the oligodendrocyte (Barbarese, 1991; Campagnoni et al., 1990). This is where the MBP synthesis takes place on free ribosomes to subsequently incorporate into the myelin membrane (Colman et al., 1982). Some of the proteins identified in these RNP granules include arginyl-tRNA synthetase, elongation factor 1α, ribosomal RNA, and hnRNP A2 (Ainger et al., 1997; Barbarese et al., 1995). Our simplified model during myelination is that QKI-5 retains the MBP mRNA into the nucleus, until expression of the QKI cytoplasmic components at the

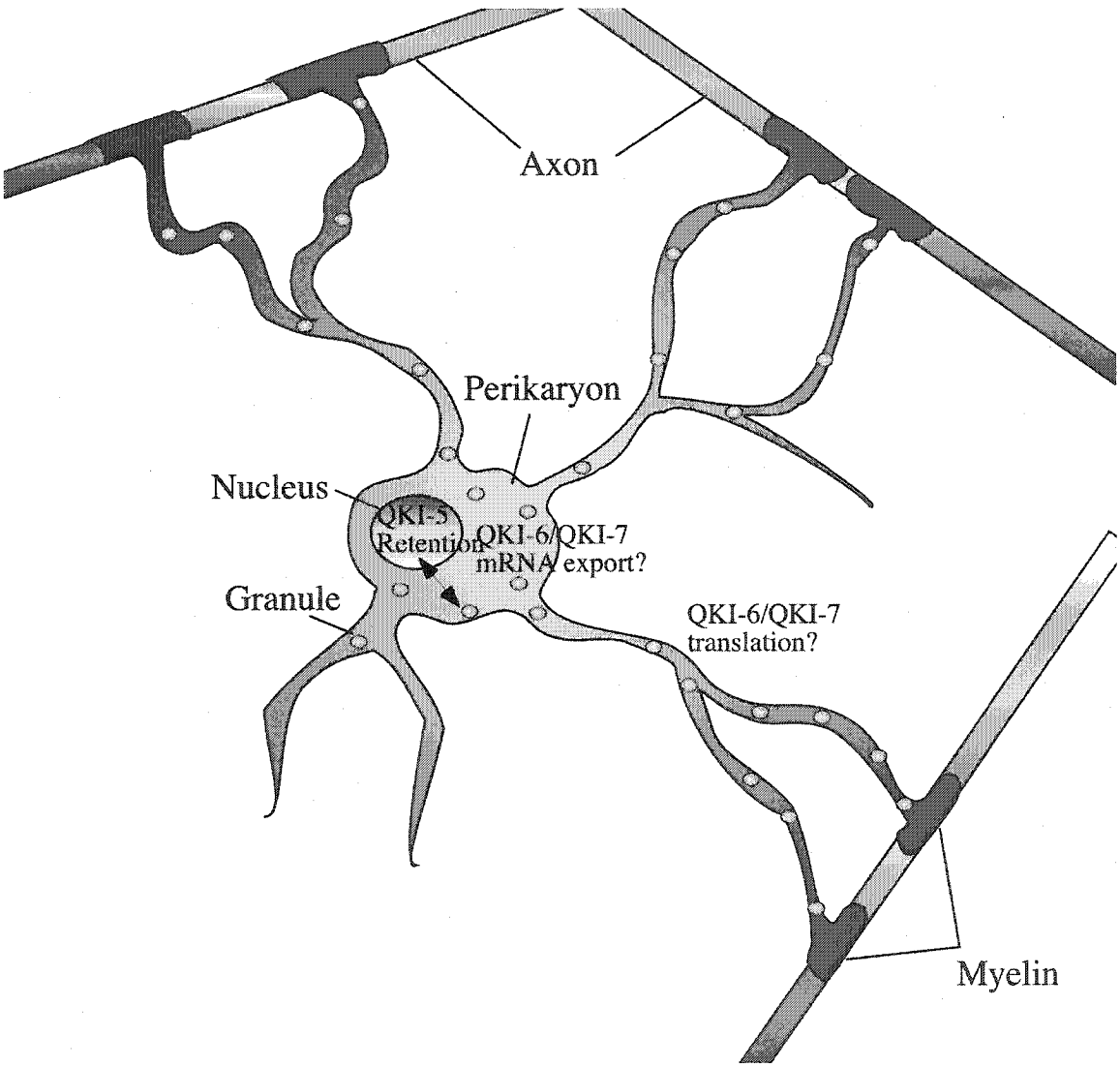

Figure 4-1. Model of the possible roles of QKI in oligodendrocytes.

See section 4.6 for a detailed description.

beginning of myelination (see Figure 4-1). The QKI-6 and QKI-7 cytoplasmic isoforms would regulate the export of the MBP mRNA out of the nucleus, where it presumably associates with other mRNP factors responsible for MBP mRNA transport. The RNP complex could then travel down microtubules to the periphery of oligodendrocytes. Due to the adhesive properties of the MBP protein, it is thought that there is a need for translational repression during this transport, where QKI-6 and QKI-7 could participate in this process. Once they arrive at their destination, the QUAKING proteins are probably displaced from the polyribosome, where the MBP messenger is subsequently translated. QKI proteins associate with RNP granules within the oligodendrocyte extensions along with the MBP mRNA, and the QKI proteins seem to be absent in the myelin periphery (Hardy et al., 1996; Wu et al., 2001). Strengthening the concept that cytoplasmic QKI isoforms are responsible for mRNA transport from the cell body of the oligodendrocyte to the myelin periphery is the fact that an intact cytoskeleton seems to be important for QKI-6 and QKI-7 localization (Wu et al., 2001). Disruption of microtubules with nocadozole resulted in mislocalization of the QKI proteins, and disruption of actin filaments also interferes with QKI-6 localization.

We presume that other components of myelin are targets of QUAKING as well, and that this transport mechanism might be a more general effect. Golli-MBP as well as MOBP resemble MBP and could be other mRNA targets. Unfortunately, the QRE sequence only shows similarity between MBPs of different species by BLAST searches. The secondary structure is probably more important in defining the area necessary for binding to the KH domain of QUAKING. We have identified a 10 nucleotides stretch predicted to form a stable loop that is probably responsible for binding. Recently, more

RNA targets have been identified in GLD-1 and FMR, where comparisons might lead to the identification of other possible QUAKING targets (Darnell et al., 2001; Lee and Schedl, 2001; Brown et al., 2001).

4.7 Concluding remarks

QUAKING belongs to the very exciting field of RNA and diseases. One of the most inherited disease of mental retardation is the Fragile-X syndrome, where one of the most severe cases documented have shown a mutation in the second KH domain of the FMR1 protein (DeBoulle et al., 1993). Then there are demyelinating diseases, such as Multiple Sclerosis and Perlizaeus-Merzbacher, which remain hopeful in finding treatments or cures by remyelination. *Quaking* has never been characterized in human diseases thus far, although mutations in the *quaking* gene would probably result in embryonic death as seen from *quaking* ENU-induced mutations (Justice and Bode, 1988). We have presented in this thesis a better understanding of QKI function in oligodendrocytes. The balance between the different alternatively-spliced QKI isoforms influenced apoptosis as well as the MBP mRNA export, strengthening the importance of the different QKI isoforms. Since oligodendrocyte differentiation and myelination are spatial and temporal events, understanding exactly when and where the QKI proteins are expressed during this crucial time, as well as their function with RNA, will help in understanding not only the function of QKI but of myelination in general.

References (Chapters 1 & 4)

Adams, J. M., and Cory, S. (1998). The Bcl-2 protein family: arbiters of cell survival. Science *281*, 1322-1326.

Ainger, K., Avossa, D., Diana, A. S., Barry, C., Barbarese, E., and Carson, J. H. (1997). Transport and localization elements in myelin basic protein mRNA. J. Cell. Biol. *138*, 1077-87.

Ainger, K., Avossa, D., Morgan, F., Hill, S. J., Barry, C., Barbarese, E., and Carson, J. H. (1993). Transport and localization of exogenous myelin basic protein mRNA microinjected into oligodendrocytes. J. Cell. Biol. *123*, 431-441.

Allinquant, B., Staugaitis, S. M., D'Urso, D., and Colman, D. R. (1991). The ectopic expression of MBP isoforms in shiverer oligodendrocytes: implications for myelinogenesis. J. Cell Biol. *113*, 393-403.

Alnemri, E. S., Fernandes-Alnemri, T., and Litwack, G. (1995). Cloning and expression of four novel isoforms of human interleukin-1β converting enzyme with different apoptotic activities. J. Biol. Chem. *270*, 4312-4317.

Arning, S., Gruter, P., Bilbe, G., and Kramer, A. (1996). Mammalian splicing factor SF1 is encoded by variant cDNAs and binds to RNA. RNA *2*, 794-810.

Arquint, M., Roder, J., Chia, L. S., Down, J., Wilkinson, D., Bayley, H., Braun, P., and Dunn, R. (1987). Molecular cloning and primary structure of myelin-associated glycoprotein. Proc. Natl. Acad. Sci. USA *84*, 600-604.

Ashkenazi, A., and Dixit, V. M. (1998). Death receptors: signaling and modulation. Science *281*, 1305-1308.

Baehrecke, E. H. (1997). Who encodes a KH RNA binding protein that functions in muscle development. Development *124*, 1323-1332.

Barbarese, E. (1991). Spatial distribution of myelin basic protein mRNA and polypeptide in Quaking oligodendrocytes in culture. J. Neurosci. Res. *29*, 271-281.

Barbarese, E., Brumwell, C., Kwon, S., Cui, H., and Carson, J. H. (2000). RNA on the road to myelin. J. Neurocyto. *28*, 263-270.

Barbarese, E., Koppel, D. E., Deutscher, M. P., Smith, C. L., Ainger, K., Morgan, F., and Carson, J. H. (1995). Protein translation components are colocalized in granules in oligodendrocytes. J. Cell Sci. *108*, 2781-2790.

Barres, B. A., Hart, I. K., Coles, H. S. R., Burne, J. F., Voyvodic, J. T., Richardson, W. D., and Raff, M. C. (1992). Cell death and control of cell survival in the oligodendrocyte lineage. Cell *70*, 31–46.

Barres, B. A., and Raff, M. C. (1994). Control of oligodendrocyte number in the developing rat optic nerve. Neuron *12*, 935–942.

Barres, B. A., and Raff, M. C. (1993). Proliferation of oligodendrocytes precursor cells depends on electrical activity in axons. Nature *361*, 258–260.

Barry, M., Heibein, J. A., Pinkoski, M. J., Lee, S. F., Moyer, R. W., Green, D. R., and Bleackley, R. C. (2000). Granzyme B short-circuits the need for caspase-8 activity during granule-mediated cytotoxic T-lymphocyte killing by directly cleaving Bid. Mol. Cell. Biol. *20*, 3781-3794.

Bartoszewicz, Z. P., Noronha, A. B., Fujita, N., Sato, S., Bo, L., Trapp, B. D., and Quarles, R. H. (1995). Abnormal expression and glycosylation of the large and small isoforms of myelin-associated glycoprotein in dysmyelinating quaking mutants. J. Neurochem. Res. *41*, 27-38.

Bashirullah, A., Halsell, S. R., Cooperstock, R. L., Kloc, M., Karatraniskakis, A., Fisher, W. W., Fu, W., Hamilton, J. K., Etkin, L. D., and Lipshitz, H. D. (1999). Joint action of two RNA degradation pathways controls the timing of maternal transcript elimination at the midblastula transition in Drosophila melanogaster. EMBO J. *18*, 2610–2620.

Baumann, N., and Pham-Dinh, D. (2001). Biology of oligodendrocyte and myelin in the mammalian central nervous system. Phys. Rev. *81*, 871-925.

Baumann, N. A., Jacque, C.M., Pollet, S.A., and Harpin, M.L. (1968). Fatty acid and lipid composition of the brain of a myelin deficient mutant, the "quaking" mouse. Eur. J. Biochem. *4*, 340-344.

Beelman, C. A., and Parker, R. (1995). Degradation of mRNA in eukaryotes. Cell *81*, 179-183.

Bennett, W. I., Gall, A.M., Southard, J.L., and Sidman, R.L. (1971). Abnormal spermiogenesis in quaking, a myelin-deficient mutant mouse. Biol. Reprod. *5*, 30-58.

Bentley, D. (2002). The mRNA assembly line: transcription and processing machines in the same factory. Curr. Cell Biol. *14*, 336-342.

Berger, T., Walz, W., Schnitzer, J., and Kettenmann, H. (1992). GABA- and glutamate-activated currents in glial cells of the mouse corpus callosum slice. J. Neurosci. Res. *31*: 21–27.

Berglund, J. A., Chua, K., Abovich, N., Reed, R., and Rosbash, M. (1997). The splicing factor BBP interacts specifically with the pre-mRNA branch-point sequence UACUAAC. Cell *89*, 781-787.

Bernstein, P., Peltz, S. W., and Ross, J. (1989). The poly(A)-poly(A)-binding protein complex is a major determinant of mRNA stability in vitro. Mol. Cell. Biol. *9*, 659-70.

Biddle, F., March, E., and Miller, J. R. (1973). Research news. Mouse News Lett *48*, 24.

Billings-Gagliardi, S., Adcock, L. H., and Wolf, M. K. (1980). Hypomyelinated mutant mice: description of jpmsd and comparison with jp and qk on their present genetic backgrounds. Brain Res. *194*, 325–338.

Black, D. L. (2003). Mechanisms of alternative pre-messenger RNA splicing. Annu. Rev. Biochem. *72*, 291-336.

Black, D. L., Chabot, B., and Steitz, J. A. (1985). U2 as well as U1 small nuclear ribonucleoproteins are involved in premessenger RNA splicing. Cell *42*, 737-50.

Bo, L., Quarles, R. H., Fujita, N., Bartoszewiez, Z., Sato, S., and Trapp, B. D. (1995). Endocytic depletion of L-MAG from CNS myelin in quaking mice. J. Cell Biol. *131*, 1811-1820.

Bode, V. C. (1984). Ethylsnitrosourea mutagenesis and the isolation of mutant alleles for specific genes located in the t region of the mouse chromosome 17. Genetics *108*, 457-470.

Boise, L. H., Gonzales-Garcia, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L. A., Mao, X., Nunez, G., and Thompson, C. B. (1993). Bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell *74*, 597-608.

Bongarzone, E. R., Howard, S. G., Schonmann, V., and Campagnoni, A.T. (1998). Identification of the dopamine D3 receptor in oligodendrocyte precursors: potential role in regulating differentiation and myelin formation. J. Neurosci. *18*: 5344–5353.

Bousquet-Antonelli, C., Presutti, C., and Tollervey, D. (2000). Identification of a regulated pathway for nuclear pre-mRNA turnover. Cell *102*, 765–775.

Bronstein, J. M., Micevych, P. E., and Chen, K. (1997). Oligodendrocyte-specific protein (OSP) is a major component of CNS myelin. J. Neurosci. Res. *50*, 713–720.

Brostoff, S. W., Greenfield, S., and Hogan, E.L. (1977). The differentiation of synthesis from incorporation of basic protein in Quaking mutant mouse myelin. Brain Res. *120*, 517-520.

Brown, V., Jin, P., Ceman, S., Darnell, J. C., O'Donnell, W. T., Tenenbaum, S. A., Jin, X., Feng, Y., Wilkinson, K. D., Keene, J. D., Darnell, R. B., and Warren, S. T. (2001). Microarray identification of FMRP-associated brain mRNAs and altered mRNA translational profiles in fragile X syndrome. Cell *107*, 477-487.

Buendia, B., Santa-Maria, A., and Courvalin, J. C. (1999). Caspase-dependent proteolysis of integral and peripheral proteins of nuclear membranes and nuclear pore complex proteins during apoptosis. J. Cell Sci. *112*, 1743-1753.

Cain, K. (2000). Apaf-1 oligomerizes into biologically active approximately 700 kDa and inactive approximately 1.4 MDa apoptosome complexes. J. Biol. Chem. *275*, 6067-6070.

Campagnoni, A. T., and Macklin, W. B. (1988). Cellular and molecular aspects of myelin protein gene expression. Mol. Neurobiol. *2*, 41–89.

Campagnoni, A. T., Pribyl, T. M., Campagnoni, C. W., Kampf, K., Amur-Umar-Jee, S., Landry, C. F., Handley, V. W., Newman, S. L., Garbay, B., and Kitamura, K. (1993). Structure and developmental regulation of the Golli-mbp, a 105-kilobase gene that encompasses the myelin basic protein gene and is expressed in cells in the oligodendrocyte lineage in the brain. J. Biol. Chem. *265*, 4930–4938.

Campagnoni, A. T., Sorg, B., Roth, H. J., Kronquist, K., Newman, S. L., Kitamura, K., Campagnoni, C., and Crandall, B. (1987). Expression of myelin protein genes in the developing brain. J. Physiol. (Paris) *82*, 229-238.

Campagnoni, A. T., Verdi, J. M., Verity, A. N., and Amur-Umarjee, S. (1990). Posttranscriptional events in the expression of myelin protein genes. Ann. NY Acad. Sci. *605*, 270-279.

Campagnoni, A. T., Verdi, J. M., Verity, A. N., Amur-Umarjee, S., and Byravan, S. (1991). Posttranscriptional regulation of myelin protein gene expression. Ann. NY Acad. Sci. *633*, 178-188.

Canoll, P. D., Musacchio, J. M., Hardy, R., Reynolds, R., Marchionni, M. A., and Salzer, J. L. (1996). GGF neuregulin is a neuronal signal that promotes the proliferation and survival and inhibits the differentiation of oligodendrocyte progenitors. Neuron *17*: 229–243.

Capello, E., Voskuhl, R. R., McFarland, H. F., and Raine, C. S. (1997). Multiple sclerosis: re-expression of a developmental gene in chronic lesions correlates with remyelination. Ann. Neurol. *41*, 797–805.

Carnow, T. B., Carson, J. H., Brostoff, S. W., and Hogan, E. L. (1984). Myelin basic protein gene expression in quaking, jimpy and myelin synthesis-deficient mice. Dev. Biol. *106*, 38-44.

Carson, J. H., Cui, H., and Barbarese, E. (2001). The balance of power in RNA trafficking. Curr. Neurobiol. *11*, 558-563.

Chartrand, P., Meng, X. H., Singer, R. H., and Long, R. M. (1999). Structural elements required for the localization of ASH1 mRNA and of a green fluorescent protein reporter particle in vivo. Curr. Biol. *9*, 333–336.

Chen, P., Nordstrom, W., Gish, B., and Abrams, J. M. (1996). Grim, a novel cell death gene in Drosophila. Genes Dev. *10*, 1773-1782.

Chen, T., Boisvert, F.-M., Bazett-Jones, D. P., and Richard, S. (1999). A role for the GSG domain in localizing Sam68 to novel nuclear structures in cancer cell lines. Mol. Cell. Biol. *10*, 3015-3033.

Chen, T., Côté, J., Carvajal, H. V., and Richard, S. (2001). Identification of Sam68 arginine glycine-rich sequences capable of conferring non-specific RNA binding to the GSG domain. J. Biol. Chem. *276*, 30803-11.

Chen, T., Damaj, B., Herrerra, C., Lasko, P., and Richard, S. (1997). Self-association of the single-KH domain family members Sam68, GRP33, GLD-1 and Qk1: Role of the KH domain. Mol. Cell. Biol. *17*, 5707-5718.

Chen, T., and Richard, S. (1998). Structure-function analysis of Qk1: A lethal point mutation in mouse quaking prevents homodimerization. Mol. Cell. Biol. *18*, 4863-4871.

Chinnaiyan, A. M., O'Rourke, K., Tewari, M., and Dixit, V. M. (1995). FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell *81*, 505-512.

Cho, K. W. Y., Blumberg, B., Steinbeisser, H., and DeRobertis, E. M. (1991). Molecular nature of Spemann's organizer: The role of the Xenopus homoebox gene goosecoid. Cell *67*, 1111-1120.

Colman, D. R., Kreibich, G., Frey, A. B., and Sabatini, D. R. (1982). Synthesis and incorporation of myelin polypeptides into CNS myelin. J. Cell. Biol. *95*, 598-608.

Conradt, B., and Horvitz, H. R. (1998). The *C. elegans* protein EGL-1 is required for programmed cell death and interacts with the Bcl-2-like protein CED-9. Cell *93*, 519-529.

Couttet, P., Fromont-Racine, D., Steel, R., Pictet, R., and Grange, T. (1997). Messenger RNA deadenylation precedes decapping in mammalian cells. Proc. Natl. Acad. Sci. USA *94*, 5628-5633.

Cox, R. D., Hugill, A., Shedlovsky, A., Noveroske, J. K., Best, S., Justice, M. J., Lehrach, H., and Dove, W. F. (1999). Contrasting effects of ENU induced embryonic lethal mutations of the quaking gene. Genomics *57*, 333-341.

Cox, R. D., Shedlovsky, A., Hamvas, R., Goldsworthy, M., Whittington, J., Connelly, C. S., Dove, W. F., and Lehrach, H. (1994). A 1.2-Mb YAC contig spans the quaking region. Genomics *21*, 77-84.

Coyle, J. H., Guzik, B. W., Bor, Y. C., Jin, L., Eisner-Smerage, L., Taylor, S. J., Rekosh, D., and Hammarskjold, M. L. (2003). Sam68 enhances the cytoplasmic utilization of the intron-containing RNA and is functionally regulated by the nuclear kinase Sik/BRK. Mol. Cell. Biol. *23*, 92-103.

Craig, A. W., Haghighat, A., Yu, A. T., and Sonenberg, N. (1998). Interaction of polyadenylate-binding protein with the eIF4G homologue PAIP enhances translation. Nature *392*, 520-523.

Cruz-Alvarez, M., and Pellicer, A. (1987). Cloning of a full-length complementary cDNA for as *Artemia salina* glycine-rich protein. J. Biol. Chem. *262*, 13377-13380.

D'eustachio, P., Colman, D. R., and Salzer, J. L. (1988). Chromosomal location of the mouse gene that encodes the myelin-associated glycoproteins. J. Neurochem. *50*, 589–593.

Dahanukar, A., Walker, J. A., and Wharton, R. P. (1999). Smaug, a novel RNA-binding protein that operates a translational switch in Drosophila. Mol. Cell *4*, 209–218.

Darnell, J. C., Jensen, K. B., Jin, P., Brown, V., Warren, S. T., and Darnell, R. B. (2001). Fragile X mental retardation protein targets G quartet mRNAs important for neuronal function. Cell *107*, 489-499.

de Ferra, F., Engh, H., Hudson, L., Kamholz, J., Puckett, C., Molineaux, S., and Lazzarini, R. A. (1985). Alternative splicing accounts for the four forms of myelin basic protein. Cell *43*, 721-727.

DeBoulle, K., Verkerk, A. J. M. H., Reyniers, E., Vits, L., Hendrickx, J., Roy, B. V., Bos, F. V. D., DeGraaff, E., Oostra, B. A., and Willems, P. J. (1993). A point mutation in the FMR-1 gene associated with fragile X mental retardation. Nature Genetics *3*, 31-35.

Delassalle, A., Zalc, B., Lachapelle, F., Raoul, M., Collier, P., and Jacque, C. (1981). Regional distribution of myelin basic protein in the CNS of quaking, jimpy and normal mice during development and aging. J. Neurosci. Res. *6*, 303-313.

Deo, R. C., Bonanno, J. B., Sonenberg, N., and Burley, S. K. (1999). Recognition of polyadenylate RNA by the poly(A)-binding protein. Cell *98*, 835-845.

Derry, J. J., Richard, S., Valderrama, H., Ye, X., Vasioukhin, V., Cochrane, A., Chen, T., and Tyner, A. (2000). Sik (BRK) phosphorylates Sam68 in the nucleus and negatively regulates its RNA binding activity. Mol. Cell. Biol. *20*, 6114-6126.

Deveraux, Q. L., and Reed, J. C. (1999). IAP family proteins-suppressors of apoptosis. Genes Dev. *13*, 239-252.

Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1997). X-linked IAP is a direct inhibitor of cell-death proteases. Nature *388*, 300-304.

Di Fruscio, M., Chen, T., Bonyadi, S., Lasko, P., and Richard, S. (1998). The identification of two Drosophila KH domain proteins: KEP1 and SAM are members of the Sam68 family of GSG domain proteins. J. Biol. Chem. *273*, 30122-30130.

Di Fruscio, M., Chen, T., and Richard, S. (1999). Characterization of Sam68-like mammalian proteins SLM-1 and SLM-2: SLM-1 is a Src substrate during mitosis. Proc. Natl. Acad. Sci. USA *96*, 2710-2715.

Di Fruscio, M., Styhler, S., Wikholm, E., Boulanger, M. C., Lasko, P., and Richard, S. (2003). *kep1* interacts genetically with *dredd*/Caspase-8, and *kep1* mutants alter the balance of *dredd* isoforms. Proc. Natl. Aca. Sci. USA *100*, 1814-1819.

Ding, Y., and Brunden, K. R. (1994). The cytoplasmic domain of myelin glycoprotein P0 interacts with negatively charged phospholipid bilayers. J. Biol. Chem. *269*, 10764–10770.

Doetsch, F., Garcia-Verdugo, J. M., and Alvarez-Buylla, A. (1997). Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. J. Neurosci. *17*, 5046–5061.

Doolittle, D. P., and Schweikart, K. M. (1977). Myelin deficient, a new neurological mutant in the mouse. J. Hered. *68*, 331–332.

Dreyfuss, G., Kim, V. N., and Kataoka, N. (2002). Messenger-RNA-binding proteins and the messages they carry. Nature Reviews: Mol. Cell. Biol. *3*, 195-205.

Du, C., Fang, M., Li, Y., Li, L., and Wang, X. (2000). Smac, a mitochondrial protein that promotes cytochrome C-dependent caspase activation by eliminating IAP function. Cell *10*, 33-42.

Duchen, L. W., Eicher, E. M., Jacobs, J. M., Scaravilli, F., and Teixera, F. (1980). Hereditary leucodystrophy in the mouse: The new mutant twitcher. Brain *103*, 695.

Duncan, I. D. (1996). Glial cell transplantation and remyelination of the central nervous system. Neuropathol. Appl. Neurobiol. *22*, 87–100.

Duncan, I. D., Hammang, J. P., Goda, S., and Quarles, R. H. (1989). Myelination in the jimpy mouse in the absence of proteolipid protein. Glia *2*, 148 –154.

Duncan, I. D., Hammang, J. P., and Trapp, B. D. (1987). Abnormal compact myelin in the myelin-deficient rat: absence of proteolipid protein correlates with a defect in the intra-period line. Proc. Natl. Acad. Sci. USA *84*, 6287–6291.

Dupouey, P., Jacque, C., Bourre, J. M., Cesselin, F., and Privat, A. (1979). Immunochemical studies of myelin basic protein in shiverer mouse devoid of major dense line of myelin. Neurosci. Lett. *12*, 113–118.

Ebersole, T. A., Chen, Q., Justice, M. J., and Artzt, K. (1996). The *quaking* gene unites signal transduction and RNA binding in the developing nervous system. Nature Genetics *12*, 260-265.

Fagg, G.E., Waehneldt, T.V., and Neuhoff, V. (1979). Myelin basic proteins in myelin subfractions from normal and quaking mice. Biochem. J. *183*, 159-162.

Falconer, D. S. (1951). Two new mutants, "trembler" and "reeler", with neurological actions in the house mouse. J. Genet. *50*, 192.

Fan, X. C., and Steitz, J. A. (1998). Overexpression of HuR, a nuclear-cytoplasmic shuttling, increases the in vivo stability of ARE-containing mRNAs. EMBO J. *17*, 3448-3460.

Frail, D. E., and Braun, P. E. (1985). Abnormal expression of the myelin-associated glycoprotein in the central nervous system of dysmyelinating mutant mice. J. Neurochem. *45*, 1071-1075.

Francis, R., Barton, M. K., Kimbel, J., and Schedl, T. (1995). Control of oogenesis, germline proliferation and sex determination by the *C.elegans* gene *gld-1*. Genetics *139*, 579-606.

Francis, R., Maine, E., and Schedl, T. (1995). Gld-1 a cell-type specific tumor suppressor gene in *C.elegans*. Genetics *139*, 607-630.

Friedrich, V. L. J. (1975). Hyperplasia of oligodendrocytes in quaking mice. Anat. Embryol. (Berl) *147*, 259-271.

Friedrich, V. L. J. (1974). The myelin deficit in quaking mice. Brain Res. *20*, 168-172.

Fujita, N., Sato, S., Kurihara, T., Inuzuka, T., Takahashi, Y., and Miyatake, T. (1988). Developmentally regulated alternative splicing of brain myelin-associated glycoprotein mRNA is lacking in the quaking mouse. FEBS Lett. *232*, 323-327.

Fujita, N., Sato, S., Ishiguro, H., Inuzuka, T., Baba, H., Kurihara, T., Takahashi, Y., and Miyatake, T. (1990). The large isoform of the myelin-associated glycoprotein is scarcely expressed in the quaking mouse brain. J. Neurochem. *55*, 1056-1059.

Fumagalli, S., Totty, N. F., Hsuan, J. J., and Courtneidge, S. A. (1994). A target for Src in mitosis. Nature *368*, 871-874.

Gao, M., Wilusz, C. J., Peltz, S. W., and Wilusz, J. (2001). A novel mRNA-decapping activity in HeLa cytoplasmic extracts is regulated by AU-rich elements. EMBO J. *20*, 1134-1143.

Gibson, T. J., Thompson, J. D., and Heringa, J. (1993). The KH domain occurs in a diverse set of RNA-binding proteins that include the antiterminator NusA and is probably involved in binding to nucleic acid. FEBS Lett. *324*, 361-366.

Gont, L. K., Fainsod, A., Kim, S. H., and De Robertis, E. M. (1996). Overexpression of the Homeobox gene Xnot-2 leads to notochord formation in Xenopus. Dev. Biol. *174*, 174-178.

Gorlich, D., Kraft, R., Kostka, S., Vogel, F., Hartmann, E., Laskey, R. A., Mattaj, I. W., and Izaurralde, E. (1996). Importin provides a link between nuclear protein import and U snRNA export. Cell *87*, 21-32.

Gozani, O., Boyce, M., Yoo, L. I., Karuman, P., and Yuan, P. (2002). Life and death in paradise. Nature Cell Biol. *4*, 159-162.

Green, D. R., and Reed, J. C. (1998). Mitochondria and apoptosis. Science *281*, 1309-11.

Greenfield, S., Brostoff, S., and Hogan, E.L. (1977). Evidence for defective incorporation of proteins in myelin of the quaking mutant mouse. Brain Res. *120*, 507-515.

Griffiths, I. R., Duncan, I. D., and McCulloch, M. (1981). Shaking pups: a disorder of central myelination in the spaniel dog. II. Ultrastructural observations on the white matter of the cervical spinal cord. J. Neurocytol. *10*, 847–858.

Gross, A., McDonnell, J. M., and Korsmeyer, S. J. (1999). BCL-2 family members and the mitochondria in apoptosis. Genes Dev. *13*, 1899-1911.

Guenet, J. L. (1980). Neurological mutations affecting myelination, N. Baumann, ed. (Seillac, France: Elsevier/North-Holland).

Habib, A. A., Marton, I. S., Allwart, B., Gulcher, J. R., Mikol, D. D., Hognason, T., Chattopadhyay, N., and Stefansson, K. (1998). Expression of the oligodendrocyte-myelin glycoprotein by neurons in the mouse central nervous system. J. Neurochem. *70*, 1704–1711.

Han, J. R., Yiu, G. K., and Hecht, N. B. (1995). Testis/Brain RNA-binding protein attaches translationally repressed and transported mRNAs to microtubules. Proc. Natl. Acad. Sci. USA *92*, 9550-4.

Hardy, R., and Reynolds, R. (1993). Neuron-oligodendroglial interactions during central nervous system development. J. Neurosci. Res. *36*, 121–126.

Hardy, R. J. (1998). QK1 expression is regulated during neuron-glial cell fate decisions. J. Neurosci. Res. *54*, 46-57.

Hardy, R. J., Loushin, C. L., Friedrich Jr., V. L., Chen, Q., Ebersole, T. A., Lazzarini, R. A., and Artzt, K. (1996). Neural cell type-specific expression of QKI proteins is altered in the *quaking* viable mutant mice. J. Neurosci. *16*, 7941-7949.

Hengartner, M., Ellis, R. E., and Horvitz, H. R. (1992). C. elegans gene ced-9 protects cells from programmed cell death. Nature *356*, 494-499.

Hengartner, M. O. (2000). The biochemistry of apoptosis. Nature *407*, 770-776.

Hentze, M. W., and Kuhn, L. C. (1996). Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress. Proc. Natl. Acad. Sci. USA *93*, 8175-8182.

Hirose, Y., and Manley, J. L. (2000). RNA polymerase II and the integration of nuclear events. Genes Dev. *14*, 1415-1429.

Hoek, K. S., Kidd, G. J., Carson, J. H., and Smith, R. (1998). hnRNP A2 selectively binds the cytoplasmic transport sequence of myelin basic protein mRNA. Biochemistry *37*, 7021-7029.

Hogan, E. L., and Joseph, K.C. (1970). Composition of cerebral lipids in murine leucodystrophy: the quaking mutant. J. Neurochem. *17*, 1209-1214.

Hogan, E. L., and Greenfield, S. (1984). Animal models of genetic disorders of myelin. In Myelin, P. Morell, ed. (New York: Plenum), pp. 489-534.

Hogan, E. L., and Greenfield, S. (1984). Diffraction studies of molecular organization and membrane interactions in myelin. In Myelin, P. Morell, ed. (New York: Plenum), pp. 51-96.

Hogan, E. L., and Greenfield, S. (1984). Morphology of myelin and myelination. In Myelin, P. Morell, ed. (New York: Plenum), pp. 1-50.

Hogan, E. L., and Greenfield, S. (1984). The neuropathology of myelin diseases. In Myelin, P. Morell, ed. (New York: Plenum), pp. 259-336.

Hogan, E. L., and Greenfield, S. (1984). Physiological basis of conduction in myelinated nerve fibers. In Myelin, P. Morell, ed. (New York: Plenum), pp. 117-130.

Hoshino, S., Imai, M., Kobayashi, T., Uchida, N., and Katada, T. (1999). The eukaryotic polypeptide chain releasing factor (eRF3/GSPT) carrying the translation termination signal to the 3'-poly(A) tail of mRNA. Direct association of erf3/GSPT with polyadenylate-binding protein. J. Biol. Chem. *274*, 16677-16680.

Huang, D. C., and Strasser, A. (2000). BH3-Only proteins-essential initiators of apoptotic cell death. Cell *103*, 839-842.

Huang, Y., and Carmichael, G. C. (1996). Role of polyadenylation in nucleocytoplasmic transport of mRNA. Mol. Cell. Biol. *16*, 1534-1542.

Jacobs Anderson, J. S., and Parker, R. (1998). The 3' to 5' degradation of yeast mRNAs is a general mechanism for mRNA turnover that requires the SKI2 DEVH box protein and 3' to 5' exonucleases of the exosome complex. EMBO J. *17*, 1497–1506.

Jacque, C.M., Jorgensen, O.S. and Bock, E. (1974). Quantitative studies on the brain specific antigens S-100, GFA, 14-3-2, D1, D2, D3 and C1 in Quaking mouse. FEBS Lett. *49*, 264-266.

Jan, E., Motzny, C. K., Graves, L. E., and Goodwin, E. B. (1999). The STAR protein, GLD-1, is a translational regulator of sexual identity in Caenorhabditis elegans. EMBO J. *18*, 258-269.

Jan, Y. N. (2000). Staufen: a common component of mRNA transport in oocytes and neurons? Trends Cell Biol. *10*, 220–224.

Jaramillo, M. L., Afar, D. E., Almazan, G., and Bell, J. C. (1994). Identification of tyrosine 620 as the major phosphorylation site of myelin-associated glycoprotein and its implication in interacting with signaling molecules. J. Biol. Chem. *269*: 27240-27245.

Jensen, T. H., Dower, K., Domenico, L., and Rosbash, M. (2003). Early formation of mRNP: License for export of quality control? Mol. Cell *11*, 1129-1138.

Jones, A. R., and Schedl, T. (1995). Mutations in GLD-1, a female germ cell-specific tumor suppressor gene in *C.elegans*, affect a conserved domain also found in Sam68. Genes Dev. *9*, 1491-1504.

Jurica, M. S., and Moore, M. J. (2003). Pre-mRNA splicing: awash in a sea of proteins. Mol. Cell *12*, 5-14.

Justice, M. J., and Bode, V. C. (1988). Three ENU-induced alleles of the murine quaking locus are recessive embryonic lethal mutations. Genet. Res. Camb. *51*, 95-102.

Kaufmann, S. H., and Hengartner, M. O. (2001). Programmed cell death: alive and well in the new millenium. Trends Cell Biol. *11*, 526-534.

Kempf, E., Greilsamer, J., Mack, G., and Mandel, P (1973). Turnover of brain adrenergic transmitters in Quaking mice. J Neurochem *20*, 1269-1273.

Kerr, J.F., Wyllie, A.H., and Currie, A.R. (1972). Apoptosis: a biological phenomenon with wide-ranging applications in tissue kinetics. Br. J. Cancer *26*, 239-257.

Khaleghpour, K., Svitkin, Y. V., Craig, A. W., DeMaria, C. T., Deo, R. C., Burley, S. K., and Sonenberg, N. (2001). Translational repression by a novel partner of human poly(A)-binding protein, Paip2. Mol. Cell *7*, 205-216.

Kiebler, M. A., and DesGroseillers, L. (2000). Molecular insights into mRNA transport and local translation in the mammalian nervous system. Neuron *25*, 19-28.

Kim-Ha, J., Kerr, K., and Macdonald, P. M. (1995). Translational regulation of oskar mRNA by Bruno, an ovarian RNA-binding protein, is essential. Cell *81*, 403-412.

Kloc, M., Zearfoss, N. R., and Etkin, L. D. (2002). Mechanisms of Subcellular mRNA Localization. Cell *108*, 533–544.

Klugmann, M., Schwab, M. H., Pulhofer, A., Schneider, A., Zimmermann, F., Griffiths, I. R., and Nave, K. A. (1997). Assembly of CNS myelin in the absence of proteolipid protein. Neuron *18*, 59–70.

Knapp, S. E., Skoff, R. P., and Redstone, D. W. (1986). Oligodendroglial cell death in jimpy mice: an explanation for the myelin deficit. J. Neurosci. *6*, 2813–2822.

Ko, L. J., and Prives, C. (1996). p53: puzzle and paradigm. Genes Dev. *10*, 1054-1072.

Kondo, T., Furuta, T., Mitsunaga, K., Ebersole, T. A., Shichiri, M., Wu, J., Artzt, K., Yamamura, K., and Abe, K. (1999). Genomic organization and expression analysis of the mouse qkI locus. Mamm. Genome *10*, 662-669.

Kothakota, S., Azuma, T., Reinhard, C., Klippel, A., Tang, J., Chu, K., McGarry, T. J., Kirschner, M. W., Koths, K., Kwiatkowski, D. J., and Williams, L. T. (1997). Caspase-3 generated fragment of gelsolin: effector of morphological change in apoptosis. Science *278*, 294-298.

Kramer, A. (1992). Purification of splicing factor SF1, a heat-stable protein that functions in the assembly of a pre-splicing complex. Mol. Cell. Biol. *12*, 4545-4552.

Kubbutat, M. H. G., Ludwig, R. L., Ashcroft, M., and Vousden, K. H. (1998). Regulation of mdm2-directed degradation by the C terminus of p53. Mol. Cell. Biol. *18*, 5690-5698.

Kuwabara, P. E., and Kimble, J. (1992). Molecular genetics of sex determination in C. elegans. Trends Genet. *8*, 164-168.

Kwon, S., Barbarese, E., and Carson, J. H. (1999). The cis-acting RNA trafficking signal from myelin basic protein mRNA and its cognate trans-acting ligand hnRNP A2 enhance cap-dependent translation. J. Cell. Biol. *147*, 247-256.

Lai, C., Watson, J. B., Bloom, F. E., Sutcliffe, J. G., and Milner, R. J. (1987). Neural protein 1B236/myelin-associated glycoprotein (MAG) defines a subgroup of the immunoglobulin superfamily. Immunol. Rev. *100*, 129-151.

Lamond, A. I., and Earnshaw, W. C. (1998). Structure and function in the nucleus. Science *280*, 547-553.

Lang, V., Mege, D., Semichon, M., Gary-Gouy, H., and Bismuth, G. A. (1997). A dual participation of ZAP-70 and src protein tyrosine kinases is required for TCR-induced tyrosine phosphorylation of Sam68 in Jurkat T cells. Eur. J. Immunol. *27*, 3360-3367.

Lee, M.-H., and Schedl, T. (2001). Identification of in vivo mRNA targets of GLD-1, a maxi-KH motif containing protein required for C. elegans germ cell development. Genes Dev. *15*, 2408-20.

Lendahl, U., Zimmerman, L. B., and McKay, R. D. (1990). CNS stem cells express a new class of intermediate filament protein. Cell *60*, 585–595.

Li, H., Zhu, H., Xu, C. J., and Yuan, J. (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell *94*, 491-501.

Li, L. Y., Luo, X., and Wang, X. (2001). Endonuclease G is an apoptotic DNase when released form the mitochondria. Nature *412*, 95-9.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell *91*, 479-489.

Li, Z., Zhang, Y., Li, D. and Feng, Y. (2000). Destabilization and mislocalization of the myelin basic protein mRNAs in quaking dysmyelination lacking the Qk1 RNA-binding proteins. J. Neurosci. *20*, 4944-4953.

Li, Z. Z., Kondo, T., Murata, T., Ebersole, T. A., Nishi, T., Tada, K., Ushio, Y., Yamamura, K., and Abe, K. (2002). Expression of Hqk encoding a KH RNA binding protein is altered in human glioma. Jpn J. Cancer Res. *93*, 167-177.

Lin, Q., Taylor, S. J., and Shalloway, D. (1997). Specificity and determinants of Sam68 RNA binding. J. Biol. Chem. *272*, 27274-27280.

Livingstone, L. R., White, A., Sprouse, J., Livanos, E., Jacks, T., and Tisty, T. D. (1992). Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. Cell *70*, 923-935.

Lo, P. C. H., and Frasch, M. (1997). A novel KH-domain in protein mediates cell adhesion processes in *Drosophila*. Dev. Biol. *190*, 241-256.

Lock, P., Fumagalli, S., Polakis, P., McCormick, F., and Courtneidge, S. A. (1996). The human p62 cDNA encodes Sam68 and not the ras-GAP-associated p62 protein. Cell *84*, 23-24.

Lu, Z., Zhang, Y., Ku, L., Wang, H., Ahmadian, A., and Feng, Y. (2003). The quaking viable mutation affects qkI mRNA expression specifically in myelin-producing cells of the nervous system. Nucl. Acids Res. *31*, 4616-4624.

Luhrmann, R., Kastner, B., and Bach, M. (1990). Structure of spliceosomal snRNPs and their role in pre-mRNA splicing. Biochim. Biophys. Acta Gene Struct. Express. *1087*, 265-292.

Lumsden, A., and Krumlauf, R. (1996). Patterning the vertebrate neuroaxis. Science *274*, 1109-1115.

Macklin, W. B., Gardinier, M. V., Obeso, Z. O., King, K. D., and Wight, P. A. (1991). Mutations in the myelin proteolipid protein gene alter oligodendrocyte gene expression in jimpy and jimpy msd mice. J. Neurochem. *56*, 163-171.

Mader, S., Lee, H., Pause, A., and Sonenberg, N. (1995). The translation initiation factor eIF4E binds to a common motif shared by the translation factor eIF4G and the translational repressors 4E-binding proteins. Mol. Cell. Biol. *15*, 4990-4997.

Madhani, H. D., and Guthrie, C. (1992). A novel base-pairing interaction between U2 and U6 snRNAs suggests a mechanism for the catalytic activation of the spliceosome. Cell *71*, 803-817.

Mangus, D. A., Evans, M. C., and Jacobson, A. (2003). Poly(A)-binding proteins: multifunctional scaffolds for the post-transcriptional control of gene expression. Gen. Biol. *4*.

Martinez, J., Ren, A. C., Thuresson, U., Hellman, U., Astrom, J., and Virtanen, A. (2000). A 54-kDa fragment of the poly(A)-specific ribonuclease is an oligomeric, processive, and cap-interacting Poly(A)-specific 3' exonuclease. J. Biol. Chem. *275*, 24222-24230.

Martini, R. (1997). Animal models for inherited peripheral neuropathies. J. Anat. *191*: 321-336.

Martinon, F., Hofmann, K., and Tschopp, J. (2001). The pyrin domain: a possible member of the death domain-fold family implicated in apoptosis and inflammation. Curr. Biol. *11*, R118-R120.

Matter, N., Herrlich, P., and Konig, H. (2002). Signal-dependent regulation of splicing via phosphorylation of Sam68. Nature *420*, 691-695.

Mayford, M., Bach, M. E., Huang, Y. Y., Wang, L., Hawkins, R. D., and Kandel, E. R. (1996). Control of memory formation through regulated expression of a CaMKII transgene. Science *274*, 1678–1683.

McBride, A. E., Schlegel, A., and Kirkegaard, K. (1996). Human protein Sam68 relocalization and interaction with poliovirus RNA polymerase in infected cells. Proc. Natl. Acad. Sci. USA *93*, 2296-2301.

McBride, A. E., Taylor, S. J., Shalloway, D., and Kirkegaad, K. (1998). KH domain integrity is required for wild-type localization of Sam68. Exp. Cell Res. *241*, 84-95.

McMorris, A. and Dubois-Dalcq, M. (1988). Insulin-like growth factor 1 promotes cell proliferation and oligodendroglial commitment in rat glial progenitor cells developing in vitro. J. Neurosci. Res. *21*: 199-209.

McTigue, D. M., Horner, P. J., Stokes, B. T., and Gage, F.H. (1998). Neurotrophin-3 and brain-derived neurotrophic factor induce oligodendrocyte proliferation and myelination of regenerating axons in the contused adult rat spinal cord. J. Neurosci. *18*: 5354-5365.

Metztein, M. M. (1998). Genetics of programmed cell death in *C. elegans*: past, present, and future. Trends Genet. *14*, 410-416.

Mezquita, J., Pau, M., and Mezquita, C. (1998). Four isoforms of the signal-transduction and RNA-binding protein Qk1 expressed during chicken spermatogenesis. Mol. Reprod. Dev. *50*, 70-78.

Michael, W. M., Choi, M., and Dreyfuss, G. (1995). A nuclear export signal in hnRNP A1: a signal-mediated, temperature-dependent nuclear protein export pathway. Cell *83*, 415-422.

Micklem, D. R., Adams, J., Grunert, S., and St Johnston, D. (2000). Distinct roles of two conserved Staufen domains in oskar mRNA localization and translation. EMBO J. *19*, 1366–1377.

Mitchell, P., Petfalski, E., Shevchenko, A., Mann, M., and Tollervey, D. (1997). The exosome: a conserved eukaryotic RNA processing complex containing multiple 3'-5' exoribonucleases. Cell *91*, 457–466.

Mitclman, F., Mertens, F., and Johansson, B. (1997). A breakpoint map of recurrent chromosomal rearrangement in human neoplasia. Nat. Genet. 417-474.

Miyakawa, A., Ichimura, K., Schmidt, K., Vmeh-Ziaie, E. E., and Collins, V. P. (2000). Multiple deleted regions on the long arm of chromosome 6 in astrocytic tumors. Br. J. Cancer *82*, 543-549.

Moore, M. J., Query, C. C., and Sharp, P. A. (1993). Splicing of precursors to mRNAs by the spliceosome. In The RNA world., R. F. Gesteland and J. F. Atkins, eds. (Cold Spring Harbor: Cold Spring Harbor Laboratory Press), pp. 303-357.

Musco, G., Stier, G., Joseph, C., Morelli, M. A., and Pastore, A. (1996). Three-dimensional structure and stability of the KH domain: molecular insights into the fragile X syndrome. Cell *85*, 237-245.

Nabel-Rosen, H., Dorevitch, N., Reuveny, A., and Volk, T. (1999). The balance between two isoforms of the Drosophila RNA-binding protein How controls tendon cell differentiation. Mol. Cell *4*, 573-584.

Nabel-Rosen, H., Volohonsky, G., Reuveny, A., Zaidel-Bar, R., and Volk, T. (2002). Two isoforms of the Drosophila RNA binding protein, How, act in opposing directions to regulate tendon cell differentiation. Dev. Cell *2*, 183-193.

Nagata, S. (2000). Apoptotic DNA fragmentation. Exp. Cell Res. *256*, 12-18.

Nakano, K., and Woudsen, K. H. (2001). PUMA, a novel proapoptotic gene, is induced by p53. Mol. Cell *7*, 683-694.

Nave, K. A., Bloom, F. E., and Milner, R. J. (1987). A single nucleotide difference in the gene for myelin proteolipid protein defines the jimpy mutation in the mouse. J. Neurochem. *49*, 1873-1877.

Nave, K. A., Lai, C., Bloom, F. E., and Milner, R. J. (1986). Splice site selection in the proteolipid protein (PLP) gene transcript and primary structure of the DM-20 protein of the central nervous system myelin. Proc. Natl. Acad. Sci. USA *84*, 5665–5669.

Newman, A. J. (1997). The role of U5 snRNP in pre-mRNA splicing. EMBO J. *16*, 5797-5800.

Norvell, A., Kelley, R. L., Wehr, K., and Schupbach, T. (1999). Specific isoforms of squid, a Drosophila hnRNP, perform distinct roles in Gurken localization during oogenesis. Genes Dev. 864–876.

Noveroske, J. K., Lai, L., Gaussin, V., Northrop, J. L., Nakamura, H., Hirschi, K. K., and Justice, M. J. (2002). Quaking is essential for blood vessel development. Genesis *32*, 218-230.

Nussbaum, J. L., and Mandel, P. (1973). Brain proteolipids in neurological mutant mice. Brain Res. *26*, 295-310.

Nylandsted, J., Rohde, M., Brand, K., Bastholm, L., Elling, F., and Jaattela, M. (2000). Selective depletion of heat shock protein 70 (Hsp70) activates a tumor-specific death program that is independent of caspases and bypasses Bcl-2. Proc. Natl. Acad. Sci. USA *97*, 7871-7876.

Osterhout, D. J., Wolven, A., Wolf, R. M., Resh, M. D., and Chao, M. V. (1999). Morphological differentiation of oligodendrocytes requires activation of Fyn tyrosine kinase. J. Cell Biol. *145*, 1209-1218.

Otero, G. C. and J. E. Merrill (1997). Response of human oligodendrocytes to interleukin-2. Brain Behav. Immun. *11*: 24–38.

Pedraza, L., Fidler, L., Staugaitis, S. M., and Colman, D. R. (1997). The active transport of myelin basic protein into the nucleus suggests a regulatory role in myelination. Neuron *18*, 579-89.

Peyron, F., Timsit, S., Thomas, J. L., Kagawa, T., Ikenaka, K., and Zalc, B. (1997). In situ expression of PLP/DM-20, MBP, and CNP during embryonic and postnatal development of the jimpy mutant and of transgenic mice overexpressing PLP. J. Neurosci. Res. *50*, 190–201.

Pfeiffer, S. E., Warrington, A. E. and Bansal, R. (1993). The oligodendrocyte and its many cellular processes. Trends Cell Biol. *3*: 191-197.

Pfrieger, F. W., and Barres, B. A. (1995). What the fly's glia tell the fly's brain. Cell *83*, 671–674.

Phillips, J. S. R. (1954). Jimpy: a new totally sex-linked gene in the house mouse. Arch. Indukt. Abstammungs. Verebungsl. *86*, 322.

Prives, C. (1998). Signaling to p53: Braking the MDM2-p53 circuit. Cell *95*, 5-8.

Proudfoot, N. J., Furger, A., and Dye, M. J. (2002). Integrating mRNA processing with transcription. Cell *108*, 501-512.

Quarles, R. H. (1997). Glycoprotein of myelin sheaths. J. Mol. Neurosci. *8*, 1–12.

Rain, J. C., Rafi, Z., Rhani, Z., Legrain, P., and Kramer, A. (1998). Conservation of functional domains involved in RNA binding and protein-protein interactions in human and Saccharomyces cerevisiae pre-mRNA splicing factor SF1. RNA *4*, 551-565.

Reddy, T. R., Suhasini, M., Xu, W., Yeh, L. Y., Yang, J. P., Wu, J., Artzt, K., and Wong-Staal, F. (2002). A role for KH domain proteins (Sam68-like mammalian proteins and quaking proteins) in the post-transcriptional regulation of HIV regulation. J. Biol. Chem. *277*, 5778-5784.

Reddy, T. R., Xu, W., Mau, J. K. L., Goodwin, C. D., Suhasini, M., Tang, H., Frimpong, K., Rose, D. W., and Wong-Staal, F. (1999). Inhibition of HIV replication by dominant negative mutants of Sam68, a functional homolog of HIV-1 Rev. Nature Med. *5*, 635-642.

Reed, J. C. (1998). Bcl-2 family proteins. Oncogene *17*, 3225-3236.

Richard, S., Yu, D., Blumer, K. J., Hausladen, D., Olszowy, M. W., Connelly, P. A., and Shaw, A. S. (1995). Association of p62, a multi-functional SH2- and SH3-binding protein, with src-family tyrosine kinases, Grb2, and phospholipase Cγ-1. Mol. Cell. Biol. *15*, 186-197.

Roach, A., Boylan, K., Horvath, S., Prusiner, S. B., and Hood, L. E. (1983). Characterization of cloned cDNA representing rat myelin basic protein: absence of expression in brain of shiverer mutant mice. Cell *34*, 799–806.

Roach, A., Takahashi, N., Pravtcheva, D., Ruddle, F., and Hood, L. E. (1985). Chromosomal mapping of mouse myelin basic protein gene and structure and transcription of the partially deleted gene in shiverer mutant mouse. Cell *42*, 149–155.

Roy, G., De Crescenzo, G., Khaleghpour, K., Kahvejian, A., O'Connor-McCourt, M., and Sonenberg, N. (2002). Paip1 interacts with poly(A)-binding protein through two independent binding motifs. Mol. Cell. Biol. *22*, 3769-3782.

Rozen, F., Edery, I., Meerovitch, K., Dever, T. E., Merrick, W. C., and Sonenberg, N. (1990). Bidirectional RNA helicase of eucaryotic translation initiation factors 4A and 4F. Mol. Cell. Biol. *10*, 1134-1144.

Rudel, T., and Bokoch, G. M. (1997). Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2. Science.

Saccomanno, L., Loushin, C., Jan, E., Punkay, E., Artzt, K., and Goodwin, E. B. (1999). The STAR protein Qk1-6 is a translational repressor. Proc. Natl. Acad. Sci. USA *96*, 12605-12610.

Sachs, A., and Wahle, E. (1993). Poly(A) tail metabolism and function in eucaryotes. J. Biol. Chem. *268*, 22955-22958.

Sachs, A. B., Sarnow, P., and Henzte, M. W. (1997). Starting at the beginning, middle and end: translation initiation in eukaryotes. Cell *89*, 831-838.

Sapirstein, V. S. (1982). Development of membrane-bound carbonic anhydrase and glial fibrillary acidic protein in normal and quaking mice. Brain Res. *282*, 13-19.

Scherer, S. S., Deschenes, S. M., Xu, Y. T., Grinspan, J. B., Fischbeck, K. H., and Paul, D. L. (1995). Connexin32 is a myelin-related protein in the PNS and CNS. J. Neurosci. *15*, 8281–8294.

Schoenherr, C. J., and Anderson, D. J. (1995). The neuron-restrictive silencer factor (NRSF): a coordinate repressor of multiple neuron-specific genes. Science *267*, 1360–1363.

Scolding, N. (1997). Strategies for repair and remyelination in demyelinating diseases. Curr. Neurol. *10*, 193–200.

Shaham, S., and Horvitz, H. R. (1996). An alternatively spliced C. elegans ced-4 RNA encodes a novel cell death inhibitor. Cell *86*, 201-208.

Shedlovsky, A., King, T. R., and Dove, W. F. (1988). Saturation germ line mutagenesis of the murine t region including a lethal allele of the quaking locus. Proc. Natl. Acad. Sci. USA *85*, 180-184.

Shimizu, S., Narita, M., and Tsujimoto, Y. (1999). Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature *399*, 483-487.

Sidman, R. L., Dickie, M. M., and Appel, S. H. (1964). Mutant mice (quaking and jimpy) with deficient myelination in the central nervous system. Science *144*, 309-311.

Siomi, H., Choi, M., Siomi, M. C., Nussbaum, R. L., and Dreyfuss, G. (1994). Essential role for KH domain in RNA binding: impaired RNA binding by a mutation in the KH domain of FMR1 that causes fragile X syndrome. Cell *77*, 33-39.

Siomi, H., Matunis, M. J., Michael, W. M., and Dreyfuss, G. (1993). The pre-mRNA binding K protein contains a novel evolutionarily conserved motif. Nucl. Acids. Res. *21*, 1193-1198.

Smith, J. C., Price, B. M. J., Grenn, J. B. A., Weigel, D., and Herrman, B. G. (1991). Expression of a Xenopus homologue of Brachyury (T) is an immediate-early response to mesoderm induction. Cell *67*, 79-87.

Solly, S. K., Thomas, J. L., Monge, M., Demerens, C., Lubetzki, C., Gardinier, M. V., Matthieu, J. M., and Zalc, B. (1996). Myelin/oligodendrocyte glycoprotein (MOG) expression is associated with myelin deposition. Glia *18*, 39–48.

Sorg, B. A., Smith, M. M., and Campagnoni, A. T. (1987). Developmental expression of the myelin proteolipid protein and basic protein mRNAs in normal and dysmyelinating mutant mice. J. Neurochem. *49*, 1146-1154.

Sorg, B. J., Agrawal, D., Agrawal, H. C., and Campagnoni, A. T. (1986). Expression of myelin proteolipid protein and basic protein in normal and dysmyelinating mutant mice. J. Neurochem. *46*, 379-387.

Sperber, B. R., Boyle-Walsh, E. A., Engleka, M. J., Gadue, P., Peterson, A. C., Stein, P. L., Scherer, S. S., and McMorris, F. A. (2001). A unique role for fyn in CNS myelination. J. Neurosci. *21*, 2039-2047.

Srinivasula, S. M., Datta, P., Kobayashi, M., Wu, J. W., Fujioka, M., Hegde, R., Zhang, Z., Mukattash, R., Fernandes-Alnemri, T., Shi, Y., Jaynes, J. B., and Alnemri, E. S. (2002). *Sickle*, a novel *Drosophila* death gene in the *reaper/hid/grim* region, encodes an IAP-inhibitory protein. Curr. Biol. *12*, 125-130.

Sternberger, N. H., Itoyama, Y., Kies, M., and Webster, H. D. F. (1978). Myelin basic protein demonstrated immunocytochemically in oligodendroglia prior to myelin formation. Proc. Natl. Acad. Sci. USA *75*, 2521–2524.

Stoss, O., Olbrich, M., Hartmann, A. M., Konig, H., Memmott, J., Andreadis, A., and Stamm, S. (2001). The STAR/GSG Family Protein rSLM-2 Regulates the Selection of Alternative Splice Sites. J. Biol. Chem. *276*, 8665 - 8673.

Sturrock, R. R. (1987). Age-related changes in the number of myelinated axons and glial cells in the anterior and posterior limbs of the mouse anterior commissure. J. Anat. *150*, 111-127.

Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D. R., Aebersold, R., Siderovski, D. P., Penninger, J. M., and Kroemer, G. (1999). Molecular characterization of mitochondrial apoptosis-inducing factor. Nature *397*, 441-446.

Suzuki, K., and Zagoren, J. C. (1975). Focal axonal swelling in cerebellum of quaking mouse: light and electron microscopic studies. Brain Res. *85*, 38-43.

Tanabe, Y., and Jessell, T. M. (1996). Diversity and pattern in the developing spinal cord. Science *274*, 1115-1123.

Tanaka, H., Abe, K., and Kim, C. H. (1997). Cloning and expression of the quaking gene in the Zebrafish embryo. Mech. Develop. *69*, 209-213.

Tartaglia, L. A., Ayres, T. M., Wong, G. H., and Goeddel, D. V. (1993). A novel domain within the 55 kd TNF receptor signals cell death. Cell *74*, 845-53.

Tartaglia, L. A., and Goeddel, D. V. (1992). Two TNF receptors. Immunol. Today *13*, 151-153.

Taylor, S. J., Anafi, M., Pawson, T., and Shalloway, D. (1995). Functional interaction between c-src and its mitotic target, Sam68. J. Biol. Chem. *270*, 10120-10124.

Taylor, S. J., and Shalloway, D. (1994). An RNA-binding protein associated with src through its SH2 and SH3 domains in mitosis. Nature *368*, 867-871.

Thiede, B., Dimmler, C., Siejak, F., and Rudel, T. (2001). Predominant identification of RNA-binding proteins in Fas-induced apoptosis by proteome analysis. J. Biol. Chem. *276*, 26044-26050.

Thornberry, N. A., and Lazebnik, Y. (1998). Caspases: enemies within. Science *281*, 1312-16.

Tian, Q., Streuli, M., Saito, H., Schlossman, S. F., and Anderson, P. (1991). A polyadenylate binding protein localized to the granules of cytolytic lymphocytes induces DNA fragmentation in target cells. Cell *67*, 629-639.

Toda, T., Iida, A., Miwa, T., Nakamura, Y., and Imai, T. (1994). Isolation and characterization of a novel gene encoding nuclear protein at a locus (D11S636) tightly linked to multiple endocrine neoplasia type 1 (MEN1). Hum. Mol. Genet. *3*, 465-470.

Tourrière, H., Chebli, K., and Tazi, J. (2002). mRNA degradation machines in eukaryotic cells. Biochimie *84*, 821-837.

Trapp, B. D., Nishiyama, A., Cheng, D., and Macklin, W. (1997). Differentiation and death of premyelinating oligodendrocytes in developing rodent brain. J. Cell Biol. *137*, 459–468.

Trapp, B. D., Peterson, J., Ransohoff, R. M., Rudick, R., Mork, S., and Bo, L. (1998). Axonal transection in the lesions of multiple sclerosis. N. Engl. J. Med. *338*, 278–285.

Trent, J. M., Stanbridge, E. J., McBride, H. L., Meese, B. U., Casey, G., Araujo, D. E., Witkown, C. M., and Nagle, R. B. (1990). Tumorigenicity in human melanoma cell lines controlled by introduction of human chromosome 6. Science *247*, 568-571.

Tropak, M. B., Johnson, P. W., Dunn, R. J., and Roder, J. C. (1988). Differential splicing of MAG transcripts during CNS and PNS development. Brain Res. *464*, 143-155.

Tsujimoto, Y., Cossman, J., Jaffe, E., and Croce, C. M. (1985). Involvement of the bcl-2 gene in human follicular lymphoma. Science *228*, 1440-1443.

Uchida, N., Hoshino, S., Imataka, H., Sonenberg, N., and Katada, T. (2002). A novel role of the mammalian GSPT/eRF3 associating with poly(A)-binding protein in Cap/Poly(A)-dependent translation. J. Biol. Chem. *277*, 50286-50292.

Umemori, H., Kadowaki, Y., Hirosawa, K., Yoshida, Y., Hironaka, K., Okano, H., and Yamamoto, T. (1999). Stimulation of myelin basic protein gene transcription by Fyn tyrosine kinase for myelination. J. Neurosci. *19*, 1393-1397.

Umemori, H., Sato, S., Yagi, T., Alzawa, S., and Yamamoto, T. (1994). Initial events of myelination involve Fyn tyrosine kinase signaling. Nature *367*, 572-576.

Verhagen, A. M., Ekert, P. G., Pakusch, M., Silke, J., Connolly, L. M., Reid, G. E., Moritz, R. L., Simpson, R. J., and Vaux, D. L. (2000). Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell *102*, 43-53.

Verity, A. N., and Campagnoni, A. T. (1988). Regional expression of myelin protein genes in the developing mouse brain: In situ hybridization studies. J. Neurosci. Res. *21*, 238-248.

Vernet, C., and Artzt, K. (1997). STAR, a gene family involved in signal transduction and activation of RNA. Trends Genet. *13*, 479-484.

Vogel, L. B., and Fujita, D. J. (1995). p70 phosphorylation and binding to p56lck is an early event in interleukin-2 induced onset of cell cycle progression in T-lymphocytes. J. Biol. Chem. *270*, 2506-2511.

Vogel, U. S., and Thompson, R. J. (1988). Molecular structure, localization and possible functions of the myelin-associated enzyme 2'3'-cyclic nucleotide-3'-phosphodiesterase. J. Neurochem. *50*, 1667–1677.

Vouyiouklis, D. A., and Brophy, P. J. (1993). Microtubules-associated protein MAP1B expression precedes the morphological differentiation of oligodendrocytes. J. Neurosci. Res. *35*, 257-27.

Vucic, D., Kaiser, W. J., and Miller, L. K. (1998). Inhibitor of apoptosis proteins physically interact with and block apoptosis induced by Drosophila proteins HID and GRIM. Mol. Cell. Biol. *18*, 330-3309.

Wan, L., Dockendorff, T. C., Jongens, T. A., and Dreyfuss, G. (2000). Characterization of dFMR1, a Drosophila melanogaster homolog of the fragile X mental retardation protein. Mol. Cell. Biol. *20*, 8536-8547.

Wang, L., Miura, M., Bergeron, L., Zhu, H., and Yuan, J. (1994). Ich-1, an Ice/ced-3-related gene encodes both positive and negative regulators of programmed cell death. Cell *78*, 739-750.

Wang, L. L., Richard, S., and Shaw, A. S. (1995). p62 association with RNA is regulated by tyrosine phosphorylation. J. Biol. Chem. *270*, 2010-2013.

Wang, S., Sdrulla, A. D., Disibio, G., Bush, G., Nofziger, D., Hicks, C., Weinmaster, G., and Barres, B. A. (1998). Notch receptor activation inhibits oligodendrocyte differentiation. Neuron *21*, 63–75.

Wang, S. L., Hawkins, C. J., Yoo, S. J., Muller, H.-A. J., and Hay, B. A. (1999). The *Drosophila* caspase inhibitor DIAP1 is essential for cell survival and is negatively regulated by HID. Cell *98*, 453-463.

Wang, Z., Day, N., Trifillis, P., and Kiledjian, M. (1999). An mRNA stability complex functions with poly(A)-binding protein to stabilize mRNA in vitro. Mol Cell Biol *19*, 4552-4560.

Weis, K. (2002). Nucleocytoplasmic transport: cargo trafficking across the border. Curr. Cell Biol. *14*, 328-335.

Wekerle, H. (1993). Experimental autoimmune encephalomyelitis as a model of immune-mediated CNS disease. Curr. Neurobiol. *3*, 779–784.

Wells, S. E., Hillner, P. E., Vale, R. D., and Sachs, A. B. (1998). Circularization of mRNA by eukaryotic translation initiation factors. Mol. Cell *2*, 135-140.

Wu, H. Y., Dawson, M. R. L., Reynolds, R. and Hardy, R. J. (2001). Expression of QKI proteins and MAP1B identifies actively myelinating oligodendrocytes in adult rat brain. Mol. Cell. Neurosci. *17*, 292-302.

Wu, J., Zhou, L., Tonissen, K., Tee, R., and Artzt, K. (1999). The Qk1-5 protein (QK1-5) has a novel nuclear localization signal and shuttles between the nucleus and the cytoplasm. J. Biol. Chem. *274*, 29202-29210.

Wu, J. I., Reed, R.B., Grabowski, P.J., Artzt, K. (2002). Function of quaking in myelination: regulation of alternative splicing. Proc. Natl. Acad. Sci. USA *99*, 4233-4238.

Wu, X.-Q., and Hecht, N. B. (2000). Mouse testis brain RNA binding protein/Translin colocalizes with microtubules and is immunoprecipitated with mRNAs encoding MBP, alpha CaMKII and protamines 1 and 2. Biol. Reprod. *62*, 720-725.

Xu, N., Chen, C. Y., and Shyu, A. B. (2001). Versatile role for hnRNP D isoforms in the differential regulation of cytoplasmic mRNA turnover. Mol. Cell. Biol. *21*, 6960-6971.

Xu, W., Manichella, D., Jiang, H., Vallat, J.-M., Lilien, J., Baron, P., Scarlato, G., Kamholz, J., and Shy, M. E. (2000). Absence of P0 leads to the dysregulation of myelin gene expression and myelin morphogenesis. J. Neurosci. Res. *60*, 714-724.

Yamamoto, Y., Mizuno, R., Nishimura, T., Ogawa, Y., Yoshikawa, H., Fujimura, H., Adachi, E., Kishimoto, T., Yanagihara, T., and Sakoda, S. (1994). Cloning and expression of myelin-associated oligodendrocytic basic protein. J. Biol. Chem. *269*, 31725–31730.

Yin, Y., Luciani, M. G., and Fahraeus, R. (2002). p53 stability and activity is regulated by Mdm2-mediated induction of alternative p53 translation products. Nature Cell Biol. *4*, 462-467.

Yuan, J., and Horvitz, H. R. (1992). The C. elegans cell death gene ced-4 encodes a novel protein and is expressed during the period of extensive programmed cell death. Development *116*, 309-320.

Yuan, J., Shaham, S., Ledoux, S., Ellis, H. M., and Horvitz, H. R. (1993). The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1beta-converting enzyme. Cell *75*, 641-652.

Yuan, X., Eisen, A. M. , McBain, C. J., and Gallo, V. A. (1998). Role for glutamate and its receptors in the regulation of oligodendrocyte development in cerebellar tissue slices. Development *125*: 2901–2914

Zaffran, S., Astier, M., Gratecos, D., and Semeriva, M. (1997). The held out wings (how) Drosophila gene encodes a putative RNA binding protein involved in the control of muscular and cardiac activity. Development *124*, 2087-2098.

Zhang, Y., and Feng, Y. (2001). Distinct molcular mechanism lead to diminished myelin basic protein and 2',3'-cyclic nucleotide 3'-phosphodiesterase in qk^{v} dysmyelination. J. Neurochem. *77*, 165-172.

Zhang, Y., Lu, Z., Ku, L., Chen, Y., Wang, H., and Feng, Y. (2003). Tyrosine phosphorylation of QKI mediates developmental signals to regulate mRNA metabolism. EMBO J. *22*, 1801-1810.

Zhu, J., and Chen, X. (2000). *MCG10*, a novel p53 target gene that encodes a KH domain RNA-binding protein, is capable of inducing apoptosis and cell cycle arrest in G_2-M. Mol. Cell. Biol. *20*, 5602-5618.

Zorn, A. M., Grow, M., Patterson, K. D., Ebersole, T. A., Chen, Q., Artzt, K., and Krieg, P. A. (1997). Remarkable sequence conservation of transcripts encoding amphibian and mammalian homologues of *quaking* a KH domain RNA-binding protein. Gene *188*, 199-206.

Zorn, A. M., and Krieg, P. A. (1997). The KH domain protein encoded by quaking functions as a dimer and is essential for notochord development in Xenopus embryos. Genes Dev. *11*, 2176-2190.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

The present study focused on the characterization of the QKI alternatively-spliced isoforms in programmed cell death, as well as its RNA-binding properties in myelination. The major discoveries by the author are summarized below.

1- The alternatively-spliced C-terminus of the QKI-7 isoforms, named the 'KILLER' sequence, confers the ability to induce apoptosis on its own.

2- The QKI isoforms heterodimerize and subsequently leads to changes in localization.

3- Nuclear translocation of the QKI-7 isoform inactivates its ability to induce apoptosis. The balance of the QKI isoforms leads to a life-or-death sensor in the cell.

4- QKI regulates the nuclear export of the MBP mRNA via its QRE element located in the 3'UTR.