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A Role for RNA Localization in the Human Neuromuscular Disease Myotonic Dystrophy

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A Role for RNA Localization in the Human Neuromuscular Disease Myotonic Dystrophy

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Dedication

To Kels and my family for their love and encouragement during this process. Especially to my parents and grandparents whose unwavering support of me and my love for science put me on this path. Thank you for always feeding my scientific curiosity.

A Role for RNA Localization in the Human Neuromuscular Disease Myotonic Dystrophy

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RNA localization, a regulated step of gene expression, is fundamentally important in development and differentiation. In multidisciplinary experiments, we discovered that RNA (mis)localization underlies the human disease myotonic dystrophy (DM). DM, the most prevalent adult muscular dystrophy, is caused independently by two alleles: DM1 is characterized by a (CTG)n expansion in the *DM kinase* (DMPK) gene 3' untranslated region while DM2 has a mutation in a small presumptive RNA binding protein. These analyses were guided by disease characteristics and have provided insights to DM's cytopathology, cell biology and molecular genetics.

Examining muscle biopsies, it is demonstrated here that *DM kinase* mRNA is specifically subcellularly localized within normal human muscle and that DM kinase mRNA harboring the 3'UTR mutation (DM1) is mislocalized in DM patient muscle to cytoplasmic areas characteristic of DM disease pathology. Thus, the disease mutation alters the cellular distribution of the effected message. *DMPK* mRNA mislocalization causes altered DM kinase protein localization, correlates with novel phosphoprotein appearance and can account for DM's diseased phenotype.

While we were fortunate to access DM patient tissue to establish these key findings, the system does not lend itself to experimental manipulation. Hence, I established a disease- relevant tissue culture system, which recapitulates DMPK trafficking, Employing this system; I elucidate a complementary role for the DM2 gene product as a localization factor for *DMPK* mRNA (DM1 gene product). Comprehensive RNA-protein interaction experiments reveal the DM2 protein specifically and selectively recognizes a small, definitive area within the *DMPK* RNA 3'UTR. Detailed biochemical, cytological and functional experiments reveal 1) the DM2 protein colocalizes with *DMPK* mRNA, 2) the small area of the *DMPK* 3'UTR bound by pDM2 acts to properly localize a reporter construct and 3) disruption of the DM2 protein results in *DMPK* mRNA mislocalization.

These data establish mRNA localization as a vital process underlying human disease etiology. Moreover, they reveal DM1 and DM2 gene products function in the same molecular pathway and that mutation of either causes *DMPK* mRNA mislocalization, leading to disease. These data have apparent application to several neuromuscular disorders and open a plethora of novel research avenues, both basic and applied.

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CHAPTER 1: INTRODUCTION

The “Central Dogma” states that the flow of genetic information in all cells examined follows a deoxyribonucleic acid (DNA) to ribonucleic acid (RNA) to protein route. DNA serves as the genetic blueprint, carrying specific sequences, genes, which contain the information necessary to produce corresponding proteins. RNA serves as an information conduit between DNA and protein, carrying the genetic information from the nucleus, where the DNA is located, to the cytoplasm, where ribosomes exist to translate the sequence into protein.

This pathway is highly regulated in all systems. Prokaryotic cells, the basal cells found in bacteria and archaebacteria, contain no nuclei and control protein production primarily at the DNA level by transcriptional regulation mechanisms. Eukaryotes, all higher organisms; separate their DNA in the cell nucleus from the translational machinery contained in the cytoplasmic compartment. They have several levels of regulation to ensure that proteins are not produced inappropriately. Like prokaryotes they use transcriptional control mechanisms, but they also employ extensive post-transcriptional and translational regulation. While all three levels of control are used, many hypothesize that RNA level, post-transcriptional regulatory mechanisms are the primary tools for gene regulation in higher eukaryotes. In fact, only 5% of RNA transcribed by RNA polymerase II (the polymerase that transcribes mRNA) in the nucleus is subsequently found in the cytoplasm, indicating extensive post-transcriptional regulation (Jackson 2000).

Post-transcriptional gene regulation

Post-transcriptional gene regulation begins in the nucleus with capping, polyadenylation, splicing and editing. Transcription of a gene by RNA polymerase II produces a nascent RNA which must be modified before it can be exported from the nucleus and translated into polypeptide. All messages require the addition of a 5' 7-methyl guanosine cap and most also require a 3' end poly-adenosine tail. A small, but vital, subset of transcripts is a substrate for RNA editing at this point in the post-transcriptional regulation pathway. Capped and tailed transcripts are capable of being exported and translated at this point; however, the vast majority of higher eukaryotic transcripts are further processed by pre-mRNA splicing before export. Once in the cytoplasm, messages may also be subjected to further post-transcriptional regulation by nonsense-mediated decay, stability and mRNA localization mechanisms. This occurs in a mix-and-match fashion with different messages being subjected to one or more of these processes. The importance of every post-transcriptional process is illustrated by the presence of human diseases that have been attributed to aberrations in these processes, with the one exception of mRNA localization. Each potential regulatory mechanism performs a crucial role in controlling protein amount, timing and location; however, only two of these steps are relevant to understanding the data presented in this dissertation. Therefore, only these two processes, pre-mRNA splicing and subcellular mRNA localization, will be discussed in further detail. Where relevant, I will highlight experimental insights gleaned from the molecular analyses of human diseases.

Post-Transcriptional Step	Disease Association	Gene/Protein Affected	Reference
Splicing			
	Acute intermittent porphyria	Porphobilinogen deaminase	Llewellyn 1996
	Spinal muscle atrophy	SMN1/2	Monani 2005
	Sandhof disease	Beta-hexaminidase beta-subunit	Fujimaru 1998
	Glanzmann thrombasthenia	Integrin GPIIIa	Jin 1996
	Hereditary tyrosinemia type 1	Fumarylacetoacetate hydrolase	Ploos van Amstel 1996
	Leigh's encephalomyelopathy	Pyruvate dehydrogenase E1 alpha	De Meirleir 1994
	Menkes disease	MNK	Das 1994
	Severe combined immunodeficiency disease	Adenosine deaminase	Santisteban 1995
	Metachromatic leukodystrophy	Arylsulfatase A	Hasegawa 1994
	Marfan syndrome	Fibrillin-1	Liu 1997
	Cerebrotenidinous xanthomatosis	CYP 27	Chen 1998
	Beta-Thalassemia	Beta-globin	Dobkin 1983a; Dobkin 1983b
	Breast and ovarian cancer	BRCA1	Liu 2001
	Neurofibromatosis type 1	NF-1	Ars, 2000a, 2000b;Hoffmeyer 1998; Messiaen 1997
	Opsoclonus-myoclonus ataxia	Nova RBP	Licatalosi 2006
	Prader-Willi syndrome	SNURF	Wang 2007
	Retinitis pigmentosa	PRPF31, PRPF8, HPRP3	Wang 2007
Editing	Rheumatoid Arthritis	B cell heavy and light chains	Meffre 2000
	Acute Myeloid Leukemia	PTPN6	Beghini 2000
	Amyotrophic Lateral Sclerosis	GluR2	Takuma 1999
	Alzheimer's Disease	GluR2	Akbarian 1995
	Schizophrenia	GluR2	Akbarian 1995
Polyadenylation			
	Oculopharyngeal muscular dystrophy	PABP2	Brais 1998
	Alpha-Thalassemia	Alpha-globin	Higgs 1983
	Beta-Thalassemia	Beta-globin	Orkin 1985
Nuclear Export			
	Hu syndrome	Hu RBP	Darnell 2003
	X-linked mental retardation	NXF5	Lin 2001
	Thromboembolic events	Prothrombin	Gehring 2001
Nonsense Mediated Decay			
	Syndromic mental retardation	UPF3B	Tarpey 1997
	Nonsyndromic mental retardation	UPF3B	Tarpey 1997
	Ataxia-telangiectasia	ATM	Couch 1996
	Breast and ovarian cancer	BRCA1	Gilad 1996
	Beta-Thalassemia	Beta-globin	Hall 1994
	Marfan syndrome	Fibrillin-1	Dietz 1993
Stability			
	Hu syndrome	Hu RBP	Darnell 2003
	Alzheimer's Disease	TfR	Guhaniyogi 2001
	Tumorigenesis	COX2	Dixon 2000
Localization	None		
	Modifed from Kiven et al 2008 and Stoilov et al 2002		

Table 1.1 Diseases associated with Aberrant Post-transcriptional Gene Regulation.

This represents a partial list of the human diseases with pathologies due to misregulation of particular steps of post-transcriptional processing. Depending on the mutations, some diseases are independently caused by abnormalities in more than one post-transcriptional step. Notice that all major post-transcriptional regulatory mechanisms have been shown to effect human disease except mRNA localization, whose role in humans is just beginning to be understood.

PRE-MRNA SPLICING

Overview

Precursor-mRNA (pre-mRNA) splicing is a key determinant of the final amino acid composition of encoded protein, second only to the primary gene sequence (reviewed in Sperling 2008, Moore 2008). In higher eukaryotes, on average, 80% of the nucleotides in the pre-mRNA are spliced out to make the mature message (Kramer 1996). Included in nascent transcripts are intervening sequences termed introns which must be removed for the flanking exons, which contain the protein coding template, to be spliced together. Pre-mRNA splicing is a highly complex and precise process which detects and excises these unwanted sequences from the mRNA to produce the mature message. Any imperfection in sequence removal could result in a non-functional protein due to frameshift in the decoded sequence and/or alterations in termination codons. Hence, dire consequences for the cell can ensue. After several decades of intensive research, the *cis*-acting signals, *trans*-acting factors and mechanism of pre-mRNA splicing are well defined and understood.

Cis-acting signals

Three specific elements within the pre-mRNA itself are required to demarcate the regions of sequence to be excised by the splicing machinery. The three canonical splice signals are the 5' splice site (AG**GU**RAGU), the 3' splice site (C**AG**GUAAGU) and the branch point A, an adenosine nucleotide that resides between 20 and 40 nucleotides upstream of the 3' splice site. In splicing reactions, the 5' most nucleotides of the intron must be GU and the 3' most nucleotides must be AG. This sequence specification is referred to as the GU-AG rule and is an absolute requirement for splicing to occur

(reviewed in Burge 1999). The splice site consensus sequences were discovered by sequence comparison of known spliced messages; however, natural mutations in these sites revealed the sensitivity of the reaction to their precise sequence.

β-thalasemia is a class of diseases in which the amount of functional β-globin protein is reduced compared to the level of the α-globin protein. To date, over thirty different mutations have been identified which result in β-thalasemia. β-globin gene cloning and primary sequence analysis of these mutations in a series of patients gave pioneer splicing researchers confirmation of the splice site sequence requirements because point mutations within the signals directed the production of a non-functional β-globin protein. Within this patient population, three naturally occurring point mutations of the *β- globin* 5' splice site were documented which changed the GU to AU or UU or altered the GU to CU (Orkin 1982, Treisman 1982, Old 1983, Kazazian 1984). Each single nucleotide change resulted in inactivation of that splice site and a corresponding inappropriately spliced *β- globin* mRNA. Similarly, a naturally occurring AG to GG mutation was discovered which renders the 3' splice site unusable and also results in an inappropriate splicing event (Antonarakis 1984). Therefore, the mutation of a single nucleotide of a splice site results in the loss of functional protein and elicits concomitant β-thalasemia in these patients. The extreme molecular consequence of a single nucleotide substitution reinforces the sequence requirements of the splicing signals and illustrates the level of precision that underlies the splicing events.

Interestingly, the β-thalasemia patients that lost a functional splice site still produced *β- globin* message with some splicing event of the mutated intron. Careful analyses of these messages revealed that nearby sequences that were similar to the mutated canonical splice sites were recruited as a back-up to perform splicing; these sites are called cryptic splice sites. Thus, when the preferred splice site was rendered non-

functional by mutation, the splicing machinery sought out secondary sites to initiate splicing (reviewed in Orkin 1984). Another set of β-thalasemia patients revealed the impact of mutations which enhance the usage of alternative splice sites (Spritz 1981, Westaway 1981). Patients were discovered with sequence changes near intron-exon junctions that shifted the preference of splice sites to naturally occurring cryptic sites. The cryptic sites were used exclusively, resulting in a decreased pool of correct beta-globulin transcript and disease (Busslinger 1981, Fukamaki 1982). Therefore, the *cis*-acting splicing sequences have a tremendous impact on the splicing event location; use of suboptimal sites greatly alters the message sequence composition, and potentially the functionality of the resulting protein (reviewed in Orkin 1984).

Another set of *cis*-acting signals have been identified called the enhancers and silencers. This class of signals is composed of approximately six nucleotide elements, located in close proximity to the splice sites (in either introns or exons) which function to either activate or repress splicing, respectively. Despite extensive study, no consensus sequences for these sites have been identified as of yet. However, their importance in splicing is well documented.

A different disease, Spinal Muscular Atrophy (SMA), illuminates these elements. This neurodegenerative disease is caused by a single point mutation on the 5'end of exon 7 of the *Smn2* gene (Lefebvre 1995). This mutation, which does not affect the amino acid coding of the cognate protein, causes exon 7 to be excised with the introns during pre-mRNA splicing, producing a *Smn2* message missing a portion of its coding sequence (Wirth 2006). It is not understood if the point mutation disrupts an exonic splicing enhancer or creates an exonic splicing silencer, but it is clear that this single nucleotide mutation dramatically changes the pre-mRNA fate. The protein made from this truncated

transcript is non-functional and unstable, thus causing disease due to the lack of the corresponding SMN protein (reviewed in Cooper 2009).

trans-acting Factors

The splicing process is carried out by the spliceosome, a very large (60S) macromolecular machine consisting of five small nuclear RNAs (U1, U2, U4-6) and as many as 300 proteins, the most recent of which were identified by gene mutations and/or classical biochemical purification (reviewed in Jurica 2003, Sperling 2008, Wahl 2009). The splicing machinery performs several functions during the splicing event: identification of the putative *cis*-acting splicing signals, the multistep enzymatic catalysis of the splicing reaction and proof reading the result. The composition of the spliceosome is extremely dynamic with a sequential and coordinated addition, rearrangement and exchange of factors throughout the splicing process to achieve its multi-functionality. In fact, from the initial assembly of the splicing machinery on each set of the splicing signals within the pre-mRNA to the completion of the splicing event, 6 distinct spliceosomal conformations, complexes E, A, B, B*, C and the post-spliceosomal complex, have been delineated (reviewed in Wahl 2009).

The core of the spliceosome revolves around the 5 evolutionarily conserved U snRNAs (U-rich small nuclear RNAs) and their seven to ten associated Sm proteins, forming the abundant U snRNPs (small nuclear ribonucleoprotein) particles. These snRNPs, which sediment at 7-12S on a sucrose gradient, serve to recognize key *cis*-acting signals and initiate spliceosomal assembly at the intron-exon junctions. Moreover, they are the scaffolding for the subsequent association of the other splicing components.

The initial identification of these snRNPs and an understanding of their role in splicing were facilitated by the discovery of Lerner and Steitz that Systemic Lupus Erythematosus (SLE) patients generate antibodies against the Sm proteins of the snRNPs (Lerner 1979). This autoimmune disease causes patients to naturally produce high titer antibodies against some combination of the snRNPs, therefore, serum from a variety of SLE patients created a library of antibodies against the snRNPs that enabled their physical characterization. Lerner et al discovered that the U1 snRNA has a region of complementarity to the 5' splice site and when the 5' end of U1 is removed, it can no longer bind RNA (Lerner 1980). The evidence for the role of the snRNPs in splicing followed shortly thereafter with the data that adenovirus RNA splicing was inhibited in nuclear extracts that were pre-treated with the anti-Sm antibodies and not in extracts that were pre-treated with antibodies to non-spliceosomal RNPs (Yang 1981) as well as binding experiments which revealed U1 binds the 5' splice site signal (Mount 1983). The SLE antibodies provided the tools necessary to identify the first known slicing *trans*-acting factor, U1 snRNP, and to conclude that the snRNPs are responsible for pre-mRNA splicing. It is now known that these snRNPs are the core of the spliceosomal machinery, but splicing is accomplished through the extensive coordination of hundreds of factors.

It is well documented that in certain situations the same pre-mRNA can be spliced slightly differently, thus producing a divergent protein product. This phenomenon, called alternative splicing, is due to the activity of situation-specific *trans*-acting factors that shift splice site usage to cause unique patterns of inclusion and/or exclusion of exons. Alternative splicing occurs in 60% of transcripts in humans (Modreck 2002) in a variety of circumstances; it can be tissue specific, cell cycle specific, specific to the differentiation state of the cell or a response to an external cell stimulus. This can be an extremely powerful process, producing multiple proteins from a single gene and

increasing the diversity of the protein pool in the cell. Indeed, it can generate a family of related proteins, each with distinct properties, from one gene. The most dramatic alternatively spliced transcript identified to date is *Drosophila Dscam*, which potentially produces 38,016 different mature transcripts (reviewed in Black 2003). Alternative splicing demonstrates the flexibility that has evolved within the splicing mechanism, in that minor *trans*-acting factor changes can produce entirely new spliced products and resulting proteins.

The understanding of splicing signals and factors were greatly aided by the discovery of human diseases that mutated or targeted components of this process, a few of which were discussed here. The complexity of splicing and its sensitivity to even slight changes in the signals and factors make splicing abnormalities a common effect identified in human disease. In fact, it has been estimated that as many as 60% of known human disease-causing mutations result in pre-mRNA splicing misregulation (Guigo 2005). Therefore, it is now common practice to specifically seek out potential splicing effects in many diseased situations, causing the number of splicing related diseases to continually increase.

MRNA LOCALIZATION

Overview

Messenger RNA trafficking is a key means of imparting specificity to cells and subcellular regions. This vital process encompasses the mRNA sorting, transport and subcellular localization functions in the establishment of axial polarity in development and determination of polarity within differentiated cells.

The RNA localization process is essential in a variety of diverse cell types and organisms (reviewed in Jansen 2001). mRNA localization is important in the normal function of nerves (reviewed in Martin 2000) and muscles (Reddy 2005) in humans and it is crucial in the development of several model systems, including *Drosphila*, *Xenopus* and yeast (reviewed in Bashirullah 1998). Localization is part of the biogenesis of only a subset of vital messages which encode a wide variety of essential molecules including transcription factors, growth factors and molecular receptors. All localized messages each play a pivotal role in imparting distinct properties to a cellular region.

Once properly sorted, transported and subcellularly positioned, the ultimate purpose of mRNA localization is to spatially and/or temporally regulate protein translation of these specific messages. Therefore, the message is held translationally silent until it reaches its final subcellular destination where, in the last step(s) of localization, other factors interact with it. This causes rearrangement of the localizing complex, release of translational repression and position-specific translation.

Protein localization in eukaryotic cells can be regulated by two means: post-translational protein sorting or localized mRNA translation. While protein sorting is more common, there are some distinct benefits to localizing the message. Localized translation can quickly and efficiently create a local protein pool from one localized message through multiple rounds of translation, rather than having to individually localize one protein at a time via protein sorting. Thus, it is a more effective method to rapidly produce large amounts of localized protein (reviewed in Martin 2009). Additionally, mRNA localization can have a stabilizing effect on the localized message by preventing the binding of destabilizing factors; therefore, the cell can have a pool of localized mRNA ready to translate at an appropriate time. One such example is the localized pool of *nanos* mRNA in the posterior pole of *Drosophila*. This pool of *nanos* message is more

than 100 times more stable then the non-localized pool of *nanos* transcript because the localization machinery prevents the binding of the Smaug protein to the *nanos* mRNA, a protein which triggers deadenylation and subsequent degradation of mRNA in *Drosophila* (Zaessinger 2006).

In *Drosophila* oocyte axis determination, mRNA localization of several crucial maternal transcripts establishes the polarity of the developing oocyte and embryo. The preeminent example of a localized mRNA is the Drosphila *bicoid* message (reviewed in Ephrussi 2004). *bicoid* is a primary anterior determinant in Drosphila embryos: b*icoid* mutants show complete loss of head and thorax (Frohnhofer 1986, 1987). The maternally encoded b*icoid* message is localized to the anterior of the oocyte and early embryo in a multistep post-transcriptional process. Once properly positioned, translation of the localized *bicoid* mRNA is initiated. This, combined with diffusion, produces a Bicoid protein morphogen gradient that extends from the anterior pole (where it is most concentrated) to diffuse within the anterior 30% of the embryo (Driever 1988 a,b). Resulting Bicoid protein functions as a transcription factor in a dose-dependent manner, regulating the anterior expression of several downstream, embryonic genes whose products are required for further polarized development, such as *hunchback* (Driever 1989, Struhl 1989) and *orthrodenticle* (Gao 1998). Bicoid also serves as the translational repressor of another localized message, *caudal* (Niessing 2002). Therefore, the localized message produces a localized protein that in turn serves to transcriptionally and translationally regulate other localized messages and loss of this function has a fatal outcome on the development of the fly embryo.

cis-acting Signals

The localization elements of a variety of localized mRNAs have been characterized; however, sequence analysis of these elements has not revealed a consensus localization sequence. In fact, this analysis only revealed how dissimilar the various localization signals seem to be. There are a few general commonalities to the signals identified thus far: 1) with few exceptions they reside in the 3' untranslated region (3'UTR) of the localized message and 2) they commonly have both a sequence and structural component required for localization (reviewed by Chabanon 2004, Jambhekhar 2007). They range is size and complexity from a simple 25 nucleotide sequence, *fatvg* mRNA (Chan 1999), to a 625 nucleotide signal with three separate hairpins in the *bicoid* mRNA (Macdonald 1990). This diversity has made bioinformatic prediction of localization elements extremely difficult and attempts have been unsuccessful, requiring identification of these signals to be experimentally determined (reviewed by Jambhekar 2007).

mRNA	Localisation (cell/organism)	*Cis*-acting signal	
		Length/ organisation	Sequence or structure
β-*actin*	Leading edge of the lamella (Chicken embryo fibroblasts)	54 nt zipcode	Tandem repeat ACACCC
Myelin basic protein, MBP	Localisation in myelin compartment (mouse oligodendrocytes)	Modular: A2RE and RLR (342 nt)	11 nt sequence = A2RE11 Secondary structure
Vimentin	Perinuclear cytoplasm (3T3 fibroblasts, CHO cells	100 nt	Secondary structure
Metallothionein-1	Perinuclear cytoplasm (CHO cells)	40 nt	Sequence repeat CACC + structure
c-myc	Perinuclear cytoplasm (fibroblasts)	86 nt	Structure + conserved AUUUA
Bicoid	Anterior (*Drosophila* oocyte/embryo)	625 nt — modular : 53 nt BLE1 and helical domains (stems III, IV, V)	Secondary and higher order structure
Vg1	Vegetal cortex (*Xenopus* oocyte)	340 nt	Clusters of YYUCU and CAC-containing motifs
VegT	Vegetal cortex (*Xenopus* oocyte)	525 nt	Clusters of YYUCU and CAC-containing motifs
Nanos	Posterior (germ plasm) (*Drosophila* oocyte/embryo)	547 nt	N/a
Oskar	Posterior (*Drosophila* oocyte/embryo)	Modular: Multiple elements of 100–200 nts	N/a
K10	Anterior (*Drosophila* oocyte/embryo)	44 nt region	Single stem–loop
Gurken	Dorsal–anterior (*Drosophila* oocyte/embryo)	Modular: Different elements in 5′UTR, Coding and 3′UTR	N/a
Hairy	Blastoderm apical cytoplasm (*Drosophila* oocyte/embryo)	125 nt region	SL1/SL2a. Secondary and higher order structure
Ash1	Daughter cell (budding yeast)	Several LE : E1, E2a, E2b, E3	Secondary structure

Table 1.2 Sequence and structural characteristics of known localization elements in several model systems.

The sequence and structural requirements for the localization of specific messages are listed. Notice that many messages have complex localization signals with multiple sequence and structural components. (modified from Chabanon 2004)

***trans*-acting Factors**

The process of mRNA subcellular localization occurs through a universal set of defined steps that are guided by *trans*-acting factors associated with the message. To initiate localization, the localization element must be recognized and bound by an RNA binding protein, commonly referred to as a zip-code binding protein (ZBP); however, only a small subset of ZBPs have been identified for known localized messages that originally appeared to be unique to each message. Emerging evidence suggests that related transcripts may co-assemble into localization particles and share localization factors (Lange 2008). ZBP binding recruits a variety of other *trans*-acting factors to the message, forming a localizing ribonucleoprotein (lRNP), which will transverse the cell through its association with the cytoskeleton until it reaches the final destination, as signaled by its interaction with protein 'anchors'. Some lRNPs are so large that they can be visually observed transporting though the cell. In fact, localizing *oskar* mRNA RNP granules have been discovered as large as 50-80S in *Drosophila* (Chekulaeva 2006).

The *trans*-acting factor composition of characterized lRNPs reveal little factor overlap between the different particles, suggesting that the entire localization complex is message specific rather than a generalized macromolecular machine like the spliceosome. However, a consensus in the functional requirements of components within the localizing RNP has emerged. These roles include: 1) translational repression of the localizing message 2) linkage of the lRNP to a motor and 3) a motor to transport the lRNP along the cytoskeleton. By definition, all localizing complexes must contain a translational repression mechanism to ensure only localization-dependent translation of the message. In many cases, such as ZBP1 localization of B–*actin* mRNA, the zip code binding protein also functions as a translational repressor (Huttelmaier 2005). In other examples a small non-coding RNA (Cheng 2006, Schratt 2006) or another RNA binding protein (Paquin

2007, Nakamura 2004) serves to translationally repress the localizing message (reviewed in Besse 2008).

Also found in the localizing RNP is a motor and an adaptor protein which connects the RNP to the motor. The double stranded RNA binding protein Staufen appears to function as a general adaptor protein in localizing complexes (reviewed in Chabanon 2004). This protein is known to be involved in the localization of three different *Drosophila* messages, *bicoid, oskar* and *prospero*, and has been isolated in several different localizing particles in mammalian neurons (reviewed in Roegiers 2000). The RNA binding of this protein is non-specific, indicating that the message specificity may be achieved through Staufen's association with other proteins in the lRNP (Ferrandon 1994). The commonality of Staufen in seemingly unrelated localization complexes, along with its lack of RNA binding specificity, suggests that it acts as a general adaptor, or even a scaffolding protein, in localizing RNPs. Growing evidence indicates that Staufen is associated with the kinesin motor in localization complexes in several systems, implying that it may specifically function to connect the motor to the localizing particle (Joeng 2007, Yoon 2004, Ohashi 2002, Brendza 2000). Staufen, and many other factors, are only associated with kinesin; however, kinesin is not the only motor known to be involved in mRNA localization. Dynein is the required motor for the localization of several messages in *Drosophila* embryos, particularly *gurken, bicoid*, *wingless* and *pair-rule* mRNAs. Also, the myosin motor is specifically required for the bud tip localization of *Ash1* mRNA in budding yeast (reviewed in Czaplinski 2006).

Once the localization complex has assembled and been actively transported within the cell to its ultimate destination, a signaling event occurs that tethers the RNP to the appropriate locale. This anchoring is known to occur, but few details are known about this step. In the bud tip localization of *Ash1* mRNA mentioned previously, mutant

analysis revealed that the presence of the She5 protein and actin polymerization is required to keep the mRNA localized once it reaches the bud tip, but the exact mechanism is not understood (Beach 2001). Data suggests that interaction with the anchoring machinery causes dramatic rearrangements within the lRNP that releases translational repression of the message, thus ensuring the translation only occurs in the proper location. In some transcripts, the anchoring signal is not enough to release the repression, so the message will be continually held as silent at the localized site until another signal is received. This allows the cell to have a localized message poised for immediate translation when the appropriate signal is received, allowing for the immediate production of localized protein. This has been shown extensively in neurons where specific messages are translated in mature dendrites only after synaptic activation (reviewed in Bramham 2007, Besse 2008).

Few mRNA localization pathways are well characterized to date, making definition of the signals and factors required for the process very difficult. The discovery of naturally occurring mutations in human disease greatly advanced scientific understanding of splicing (and several other post-transcriptional processes not discussed here). Unfortunately, to date, no human disease caused by aberrations in mRNA localization has been characterized to provide deeper insight into the requirements for this process.

Myotonic Dystrophy

CLINICAL PRESENTATION

Myotonic dystrophy (DM), also referred to as Steinert's disease, is a hereditary, autosomal dominant neuromuscular disorder. This chronic disease affects 1 in 8000 individuals worldwide, making it the most common form of muscular dystrophy in adults. It is characterized by an extremely diverse, multisystemic clinical presentation of a seemingly unrelated, yet consistent, constellation of symptoms. As the disease name suggests, the musculature, predominantly skeletal and cardiac muscle, is grossly affected in DM patients. The primary defects lie in muscle contraction that manifest clinically as myotonia (involuntary persistence of muscle contraction), hypotonia (reduced muscle tone), dystrophy (muscle wasting) and cardiac conduction defects. While the muscular symptoms are the hallmark of the disease, abnormalities exist in many other bodily systems: patients can develop posterior subcapsular iridescent cataracts and insulin resistant diabetes, with frontal balding and testicular atrophy also occurring in males. Central nervous system manifestations have been documented in DM patients as well, including hypersomnia, cognitive impairment, and avoidant social behavior. Another characteristic of the disease is anticipation, which refers to the fact that each subsequent generation exhibits earlier age of DM onset and increased symptom severity than the previous generation. Muscle biopsies of DM patients show unique histological characteristics such as its cytological hallmark sarcoplasmic masses, atrophic fibers, ring fibers and centralized nuclei in the muscle fiber (de Leon 2008, Harper 2001, Strong 1997).

DM1

Ninety-eight percent of DM cases are classified as DM type 1 (DM1) Positional cloning and sequence analysis revealed that the cause is a simple CTG repeat expansion in the 3' untranslated region (3'UTR) of the *Dystrophia Myotonica Protein Kinase* (*DMPK*) gene on chromosome 19q13.3 (Aslanidis 1992, Brook 1992, Buxton 1992, Fu 1992, Harley 1992, Mahadevan 1992). DM1 is a non-coding triplet repeat disease, making it one of a substantial group of triplet repeat diseases (reviewed in Ranum 2002) (see Illustration 1.1). Within the general population, the *DMPK* gene harbors 5 to 35 repeats, however, this repeat is expanded in DM1 patients who exhibit 50 to over 2000 repeats per individual (Harper 2001).

To date, no patients have been found harboring any mutation in the *DMPK* coding region. Therefore, the *DMPK* gene in both normal and DM1 patients encodes a fully functional DMPK protein. The DMPK protein is a serine/threonine kinase (Dunne 1994) and a founding member of the "DMPK-related family of kinases". This family includes the Rho-associated kinases, Cdc42-binding kinases, MRCKs, as well as many other kinases that are thought to regulate cytoskeletal changes as a response to external stimuli (Zhau 1997).

Several putative targets of DMPK phosphorylation have been identified *in vitro* including: DMPK itself, myosin phosphatase target subunit 1 (MYPT1), phospholemman (PLM), phospholamban (PLN), and CUG binding protein 1 (CUGBP1) (reviewed in Kaliman 2008). Three of the targets, MYPT1, PLM and PLN, are all ion release and muscle contraction regulatory proteins; therefore, loss of regulation of these proteins could directly cause muscle contraction malfunctions like those in DM patients. MYPT1 is a phosphatase that regulates the phosphorylation status of myosin light chain and is involved in smooth muscle contraction in response to calcium signals, cytokinesis and

cytoskeletal organization during cell migration (reviewed by Matsumura 2008). PLM phosphorylation due to adrenergic stimulation regulates the activity of cardiac Na/K pumps, effecting cardiac muscle contraction (Fuller 2006). PLN regulates calcium flux in an extremely similar manner to PLM in both cardiac and skeletal muscle. Adrenergic stimulation causes PLN to become phosphorylated which, in turn, stimulates calcium uptake in the sarcoplasmic reticulum via the SERCA 2a calcium pump and muscle contraction (Simmerman 1998). In contrast to the other known DMPK phosphorylation targets, CUGBP1 does not appear to have a direct role in the regulation of muscle contraction. It is an RNA binding protein with known function in alternative splicing (Ishiura 2005) and mRNA turnover (Zhang 2008). Its subcellular localization in DM patients is dependent upon its phosphorylation state (Roberts 1997); therefore, it is a reasonable assumption that DMPK kinase activity dictates the subcellular location of this post-transcriptional regulator of mRNA.

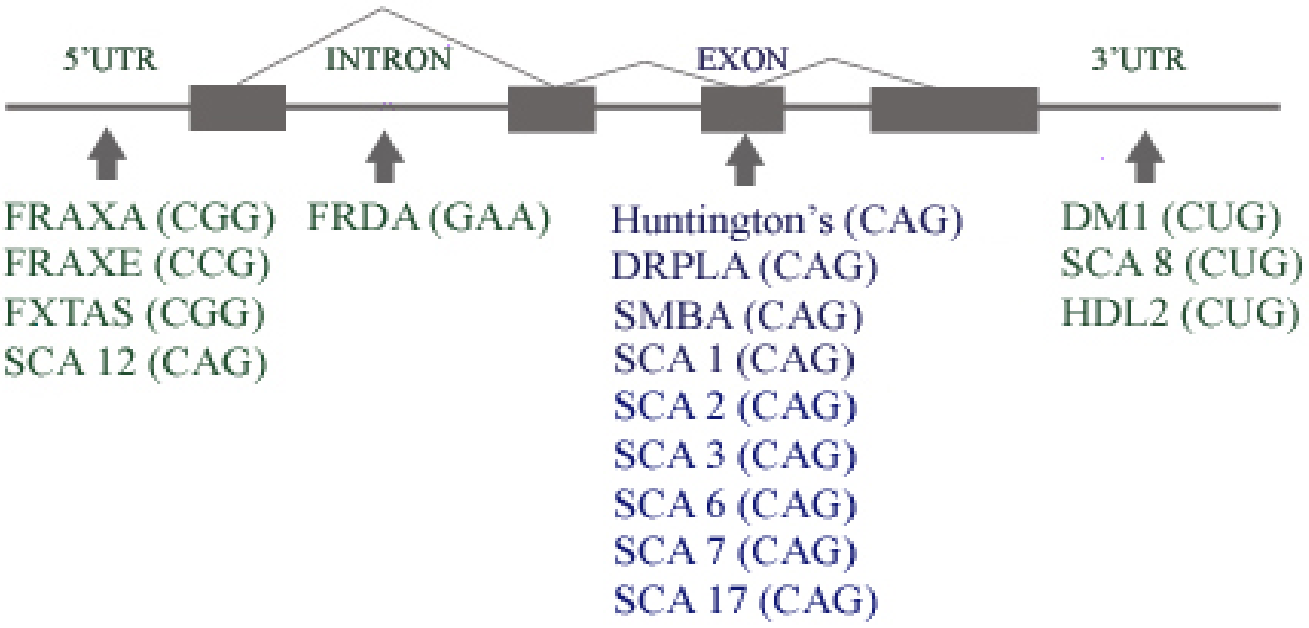

Illustration 1.1 Triplet Repeat Expansion Diseases.

Myotonic Dystrophy is a member of a class of diseases called triplet repeat expansion disorders. Most identified triplet repeat disorders have the causative simple repeat expansion occurring in exons that are translated and end up in the resulting protein. They exert a protein level effect which changes the properties of the encoded member and its molecular interaction. A small handful of disorders have their causative repeat expansions in non-coding regions (5'UTR, intron, 3'UTR). Since these expansions do not end up within the resulting protein products, an alternative model of action must be found for their effects. Diseases listed in green are non-coding and blue are coding.

DM2

A second locus known to independently cause DM (referred to as DM type 2) was mapped to chromosome 3 nine years after the mapping of DM1 (Liquori 2001).

Molecular analysis revealed the mutation to be an extremely large (11kb to 40kb) complex expansion with an abundance of (CCTG)n elements in the first intron of the *ZNF9* gene. The ZNF9 gene encodes a putative zinc-finger protein of unknown function named the DM2 protein, or pDM2. In contrast to the situation with DM1, the DM2 mutation appears to be predominantly spliced out in the nucleus (Margolis 2006, Krahe personal communication), rather than stay with the mRNA throughout its life. To date, consequences of the extremely large expansion on DM2 protein composition and expression have not been clearly delineated.

The putative DM2 protein is a very small (19kD), evolutionarily conserved protein that contains 7 predicted CCHC zinc fingers and an RGG domain (Armas 2008), both nucleic acid binding domains(reviewed in Burd 1994). Zinc fingers are a class of common protein motifs that fold into finger-like structures that make tandem contacts with the target molecule; whether the target is a protein, RNA or DNA depends on the amino acid composition affecting the fold of the domain (reviewed in Laity 2001). The CCHC type of zinc finger, as seen in the DM2 protein, is an unusual zinc finger that is only found in a small subset of zinc finger containing proteins. It is known to bind RNA (Hall 2005) with the best characterized CCHC containing proteins being the HIV-1 Nucleocapsid (NC) protein, which binds to and packages the HIV RNA genome into the capsid during viral assembly (Gorelick 1999, Summer 1992) and *Drosophila*'s Nanos protein, a translational repressor of the transcription factor *hunchback* active during *Drosophila* embryonic development (Curtis 1997, Wharton 1991). The other domain in the DM2 protein, the RGG box, is an arginine-glycine-glycine rich region highly similar to the RNA binding domain proposed to be a predictor of RNA binding activity in heterogeneous ribonuclear proteins (Kiledjian 1992). To date, the function of the DM2 protein in DM etiology is unknown.

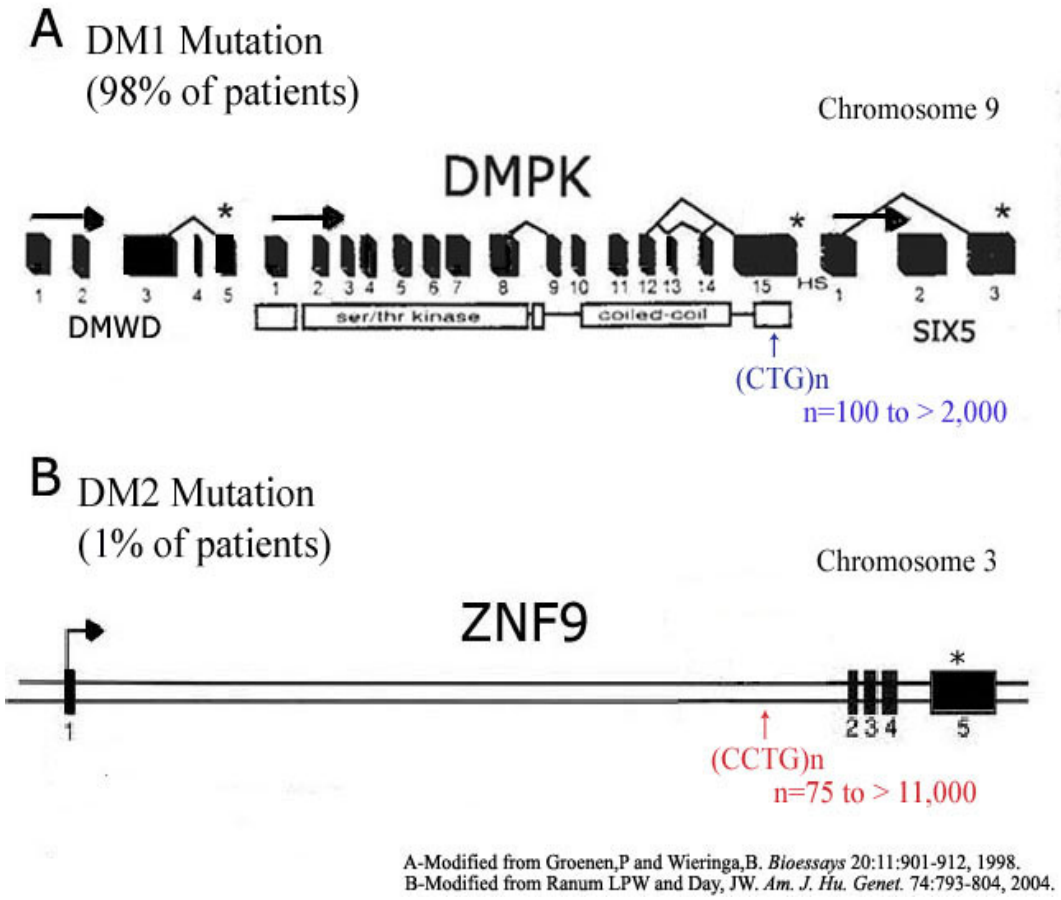

Illustration 1.2 Mutation of two distinct loci can independently cause DM.

A) Schematic of the Dystrophia Myotonica Protein Kinase (DMPK) gene and two flanking genes, DMWD and SIX5. The causative mutation of DM1 is an expanded (CTG)n tract within the 3'UTR of the DMPK gene, where n= 50 to several thousand. B) A large insertion within the first intron of the ZNF9 gene has been mapped as the causative mutation for Myotonic Dystrophy type 2. Exons are represented with black boxes. Transcriptional start sites are marked with an arrow and stop sites are demarcated with an asterisk and causative mutations are denoted.

Despite the fundamental differences in the afflicted genes and mutation characteristics causing DM1 and DM2, the clinical presentation of DM2 patients is nearly identical to the DM type 1 patients (Ricker 1994, Day 2003) (Table 1.3). One notable exception to the similarity is the large amount of misregulated mRNA splicing, 21 different spliced transcripts, that is seen in DM1 patients and is not seen in DM2 patients. In fact, only one transcript, the Insulin receptor, is known to be alternatively spliced in DM2 patients (Savkur 2004). The pathological importance of misregulated splicing in Myotonic Dystrophy will be discussed later in this chapter.

The presence of a congenital form of DM1 was a classic difference between the clinical presentations of DM1 and DM2; however, a putative case of congenital DM2 has recently been reported (Kruse 2008). Congenital DM1 is rare, occurring in only 13 of every 100,000 people (Aicardi 1998), presenting the issue that the statistical likelihood of diagnosing a rare form of DM2, which accounts for only 1% of the DM cases identified, is drastically low. However, more cases besides the case that Kruse et al recently reported

must be discovered to verify the existence of a congenital form of DM2.

SYMPTOM	DM1	DM2
Myotonia	+	+
Facial weakness	+	+
Proximal weakness	+	+
Distal weakness	+	+
Sternocleidomastoid atrophy	+	+
Iridescent cataracts	+	+
Cardiac arrhythmias	+	+
Testicular failure	+	+
Hyperinsulinemia	+	+
Hypogammaglobulinemia	+	+
Elevated creatine kinase	+	+
Retardation/Congenital abnormalities	+	+*
Misregulated mRNA Splicing	+	--

+ = present, -- = absent

Recreated from Ranum, LPW and Day JW. *Am. J. Hu. Genet.* 74:793-804, 2004.

Table 1.3 Symptoms seen in DM1 and DM2 Patients.

The clinical presentation of Myotonic Dystrophy represents a diverse constellation of multi-systemic symptoms. This unusual collection of disease manifestations is nearly identical between the two forms of DM with the exception of the misregulated mRNA splicing events seen in DM1 patients that are not seen in DM2 patients. The asterisk refers to the need for more cases to substantiate the presence of the congenital form of DM2.

MOUSE MODELS

DM1 Models

Soon after the DM1 mutation was mapped, several groups began trying to create a mouse model for DM. This is a universal step in the early stages of the research of most diseases. Mice make excellent disease models because many of their physiological systems behave similarly to humans in response to disease causing mutations. Early attempts at a DM1 mouse model began with *DMPK* knockout mouse models (Jansen 1996, Reddy 1996). These models showed only subtle defects, suggesting that effects on the DMPK protein have only a minor role in some aspects of DM pathology. Attention was then focused on the disease causing (CTG)n expansion. Two mouse models were created that expressed the (CTG)n expansion in the context of the *DMPK* gene (Seznec 2001 (300 CTG repeats), Ornego 2008 (960 interrupted CTG repeats)) and one was created which expressed 250 CTG repeats artificially inserted in the *β-actin* gene (Mankodi 2000). All three of these systems showed a variety of histological, physiological and molecular symptoms that correspond to DM patients; however, all of them failed to produce the vast majority of DM pathology (reviewed in Wansink 2003); indicating that the CTG repeat expansion does possess some pathology, but it is not sufficient to reproduce the diverse DM phenotype.

Related Models

To address the possibility that the full constellation of DM pathology is due to the affects on factors other than DMPK, several knockout mice of other proteins were created. The most significant results were achieved with the knockout/knockdown of two of the Muscleblind proteins (MBNL1 and MBNL2) and the knockdown of the DM2 protein. The Muscleblind proteins are a group of double stranded RNA binding proteins

identified as factors that specifically bind the CUG repeat expansion within the context of the *DMPK* message (Miller 2000). To examine a potential role for MBNL1 in DM pathology, a MBNL1 knockdown mouse model (Kanadia 2003) was created which specifically eliminated the MBNL1 isoforms that bind the *DMPK* (CUG)n repeat expansion (simulating a hypothesized diseased situation where MBNL1 binds to the repeat tract rather than associating with its normal splicing targets). Interestingly, this mouse model recapitulated several features of DM pathology, including many splicing abnormalities and, most notably, posterior subcapsular iridescent cataracts, a unique symptom not seen in previous models. The variety of misregulated transcripts seen in this mouse model strongly suggests that CUG binding of certain isoforms of the MBNL1 protein has a specific role in the splicing misregulation seen in DM patients. A MBNL2 knockout mouse was created (Hao 2008) that had many of the same histological abnormalities of the other DM mouse models, but did not show the MBNL1 mouse mRNA splicing abnormalities. Thus, these two proteins may share the ability to bind the DM repeat expansion, but they do not share roles in the pathology of the disease. The final DM model created was a DM2 protein knockdown (Chen 2007). The phenotype of these mice dramatically combined many of the histological and physiological disease features seen in both the DMPK mice and the MBNL1 knockout mice, with the notable exception of MBNL1 splicing abnormalities. The DM2 mouse model suggested a significant impact of the loss of DM2 in DM etiology, which appears to be distinct from the MBNL1 role. Despite the many attempts to create a comprehensive mouse model for DM, the field is left with a variety of different models that re-create various subsets of the disease, but none that represent the full constellation of DM symptoms (see Appendix 1 for a complete discussion of Myotonic Dystrophy mouse models).

Models of DM1 Disease Etiology

Presently both the mechanistic basis of DM1 and its accompanying autosomal dominance are not fully clear. Unlike other diseases where mutations in the disease gene result in an aberrant protein product or no product at all, DM1's causative mutation is not decoded into protein. Since there are no paradigms for this type of mutation, any model to adequately explain how the DMPK CUGn expansion can result in pathogenesis is likely to be novel.

DMPK HAPLOINSUFFICIENCY

Initial hypotheses of DM1 pathology proposed a mechanism based on the decrease of DMPK protein level (Fu 1993, Carango 1993). Analyses of the DMPK protein level in patient tissue produced conflicting results, with some groups detecting a reduction in total DMPK levels (Hamshere 1997, Furling 2001, Salvatori 2005) and others, including our lab (Bhagwati 1996, Raabe and Gottlieb unpublished data), finding the levels relatively unchanged. The discrepancy is likely due to differences in protein extraction techniques, tissue examined, and proteolysis (reviewed in Hofmann-Radvanyi 1993, Groenen 1998). Indeed, our findings that DMPK protein resides in sarcoplasmic masses (see Chapter 3) and that these require certain conditions for efficient extraction supports this conclusion. DMPK knockout mice were generated to determine a possible role for DMPK haploinsufficiency in DM1 pathology (Jansen 1996, Reddy 1996). Strikingly, DMPK knockout mice do not present with the multisystemic characteristics of the disease, instead only having some cardiac conduction abnormalities (see Appendix 1).

Therefore, the DMPK protein may contribute to the cardiac features of DM1, but DMPK haploinsufficiency is not responsible for the multisystemic clinical features of DM1 (Jansen 1996).

CHROMATIN REMODELING EFFECTING NEIGHBORING GENES

A second proposed mechanism for DM1 pathology stated that chromatin remodeling occurred due to the CTG repeat expansion in the *DMPK* gene. This then altered the expression of genes neighboring the *DMPK* gene. This hypothesis is supported by the observation that the CTG expansion is a strong nucleosome binding site that results in the alteration of local chromatin structure (Otten 1995). Of particular interest was the potential effect on the expression of the Six5 gene, whose 5' promoter region overlaps the CTG expansion in the *DMPK* gene. The Drosophila homologues of Six5 are known to effect eye (Serikaku 1994) and muscle development (Kirby 2001), potentially accounting for the cataracts and muscle wasting seen in DM1 patients. However, Six5 knockout mice showed no significant phenotypic overlap with DM1 symptoms, excluding this theory as a possible cause of DM1 pathology (Sarkar 2000, Klesert 2000). The subsequent mapping of the DM type 2 mutation to a different chromosome with unrelated neighboring genes completely undermined the validity of this model as well.

RNA GAIN-OF-FUNCTION THEORY

Evidence now indicates that the predominant mechanism of DM1 pathogenesis is a gain-of-function of the *DMPK* mRNA transcribed with the CTG expansion. First, transgenic mouse models expressing the repeat expansion at the RNA level showed myopathy and myotonia (Mankodi 2000, Seznec 2001, Ornego 2008) (Appendix 1).

Second, the DM1 expansion in the 3'UTR of the *DMPK* mRNA has the ability to inhibit myoblast differentiation in cell culture (Bhagavati 1999). Third, specific RNA binding proteins, CUGBP-1 and the Muscleblind proteins, show altered subcellular localization and increased expression levels upon CTG expansion expression (Roberts 1997, Fardeai 2001, Ornego 2008). Finally, recent gene expression profiling of transgenic mice expressing *actin* mRNA with a 250 CUG expansion illustrates the tremendous amount of altered gene regulation resulting from the CUG repeat (Osborne 2009).

DMPK biogenesis affects

Investigators initially focused attention on the role of the triplet repeat expansion on mRNA biogenesis, hypothesizing that the DMPK CTG mutation directly alters the creation and/or regulation of the DMPK message. However, *DMPK* mRNA transcription appears unaffected by the triplet repeat expansion because the levels of wild type and mutant unspliced *DMPK* pre-mRNA are normal in DM patients (Krahe 1995, Davis 1997). Similarly, in well executed experiments, direct splicing, stability, polyadenylation and translatability of the *DMPK* message does not seem to be affected by the triplet repeat expansion (Krahe 1995, reviewed in Groenen 1998).

Nuclear sequestration causes DM pathology

In the absence of a simple direct effect on DM biogenesis an alternative RNA-level model termed 'nuclear sequestration' emerged. This theory suggests that CUG expansion-containing transcripts are retained in nuclei and chelate CUG-binding proteins (Taneja 1995, Davis 1997). These proteins are no longer available to perform their normal functions; several are known splicing regulators, so the splicing of their

natural target mRNAs is affected and causes DM pathology (Timchenko 1996b, Philips 1998, Charlet-B 2002). Thus, the theory suggests that the (CUG)n expansion in the 3'UTR of the DMPK message acts *in trans* to chelate a subset of RNA binding proteins, indirectly resulting in splicing misregulation.

The identification of naturally occurring short CUG stretches within intronic splicing enhancers of the *cardiac troponin T* (*cTnT)* mRNA (Ryan 1996) lead Cooper and coworkers to speculate that the large DM1 CUG expansion could be competing with the binding of the *cTnT* splicing regulators. Examination of cTnT isoforms in cardiac cells from DM1 patients revealed that this message was, in fact, abnormally spliced (Philips 1998), substantiating the hypothesized binding competition between the splicing regulatory signals and the *DMPK* 3'UTR CUG repeats in DM1 patients. Since those initial studies, a variety of DM1 splicing misregulation events have been documented both *in vivo* in patient tissue and mouse models and *in vitro* in myoblasts from DM1 patients (recently reviewed in Orengo 2007, Dick 2006). The growing list of alternatively spliced transcripts in Myotonic Dystrophy type 1 stands at 21 different messages (Osborne 2006) (see Table 1.4). However, the molecular details of the observed splicing misregulation and its contribution to DM1 pathology are still being explored.

Tissue/gene	Target
Skeletal muscle	
ALP	exon 5a, 5b
CAPN3	exon 16
CLCN1	intron 2, exon 7a, 8a
FHOS	exon 11a
GFAT1	exon 10
IR	exon 11
MBNL1	exon 7
MBNL2	exon 7
MTMR1	exon 2.1, 2.2
NRAP	exon 12
RYR1	exon 70
SERCA1	exon 22
z-Titin	exon Zr4, Zr5
m-Titin	M-line exon 5
TNNT3	fetal exon
ZASP	exon 11
Heart	
TNNT2	exon 5
ZASP	exon 11
m-Titin	M-line exon 5
KCNAB1	exon 2
ALP	exon 5
Brain	
TAU	exon 2, exon 10
APP	exon 7
NMDAR1	exon 5

Modified from Osborne 2006

Table 1.4 Alternatively Spliced mRNAs in DM1 Patients.

Twenty one different messages have been identified to date in DM1 patient tissue, patient primary myoblasts or mouse models that exhibit misregulated splicing. The role of most of the inappropriate splicing events in DM1 pathology is unknown. Tissue specificity and target of the splicing event is listed.

DMPK CUG RNA binding proteins

The identification of *cTnT* mRNA splicing misregulation in DM1 provided a specific target with which to identify the key CUG RNA binding proteins that are being affected by the DM1 (CUG)n expansion (Philips 1998). The initial candidate explored was a conserved heterogeneous ribonucleoprotein called CUB-BP1. This was a known CUG RNA binding protein (Timchenko 1996a) that was subcellularly distributed in the nucleus (the site of an accumulation of the CUG containing *DMPK* message) or the cytoplasm in a phosphorylation dependent manner (Roberts 1997). Thus, Philips et al examined the potential role of CUG-BP1 in *cTnT* mRNA splicing regulation and found that CUG-BP1 does control the splicing pattern of the *cTnT* message (Philips 1998). CUG-BP1 functions in both splicing and translational regulation (Philips 1998, Timchenko 1999, Timchenko 2005) and has now been shown to bind to regions adjacent to splice sites in three DM1 misregulated transcripts (Mankodi 2002; Philips 1998; Savkur 2001). Over-expression of CUGBP-1 in cell culture results in similar defective alternative splicing patterns as seen in DM1 patients (reviewed in Faustino 2003).

Miller et al conducted a UV crosslinking experiment with DMPK 3'UTR RNA containing 54 or 90 CUG repeats to identify other CUG RNA binding proteins potentially affected by the DM1 mutation. Their work resulted in the identification of the Muscleblind (MBNL) proteins, MBNL1-3 (Miller 2000), a group of three double stranded RNA binding proteins that are known alternative splicing regulators that influence exon selection (reviewed in Pascual 2006), as well as controlling transport, stability and translation of mRNA (Timchenko 1999, Timchenko 2005, Adereth 2005). The significance of the MBNL proteins in DM pathology is discussed below.

The nuclear sequestration model gained support from 1) the discovery that mRNAs with long tracts of CUG or CCUG repeats transcribed from the mutant DM1 and

DM2 loci accumulate in discrete nuclear foci (Taneja 1995, Davis 1997, Liquori 2001) and 2) the localization of CUGBP-1 and the MBNL proteins is redistributed from the cytoplasm into the nucleus of DM tissue and myoblasts (Philips 1998, Miller 2000). Several lines of evidence strongly suggest that the nuclear shift of MBNL is a result of CUG repeat recruitment, that this binding results in a loss of function of the Muscleblind proteins, particularly MBNL1, and that this loss directly results in the abnormal regulation of alternative splicing seen in DM1 patients (reviewed in Kuyumcu-Martinez 2006, Cardani 2006, Osborne 2006). First, all three MBNL proteins co-localize to the CUG-RNA containing nuclear foci observed in DM patient cells and tissue, suggesting that their nuclear localization is a result of association with the CUG repeat RNA (Miller 2000, Mankodi 2001, Fardaei 2002). Also, expression of exogenous MBNL1 protein rescues the splicing phenotype of transgenic CUG expansion mice (Kanadia 2006), thus the splicing abnormalities caused by CUG repeat RNA are due to a reduction in free/functional MBNL. Finally, MBNL1 knockout mice presented with splicing changes that are characteristic of DM1 and seen in mice expressing CUG expansion mRNA (Kanadia 2003), so a loss of MBNL1 protein directly results in aberrant splicing regulation.

However, the role of CUGBP-1 in DM pathology is questionable; several pieces of data suggest that its role in DM pathology may be indirect. It was purified with a wild-type length *DMPK* CUG repeat, CUG_8 (Timchenko 1996a), and does not efficiently bind to the pathogenically long CUG expansions (Michalowski 1999). Also, though it does redistribute to the nucleus in DM cells, it does not colocalize with CUG containing RNA (Fardaei 2001) as MBNL1-3 proteins do, suggesting that this localization change is not due to direct recruitment by the CUG repeats. Interestingly, Roberts et al discovered that the regulation of CUGBP-1 protein subcellular localization is dependent upon its

phosphorylation by DMPK both *in vivo* and *in vitro*, hypophosphorylated CUGBP-1 is selectively localized to the nucleus while hyperphosphorylated CUGBP-1 protein is located in the cytoplasm (Roberts 1997), implying any splicing pathology due to CUGBP-1 mislocalization may be a downstream effect of DMPK kinase function misregulation.

Problems with the nuclear sequestration theory of pathology

Though the importance of the MBNL family of proteins and alternative splicing in DM1 pathology is firmly established, several problems exist with a model of DM etiology based primarily on the nuclear sequestration of RNA binding proteins. 1) It does not explain the unique clinical presentation of other non-coding (3'UTR) CTG expansion diseases, such as Spinocerebellar ataxia 8 and Huntington's disease Like-2, nor the non-pathogenic naturally occurring CTG expansions in the human genome. If the CUG expansion is sufficient to develop DM pathology, regardless of gene content, then all of these expansions should result in the wide constellation of Myotonic Dystrophy symptoms. Moreover, recent evidence suggests that the MBNL has equal binding affinity for both CAG and CUG repeat tracts (Yuan 2007), broadening the list of genes with non-coding repeats that should present identically to DM if, in fact, the triplet repeat expansion sequestration of MBNL is the primary determinant of disease. 2) If the amount of MBNL binding is directly proportional to number of repeats (Miller 2000) and the MBNL affinity for CUG repeats is the same for CCUG repeats (Warf 2008), then the tremendously higher number of CCUG repeats in the DM2 expansion (verses DM1) should result in more MBNL sequestered and increased severity of disease, when, in actuality, DM2 is the more mild form of DM. 3) Despite the large amount of effort that has been invested in characterizing mRNA alternative splicing in DM, only one

transcript, the insulin receptor, has been reported to be alternatively spliced in DM2 patients (Savkur 2004), suggesting that splicing misregulation is not at the heart of this nearly identical form of DM. 4) Recent data suggests that nuclear foci are separable from DM cellular pathology (Ho 2005, Houseley 2005, Holt 2007) and MBNL-based splicing affects are not due to its sequestration in these foci (Ho 2005), disparaging a key component of the nuclear sequestration model. This model of pathology based entirely on the non-specific RNA binding protein chelation by CUG repeats fails to address the unique and complex nature of the DM symptoms. Knockout models of MBNL proteins fail to reproduce major DM-specific characteristics and the catalog of mis-spliced transcripts do not explain many characteristic DM symptoms. Thus, despite the elegant data revealing the importance of alternative splicing in DM pathology, it cannot be the sole mechanistic basis of Myotonic Dystrophy.

RNA localization theory of DM etiology

Experience researching the process of RNA localization allowed us to recognize another potential RNA level molecular consequence of the *DMPK* mRNA disease-causing 3'UTR triplet repeat expansion. We considered that many 3'UTRs harbor signals for mRNA localization, the evolutionarily conserved post-transcriptional process whereby particle messages are positioned within the cytoplasm to impart distinct cellular or subcellular properties. Is it possible that the triplet repeat expansion could be disrupting or blocking an RNA localization *cis*-acting signal in the 3' untranslated region of the *DMPK* transcript? The developmental requirement for RNA localization has been established in many model systems (flies, frogs, yeast; reviewed in Jansen 2001), but it is also know to be required for the normal functioning of human nerves and muscles (Martin 2000, Reddy 2005). Thus, it is reasonable to hypothesize that the *DMPK* message

may be subcellularly localized in normal muscle. Disrupted mRNA localization results in developmental and birth defects in model systems, therefore, by analogy, it seems reasonable that disruption of the normal localization of certain messages in nerves and muscles might result in a neuromuscular disease, such as Myotonic Dystrophy. Notably, to date no human diseases characterized have been attributed to aberrant RNA localization.

Therefore, we hypothesized that the *DMPK* mRNA might be a localized message in muscle tissue and the CUG repeat expansion might disrupt that wild-type localization. Since the protein coding region of the message is unaffected, the mislocalization of the *DMPK* mRNA would result in the translation of a fully functional serine-threonine kinase in an inappropriate subcellular location. At the new locale, this kinase would then be exposed to novel substrates which it could potentially phosphorylate, altering signal transduction cascades and leading to DM's diverse symptoms. This is the simplest and most direct current model of DM etiology, as the mutation acts in *cis* to cause pathology, rather than only effecting *trans*-acting factors. It takes into account that the CUG disease causing repeat leads to Myotonic Dystrophy only when attached to the DMPK coding region. This vital piece of information is not accommodated by the nuclear sequestration/alternative splicing model.

This still leaves the etiology of DM2 unanswered. How can mutations in genes of functionally unrelated proteins, on different chromosomes, cause the same seemingly unrelated set of symptoms? The predicted RNA binding characteristics of the DM2 protein led us to further hypothesize that DM2 functions in the localization pathway of the *DMPK* message. If DM2 is a localization factor for *DMPK* transcripts, then the proposed protein effect of the DM2 intronic expansion would disrupt the *DMPK* mRNA localization pathway, causing mislocalization of the *DMPK* mRNA. Thus, the same

molecular outcome would be seen by the disruption of the proper localization signal in the 3'UTR of the *DMPK* mRNA by the CUG repeat expansion and disruption of the localization factor, the DM2 protein, by altered DM2 splicing of the intronic insertion. In this model, the independent mutations leading to DM1 and DM2 represent aberrations in the same molecular pathway, causing the same cellular consequences and resulting in nearly identical clinical symptoms. Therefore, the *DMPK* mRNA (mis)localization model is the first RNA based model to take into account the message-specific context of the mutations and fully incorporate the mutational differences between the two forms of DM into a cohesive model of etiology.

Prospectus and Overview

Given that 1) RNA localization is at the heart of development in model systems 2) that disruption of location in these systems causes birth defects and 3) that RNA localization is prominent in higher eukaryotic (human rat, mice) nerves and muscles, we initially reasoned that disruption of RNA localization might be involved in certain neuromuscular disorders. Consequently, we (the Gottlieb lab) set out to find a human disease where RNA localization functioned in its etiology/pathology. The criteria we established were three fold: 1) a hereditary disease of nerves and/or muscles 2) a disease characterized/caused by mutations in the 3'UTR of a causative gene since that is where RNA localization signals reside and 3) a disease caused by one or only a few genes to simplify our analyses. Our search led us to the candidate disease Myotonic Dystrophy. Via positional cloning and locus analyses, this disease was revealed to be caused by a triplet repeat expansion (CTG)n in the 3'UTR of the *DM Kinase* gene in 1992 (Aslanidis 1992, Brook 1992, Buxton 1992, Fu 1992, Harley 1992, Mahadevan 1992).

In Chapter 3, in actual patient tissue we show that *DMPK* RNA is subcellularly localized in the sarcoplasmic reticulum the muscle equivalent of the endoplasmic reticulum. Additionally, *DMPK* mRNA is mislocalized to the site of ultimate disease pathology characteristic of this disorder. We show that this has links to the disease's molecular genetics, cytopathology and cell biology. These data highly implicate RNA (mis)localization as a vital process underlying this important disease. The majority of the tissue work presented in this chapter was performed by Dr. Tobias Raabe, a former postdoctoral fellow in the Gottlieb Lab, and I conducted the remaining experiments that were requested by reviewers.

While we were truly fortunate to have access to actual muscle tissue from multiple DM patients to reveal this phenomena and establish these findings, the system does not lend itself to testing hypotheses. Therefore, in Chapter 4, we establish a disease-relevant tissue culture system using C_2C_{12} cells. These cells are of muscle origin and can be differentiated at will. Thus, they allow us to examine the undifferentiated and/or differentiated muscle states. Using this system and synthetic transcripts we extend our findings from patient tissue and establish function. We prove that 1) the *DMPK* mRNA is, indeed, subcellularly localized; 2) that localization is dictated by signals in the *DMPK* 3'UTR and is disrupted by the DM1 disease causing (CTG)n expansion and that 3) the presence of the DM2 protein is required to achieve and/or maintain that subcellular localization. These data establish that *DMPK* mRNA targeting/mistargetting underlies the Myotonic Dystrophy disease process and implicate a role for the DM2 protein, the second gene identified in DM, in the *DMPK* mRNA localization process.

In Chapter 5, we examine the role of the DM2 protein in the context of the localization/mislocalization process. Taking an interdisciplinary approach, we show that the protein encoded by the *Znf9* gene, a small, zinc finger containing protein, colocalizes

with the *DMPK* mRNA and associates with the *DMPK* 3'UTR, where the RNA localization signals reside. A small subset of the *DMPK* 3'UTR that comprises the pDM2 binding site, is sufficient to impart wild type localization onto a reporter construct and deletion of this small portion of the *DMPK* 3'UTR alters the wild-type localization. Finally, we show that the DM1 (CTG)n expansion in a synthetic *DMPK* 3'UTR RNA is sufficient to disrupt the interaction of the DM2 protein and gain a new association with the MBNL proteins, suggesting that DM etiology involves both the loss of normal pDM2 interaction and the addition of MBNL protein association with the *DMPK* message.

Together these data show that the two identified genes which, when mutated, independently caused DM act as two different components within the same molecular pathway. For the first time, we establish that the *DMPK* mRNA and DM2 protein physically interact, the *DMPK* 3'UTR sequence protected by pDM2 acts as an mRNA localization signal, the DM2 protein functions in RNA localization and mutation of either component, *DMPK* mRNA or pDM2, presumably can lead to Myotonic Dystrophy disease etiology.

These data establish RNA localization as a key process underlying the debilitating neurodegenerative disease Myotonic Dystrophy. This is the first time anyone has sought out and successfully identified a human disease as an RNA localization disorder. Our findings have major implications for Myotonic Dystrophy and, potentially, for other select human nerve and muscle diseases, as well as open new avenues for development of therapeutics for these diseases. The implications of this work are explored in Chapter 6.

CHAPTER 2: MATERIALS AND METHODS

Materials and Methods for Chapter 3

PATIENT MUSCLE

Fresh human biceps biopsies were frozen in –160 ^{o}C isopentane-liquid nitrogen (40 sec) and stored at –80 ^{o}C. 10 *um* sections were generated using a Hacker model H/I Bright OTF/AS/MR/EC/1020 cryostat at –28 ^{o}C, transferred to slides and attached by heating (5 min; 45 ^{o}C). Biopsies from 3 DM patients and 2 "normal" individuals were employed. All DM patients possess DMPK $(CTG)_n$ expansions (500nts to >4 kb; not shown) as determined via combined PCR amplification and Southern blot (Gennarelli 1998) consistent with DM diagnosis. Repeat length was assessed using the same biopsy material that was used for *in situ* hybridization. The average triplet repeat length of the *DMPK* mRNA in the muscle for these 3 DM patients was approximately 1800nts (DM1), 1050nts (DM2) and 1500nts (DM3). Note that the *DMPK* triplet repeat is more heterogeneous and often larger in muscle than in blood (reviewed in Groenen 1998). “Normal” denotes control tissue from individuals with no known muscle disorders or histological evidence of muscle abnormality. Aliquots of identical tissue samples were used to generate muscle extracts (see below).

***IN SITU* HYBRIDIZATION**

Muscle sections were fixed and subject to *in situ* hybridization (Tautz 1989) by a modification of Jostarndt et al (1996). Basically, frozen sections attached to slides were fixed (4% paraformaldehyde / 1x PBS; 5 min. RT), washed (PBS, 5min), proteinase K treated (15ug/ml in PBS; 3 min RT), washed (PBS, 5 min), fixed again (4% paraformaldehyde / 1x PBS, 5 min), washed (1x PBS, 2 min) and washed again (2x SSC, 2 min). After incubation in 0.25% acetic anhydride-0.1M triethanolamine (10 min) and then 0.1M tris-glycine pH 7 (30 min), excess fluid was removed.

Sections were pre-hybridized in 200ul (5x SSC, 40% formamide, 0.02% Ficoll, 0.02% polyvinylpyrolidone, 0.02% BSA, 200ug/ml yeast tRNA, 10% dextran sulfate; 30 min). Solution was removed and replaced with RNA probe (1ng/ul) in hybridization solution (100ul) that was preheated at 65°C (5 min). Sections were covered with a coverslip, sealed with Gurr (BDH, Poole UK) and hybridized at 47°C for 16^{+} hrs in a sealed chamber.

Coverslips were floated off in 5x SSC (3x 20min, room temperature), incubated in 20% formamide-0.5x SSC (60°C, 20 min), RNase A treated in 2x SSC (Roche 1ug/ml, 30 min, 37 ^{o}C), washed (20% formamide / 0.5x SSC, 60°C 10 min) and washed again (2x SSC, 5 min, room temperature).

RNA detection involved blocking with 5% Carnation non-fat dry milk / 1x PBS (30 min), anti-DIG Fab fragment antibody-alkaline phosphatase conjugate generation (Roche: sheep polyclonal sera, 1:3000 dilution in 0.2% non-fat dry milk / 1x PBS) and NBT / BCIP development (see Roche). The NBT / BCIP reaction occurred in 100mM NaCl, 50mM $MgCl_2$, 100mM Tris pH 9.5 in a sealed chamber in the dark at room

temperature for 24-72 hrs because of the low abundance of mRNAs in human skeletal muscle (Jostarndt *et al*, 1996). Sections were mounted (90% glycerol / 1x PBS) and used for confocal microscopy or immunostaining. Roughly 10% (patient 1, 3) and 2% (patient 2) of the biopsies' area contained mislocalized *DMPK* RNA. This is compatible with localization in other systems and underscores the specificity of our results.

RNA PROBES FOR *IN SITU* HYBRIDIZATION

RNA probes were generated by *in vitro* transcription from plasmids with DIG-UTP and the appropriate RNA polymerase (T3, T7). They were subjected to controlled alkaline hydrolysis (60 ˚C, 20 min in the presence of 1M carbonate) to yield an average probe length of 100-200 nucleotides as judged by polyacrylamide-urea gel. Alkaline hydrolysis was stopped by sodium acetate (pH 5) addition and lithium chloride precipitation. For the $(CUG)_n$ probe every third templated A was replaced with a U allowing DIG-UTP incorporation. This should not compromise probe-repeat hybridization since U-U base pairs can be accommodated in RNA-RNA base pairing and provide equivalent hydrogen binding to A-U base pairs. The $(CUG)_n$ probe detects significant RNA amounts in patient but not normal muscle. This is notable since *DMPK* RNA in patient but not normal tissue harbors large $(CUG)_n$ expansions. Sense probes corresponding to DMPK and every mRNA used here exhibited only background stain on normal and patient sections (not shown).

Notably, we initially tried this analysis on archived, frozen DM muscle and obtained variable to no RNA results. Thorough analysis of the tissue including propidium iodine staining +/- RNase revealed the original stored biopsies collected over a span of 10

years had little to no intact RNA. Hence, no distinct RNA localization pattern. This was likely due to the biopsy fixation and/or storage conditions. We subsequently determined proper conditions for fixation and storage (see above) and obtained fresh patient biopsies which gave the reproducible conditions documented here. Our experiments use DIG probes; repeated attempts to detect cytoplasmic *DMPK* RNA in patient tissue with fluorescent probes were unsuccessful. Using our DIG *DMPK* probes, we could repeat the results of Taneja et al (1995) on DM vs. wild type fibroblasts providing independent verification of the specificity of our probes.

IMMUNOFLUORESCENCE

Immunofluorescence was by standard means using a 30 min block (5% non-fat dry milk) and a 1 hr (room temperature) antibody incubation to stain. Antibodies were against DMPK amino acid regions 612-625, 259-271 and 1-542. Anti-DMPK1 (rabbit polyclonal) was purified over a peptide column Ser-Gly-Ala-Ala-Gln-Glu_Pro-Pro-Ala-Leu-Pro-Glu-Pro. Anti-DMPK2 and anti-DMPK3 (corresponding to #8391, #10033; Timchenko 1995; Roberts 1997) were against peptide Pro-Gly-Thr-Gly-Ser-Tyr-Gly-Pro-Glu-Cys-Asp-Trp and bacterially expressed DMPK protein, respectively. Some antibodies were provided by L. Timchenko (anti-DMPK 2 and 3; anti-CUG-BP) or obtained commercially (anti-SERCA, Affinity Bioreagents; anti–alpha actin, Imogenex; anti-utrophin). Secondary antibodies were goat anti-rabbit rhodamine (ICN) and anti-mouse FITC (ICN) for DMPK and other antibodies, respectively.

MICROSCOPY AND IMAGING

Laser confocal microscopy employed a Leica TCS 4D microscope. Fluorescent dyes (TRITC, red; FITC, green) and NBT/BCIP detected protein and RNA, respectively. For transverse (40X) and longitudinal (100X) sections, RNA and protein were visualized in identical or consecutive sections, respectively. Image processing used the Leica TCS maximal projection algorithm. In all figures, NBT/BCIP appears black (except Figure 2), TRITC red and FITC green. In Figure 2B, NBT/BCIP stain appears black or blue depending on the panel. Digital color conversion was done in RGB mode using Adobe Photoshop, allowing digital merging of *DMPK* RNA and protein.

IMMUNOBLOTS

Biceps extract were frozen tissue slices pulverized under liquid nitrogen. Resulting powder was boiled in sample loading buffer, centrifically cleared, gel fractionated, transferred to nitrocellulose and probed (Towbin 1979). Primary antibodies were anti-DMPK, anti-phosphoserine (Zymed) or anti-phosphothreonine (Zymed). Detection used anti-rabbit IgG-HRP (ICN) and chemiluminescence (National Diagnostics; Pierce).

Materials and Methods for Chapter 4

CELL CULTURE

C_2C_{12} mouse myoblasts cells were purchased from the American Type Culture Collection (ATCC, Camden, NJ). They were grown in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA)) supplemented with 20% Fetal Bovine Serum (Gemini BioProducts, Woodland, CA) and 1xPenicillinStreptomycin (Invitrogen, Carlsbad, CA) at 37'C in a humidified 8% CO_2 atmosphere. The myoblasts were never allowed to grow over 70% confluent to prevent unwanted drifting toward differentiation due to overgrowth. To differentiate the myoblasts into myotubes, actively growing cells were passed to 70% confluency overnight then the growth media was completely replaced with differentiation media (DMEM + 2% horse serum) (horse serum from Gemini BioProducts, Woodland, CA) rather than FBS. The media was replaced with fresh differentiation media daily until myotubes were observed visually, usually about ten days.

When C_2C_{12} cells were grown at 5% CO_2 rather than 8% CO_2, I found that it required about half of the time to differentiate them into myotubes (5 days verses 10 days), suggesting that the myoblasts are maintained in a more differentiated state than when grown at 8% CO_2. Helen Blau's website discusses the CO_2 requirement for C_2C_{12} cells in culture (http://www.stanford.edu/group/blau/protocols/c2lineprotocol.html). It indicates that 10% CO_2 is optimal and 8% CO_2 is functional, since I had access to an 8% CO_2 environment they were grown at 8% CO_2. The differentiation difference between 5% CO_2 and 8% CO_2 growth conditions may account for variability of observation seen in the literature.

GENERATION OF DM2 ANTIBODY, C_2C_{12} CELL EXTRACT PREPARATION AND WESTERN BLOT ANALYSIS

The DM2 protein is small and has a large number of predicted molecular binding domains. Thus, it possessed limited regions for potential epitopes that would yield antibodies that did not cross react with other proteins. After analysis, a 19 amino acid peptide to the DM2 protein Carboxyl terminus (amino acids 158-177) was synthesized (UT Austin Protein Core Facility). This peptide was HPLC purified and conjugated to KLH (Pierce, Rockford, IL, Imject (R) Malemide Activated McKLH Kit).We routinely employ KLH conjugation, rather than BSA conjugation, for all of our generated antibodies since we tend to use BSA as a carrier for our purified materials in some of our analytical methods. This was then sent to AnimalPharm Services (Healdsburg, CA) for injection into rabbits that we had prescreened to ensure that they had no substantial reaction to any component of the antibody creation material. Bleeds from the two rabbits at various time points were received and analyzed for specificity and strength of binding via western blot analysis of C_2C_{12} whole cell extract. Extract was prepared by harvesting overnight cultures of 70% confluent C_2C_{12} cells by 1xPBS rinse, followed by trypsinization with 1mL room temperature 0.25% Trypsin-EDTA (Invitrogen Corporation, Carlsbad, CA) for two minutes at 37'C. The cells were then collected and spun at 1500 RPM for five minutes in a clinical centrifuge, the supernatant aspirated off and the pellet resuspended in 1xPBS. After another five minute centrifugation, the supernatant was removed and the pellet was resuspended in ice cold extract buffer (150mM NaCl, 20mM NaPO4, 0.1% NP-40, .2mM EDTA, 1 mM PMSF, 1mM DTT) at a concentration of $5x10^4$ cells per microliter. This was then sonicated 3 times for 20 seconds, taking care to chill the extract on ice for 30 seconds after each sonication to minimize temperature increase (resulting in component denaturation) during this process.

The sonicated material was then centrifuged at 10,000 RPM for 30 seconds in a pre-chilled 4'C microcentrifuge and the supernatant was collected. If the extract was not to be immediately used, it was frozen in liquid nitrogen in 20% glycerol. This extract was boiled at a final concentration of 1xLaemmli (Laemmli 1970) and electrophoretically separated on a 5% separating, 14% resolving SDS mini-gel for approximately 1.5 hours at 100V before being transferred onto pre-soaked (5 minutes at room temperature in transfer buffer) Optitran nitrocellulose membrane (Schleicher and Schuell Bioscience, Keene, NH) in 1x western transfer buffer, 25% methanol at 125mA at room temperature overnight. Following transfer, the membrane was washed in 1xTBS for five minutes and blocked in blotto (2% Carnation Instant Milk, 0.2% Tween 20, 1xTBS) for thirty minutes at room temperature. It is essential to use Carnation Instant Milk rather than generic brand milk for blocking available sites as the former results in more highly specific, cleaner results in our hands. The membrane was then incubated in blotto with the anti-DM2 primary antibody (1:1000 dilution) for one hour at room temperature with shaking. Three five minute washes (1xTBS, 0.1% Tween 20) were followed by the one hour incubation with shaking in fresh blotto with Peroxidase-conjugated goat anti-rabbit secondary antibody (MP Biomedicals, Solon, OH) at a dilution of 1:2000 added. Three more five minute washes (1xTBS, 0.1 % Tween 20) and two subsequent ten minute washes (1xTBS) preceded membrane development with Western Lightning Chemiluminescent Reagent Plus detection system (PerkinElmer Life Sciences, Boston, MA). Results were visualized by exposure to autoradiographic film (ISO-MAX film, SciMart Inc., St. Louis, MO).

TISSUE IMMUNOFLUORESCENCE

Human muscle tissue mounted on slides were defrosted at room temperature for five minutes and then rehydrated in 1XPBS for ten minutes. After PBS washing, the cells were fixed with 4% paraformaldehyde in PBS for twenty minutes at room temperature. The cells were then blocked for 30 minutes at room temperature in immunofluorescence blotto, 2 % Carnation Instant Milk, .2% Tween 20, 1xTBS, before primary antibody was added for one hour. After washing, secondary antibody was added for one hour in immunofluorescence blotto. Finally, the samples were washed in PBS and mounted onto slides in 90% Glycerol in 1xPBS.

C_2C_{12} IMMUNOFLUORESCENCE

Cells were grown to 70% confluency overnight on autoclaved glass coverslips (#1 thickness). After a room temperature 1xPBS rinse, the cells were fixed (4% paraformaldehyde, 1xPBS) for ten minutes at room temperature, rinsed three times in 1xPBS and permeabalized (0 .1% Triton X 100 in water) for ten minutes at room temperature. Blocking and antibody incubations were performed as described for tissue immunofluorescence.

IN SITU HYBRIDIZATION IN C_2C_{12} CELLS

Exon 15 of the human DMPK gene was cloned into pBluescript SK- between the KpnI and SacI restriction sites. The resulting plasmid (termed UTR12) was digested with SacI and transcribed with T7 RNA Polymerase (New England Biolabs, Beverly, MA) to generate antisense probe or digested with KpnI and transcribed with T3 RNA Polymerase (Roche Diagnostics, Indianapolis, IN) to generate sense probe. The human 3'UTR was used because humans are the only species that actually get diseases caused by triplet

repeat expansion in 3'UTRs and other species (including mice) have mechanisms to rapidly eliminate these expansions. Antisense human desmin and myosin light chain probes were generated using T7 RNA Polymerase transcription of the EcoRI cut desmin or myosin light chain plasmid, respectively. These plasmids were generated by cloning desmin or myosin light chain sequences into the EcoRI and XhoI sites of pBluescript SK-. Probes were internally labeled by incorporation of Digoxeginin-uridine triphosphate (DIG-UTP, Roche Diagnostics, Indianapolis, IN) via *in vitro* transcription. Transcription reactions containing: 1ug linearized plasmid DNA, 40u rRNasin (Promega, Madison, WI), 10mM DTT, 2.5mM rATP, 2.5mM rGTP, 2.5mM rCTP, 1mM rUTP, 1xTranscription Buffer (supplied with RNA Polymerase), 25u RNA Polymerase and 1mM DIG-UTP to a volume of 20ul with water, was assembled on ice and incubated at 37'C for one hour to generate product. The probes were then PCA extracted once, ethanol precipitated and resuspended in 20ul TE. Prior to use, a collection of smaller sized probes were generated by partial hydrolysis of half of the transcription reaction with 1ul of 1M Sodium Hydroxide (1 minute, 37'C) and quenched with 1M Tris, pH 7. These smaller probes tend to be more accessible to the target and increase *in situ* hybridization signal intensity.

C_2C_{12} cells were grown on coverslips overnight and fixation, permeabalization and *in situ* hybridization was performed as described in Lerner et al (2003). Following probe hybridization, 14-16 hours at 55'C of 150ng probe/100ul hybridization solution) and washing, cells were blocked for one hour (3% Carnation Instant Milk, 1xPBS). Hybridized probes were visualized either fluorescently with anti-DIG-Fluorescein antibodies (Roche Diagnostics, Indianapolis, IN) at 1:250 dilution or chemically with anti-DIG Alkaline Phosphatase antibodies (Roche Diagnostics, Indianapolis, IN) at a

1:2000 dilution. Either antibody was added to the blocked samples and incubated for one hour at room temperature with shaking. After three washes with 1xPBS, immunofluorescence was performed as above (for anti-DIG-Fluorescein) or reactions were developed with Nitro blue tetrazolium chloride/ 5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt (NBT/BCIP, Roche Diagnostics, Indianapolis, IN) (for anti-DIG- Alakine Phosphate). In the latter case, NBT/BCIP solution (200ul NBT/BCIP Stock Solution in 10mL 100 mM Tris pH 9.5, 100 mM NaCl, 5 mM MgCl2) was added and samples were incubated for 48 hours in the dark. Samples were the subjected to immunofluorescence. Samples were mounted in 90% glycerol, 1xPBS and covered with a coverslip before being visualized on a Zeiss Axiophat Microscope and analyzed using IP Lab and Adobe Photoshop software or a Leica SP2 AOBS Confocal Microscope and analyzed using LCS Lite and Adobe Photoshop software.

EGFP CONSTRUCTS - GENERATION, TRANSFECTION AND VISUALIZATION

Portions of the DMPK 3'UTR were inserted into the multiple cloning site of EGFP-C1 with the SV40 UTR (between the KpnI and MluI cut sites) removed. EGFP-3'UTR corresponds to the insertion of human DMPK DNA from the NarI site to the end of the gene, an artificially inserted Xba site, into the EGFP vector multiple cloning site between the EcoRI and KpnI sites. EGFP-CTG90 is identical to EGFP-3'UTR except is has 90 uninterrupted CTG repeats verses the 5 CTG repeats in the standard UTR. This corresponds to the diseased and wild type 3'UTRs respectively. EGFP-CTG90 was created by digesting the plasmid pcDNA3-CAT-3'UTR (CTG90) (Quintero-Mora 2002), a gift from Drs. Bulmaro Cisneros and Robert G. Korneluk, with SacII and PstI, positions 183 and 305 from the DMPK stop codon respectively, and ligating this CTG repeat containing fragment in EGFP-3'UTR which had been digested with SacII and PstI.

EGFP-ΔHAIRPIN corresponds to the wild type 3'UTR construct from above (EGFP-3'UTR) with the sequence between SacII and PstI sites removed (a 122 nucleotide deletion). EGFP-HAIRPIN corresponds to the human DMPK 3'UTR from the DMPK stop codon to the HypCH4III cut site (14 nucleotides) blunted and ligated to the DMPK 3'UTR region between SacII and PstI sites (122 nucleotides) then blunted and ligated to the sequence between the AvrII site, position 678 from the stop codon, to the end of the UTR, an artificially inserted XbaI site (37 nucleotides). All construct stocks were prepared via the alkaline lysis plasmid prep method.

As needed, these constructs were transfected into 50% confluent overnight cultures of C_2C_{12} cells grown on coverslips in 6 well plates with TransIT-LT1 Transfection Reagent (Mirus Bio, Madison, WI) according to the Mirus protocol (3ug DNA to 9ul LT1 reagent per well in overnight media (2ml)) and incubated for 24 hours (37'C, 8% CO_2) before collecting the samples and performing immunofluorescence as described previously. Twenty four hours is sufficient to allow the expression, localization and translation of the EGFP protein in these cells; however, incubation time can be extended as long as 36 hours if needed (as was the case for the pDM2 RNAi knockdown effect on EGFP localization experiment) without reduction or diffusion of the signal.

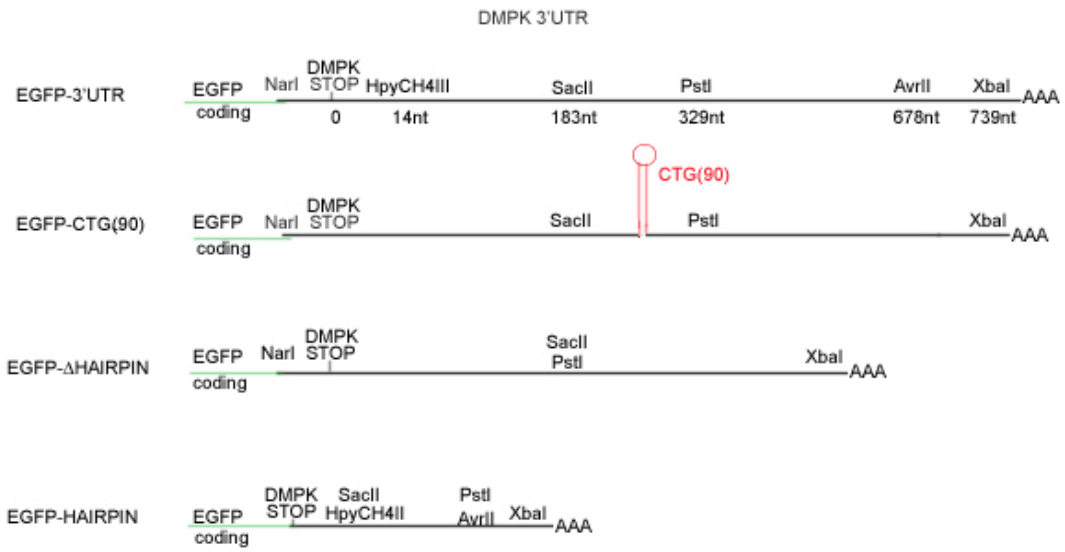

Illustration 2.1 EGFP Reporter Constructs used to Determine the Localization Capability of the DMPK 3'UTR.

Several EGFP reporter constructs were created that contained all of or portions of the *DMPK* 3'UTR instead of the SV-40 3'UTR that is usually contained in the EGFP-C1 plasmid. EGFP-3'UTR contains the *DMPK* 3'UTR in its entirety and EGFP-(CTG)90 has a CTG repeat expansion (90 repeats) precisely inserted into EGFP-3'UTR, recapitulating the DM1 disease expansion. EGFP-ΔHairpin is the entire *DMPK* 3'UTR except for the region protected by pDM2 binding. EGFP-Hairpin is the *DMPK* stoop codon followed by the pDM2 protected region and the *DMPK* poly A site.

RNAi Knockdown and Transfections

shRNA (short hairpin RNA) inserts were designed to three different areas of the CNBP mRNA, and were then cloned into pcDNA3-H1 expression vector (Invitrogen, Carlsbad, CA) between Bgl II and Hind III. The shRNA inserts contained a transcription start site and transcription termination sequence immediately flanking the shRNA 5' and 3' end, respectively. The regions used for RNAi design were 49nt in length, containing the 21nt sequence of interest, as well as its complimentary sequence to form the dsRNAi and a loop forming sequence in between. These sequences contained GC content between

30%-50%, started with a naturally occurring AA in the DNA sequence, and did not contain more than three consecutive Ts. Upon BLAST searching the mouse and human genome there was no homology to the sequences of interest chosen, preventing cross-reactivity with a message other than the one targeted. They were also to regions devoid of known protein binding sequences, were present in all mRNA spliced isoforms and were not predicted to form any major secondary structure. The sequences of the three inserts were as follows: CNBP1 5'GAAGATCTCCCAGTGTGGACGATCTGGCCATTCAAGAGATGGCCAGATCGTCCACACTTTTTTGGAACAAGCTTCTC, CNBP2 5'GAAGATCTCCCAGGACTGCAAGGAGCCCAATTCAAGAGATTGGGCTCCTTGCAGTCCTTTTTTGGAACAAGCTTCTC, CNBP3 5'GAAGATCTCCCACTGGTCATGTAGCCATCATTCAAGAGATGATGGCTACATGACCAGTTTTTTGGAACAAGCTTCTC. The three DM2 mRNA constructs target the 5'UTR, exon 2 and exon 3, respectively.

The complementary DNA of these oligonucleotides (Integrated DNA Technologies, Coralville, IA) were produced by a Klenow reaction (10ul reaction-2ug antisense oligo, 0.2 ug universal antisense primer, 2mM dNTPs, 1X Klenow buffer, 90'C 2min then 37'C 2min, add 1ul Klenow enzyme, 37'C 30min, 75'C 20min) and BglII/Hind III digested for ligation into the BglII/Hind III cut pcDNA3-H1 vector. The confirmed constructs were prepped using the Qiagen Plasmid Maxi Kit according to Qiagen instructions (Qiagen Inc., Valencia, CA).

Plasmids were independently transfected into overnight cultures of 50% confluent C_2C_{12} cells as described above and incubated for 36 hours, 37'C 8% CO_2, before harvesting the samples for subsequent *in situ* hybridization, immunofluorescence or western blot analysis. Knockdown efficacy was determined by subsequent western blot

analysis with anti-DM2 anti-sera. While efficiency varied slightly between experiments, the CNBP2 construct consistently gave the highest reduction of DM2 protein at these conditions, averaging approximately 75-80% reduction as compared to empty pcDNA3-H1 vector controls. Visualization of the DMPK or EGFP proteins after pDM2 knockdowns were performed as normal except the exposure time was increased for these knockdown samples in order to see the pattern of the remaining protein. The reduction in DMPK and EGFP proteins upon knockdown may indicate a role for the DM2 protein in the stability or translational pathway for the DMPK and EGFP-DMPK 3'UTR messages. When pDM2 RNAi plasmids were co-transfected with EGFP constructs the protocol was followed as described except only 2ug of the RNAi plasmid DNA was simultaneously transfected along with 1ug of the EGFP reporter construct.

Materials and Methods for Chapter 5

SUCROSE GRADIENTS

The 4.8mL 10-40% continuous sucrose gradients were generated in 150mM NaCl, 20mM NaPO4, pH 7 and chilled at 4'C, along with the SW 55 Ti rotor and buckets, 14-18 hours, being careful not to move or disturb them as they chilled. Sucrose gradient analyses were performed on dounced, rather than sonicated, C_2C_{12} whole cell extract. This extract was prepared by washing, trypsinizing and counting the cells as usual. However, after spinning down the cell pellet, it was resuspended in 500ul of 4'C dounce buffer (10mM KCl, 10mM Hepes, 1mM MgCl2), pipetted into a pre-chilled 1mL glass dounce. The cells were then dounced with 10 strokes on ice and 250mM sucrose was added. The solution was transferred to a chilled eppendorf tube, spun in a 4'C microfuge for 30 seconds at 10,000 RPM, and resuspended in 4'C glycerol to 8%. 200ul of freshly prepared extract ($1x10^6$ cell equivalents) was delicately layered onto pre-poured and chilled 10-40% sucrose gradient, so as not to disturb any of the sucrose layers. The gradient was run at 4'C for 14 hours, 5 min at 21,200 RPM in an ultracentrifuge. It was then fractionated by sequential removal of 400ul fractions from the top of the gradient down. Half of each fraction was increased in volume by adding 200ul TE before chloroform extracting twice and then were ethanol precipitated overnight (2.5 volumes 100% ethanol, 0.1 volume 3M NaOAc, pH 5.2, and 20ug Glycogen (Roche Diagnostics, Indianapolis, IN)). The samples were pelleted for 10 minutes at 13,000 rpm in a microfuge and the pellets were ethanol washed in 70% ethanol before being resuspended in 20ul TE and subjected to northern blot analysis. The remaining half of each fraction was suspended in SDS Loading Buffer to a final concentration of

1xLaemmli buffer and analyzed by western blot analysis. Under these gradient conditions, ribosomes should pellet at the bottom (fraction 12), hnRNPs should run just above the middle (approximately fractions 4/5) and free protein should sit at the top of the gradient in fraction 1.

NORTHERN BLOT ANALYSIS

Phenol chloroform extracted, ethanol precipitated extract, the amount varied between 5 and 15ul depending on the sample concentration, was suspended in 1X RNA loading buffer, heated at 65'C for 3 minutes and electrophoretically fractionated through a pre-run 20x20cm, 3mm thick, 4% polyacrylimide (40:2) 8M Urea gel in 1/2xTBE running buffer. The gel was run hot (approximately 1000V) until the xylene cyanol dye in the RNA loading buffer reaches the bottom of the gel and then more RNA sample buffer was loaded into the wells (5ul of plain buffer with no RNA resuspended in it) and the gel was continued to run until this second xylene cyanol dye front reached the bottom of the gel. The gel was then electrophoretically transferred to pre-soaked (5 minutes in transfer buffer) nylon membrane (Amersham HyBond N+, GE Healthcare, Piscataway, NJ) 14 to 18 hours in 25mM Sodium Phosphate pH 7.0 at 12V at room temperature. The membrane was blotted dry, wrapped in plastic wrap and crosslinked approximately 10cm from the UV source on the "Auto Crosslink" setting of the Stratalinker (UV Stratlinker 2400, Stratagene, La Jolla, CA) . It was then rehydrated in 6xSSC for ten minutes at room temperature and pre-hybed at 60'C for 3-6 hours, shaking or rotating in Northern Hybe solution (5x SSC, 50% deionized formamide, 5xDenhardt's Solution, 1% SDS and 500ug denatured salmon sperm DNA).

In vitro transcribed internally radiolabelled sense DMPK riboprobe was generated using NcoI restriction of the Weiringa 1.1 plasmid (Jansen 1992) in a transcription

reaction containing: 1ug linearized plasmid DNA, 40u rRNasin (Promega, Madison, WI), 10mM DTT, 2.5mM rATP, .1mM rGTP, 2.5mM rCTP, 2.5mM rUTP, 1xTranscription Buffer (supplied with RNA Polymerase), 25u T7 RNA Polymerase (New England Biolabs, Beverly, MA) and 5 uCi of [alpha-32P]GTP (800 Ci/mmol) (GE Healthcare, Piscataway, NJ) to a volume of 20ul with water, was assembled on ice and incubated at 37'C for one hour to generate product. The entire transcription reaction was added to the pre-Hybed reaction and incubated at 60'C for 14-18 hours shaking or rotating. After removal of the Hybe solution and probe, the nylon was washed twice in .2xSSC, .1%SDS followed by two 2xSSC, .1% SDS washes and a 2xSSC wash, for five minutes each at room temperature with shaking. The nylon membrane was then blotted to remove excess liquid, covered with plastic wrap and placed on a PhosphoImager screen overnight. The data was read by a GE Typhoon Scanner (GE Healthcare, Piscataway, NJ) and analyzed using Quantity One Software (BioRad Laboratories, Hercules, CA).

Alternatively, for a slot blot Northern, nucleic acid pools from each fraction was treated with RQ1 RNase- free DNase (Promega, Madison, WI) according to manufacturer's instructions. The RNA was then suspended in 3 volumes of denaturing solution (500ul deionized formamide, 162ul 12.3M formaldehyde, 100ul MOPS buffer) and denatured for 15 minutes at 65'C. Two volumes of ice cold 20xSSC was then added to each sample and they were loaded into individual wells of the Slot Blotter (Schleicher and Schuell Bioscience, Keene, NH) for transfer onto a 10xSSC wetted nylon membrane (Amersham HyBond N+, GE Healthcare, Piscataway, NJ). The wells were washed with 2mL of 10xSSC and the membrane was allowed to air dry. From this point the procedure is identical to that described above.

IMMUNOPRECIPITATIONS

Antibody Conjugates

Antibody conjugates were prepared by incubating 2.5 mg Protein A Sepharose 4B (Pharmacia,) in 500 ul NET-2 for 10 min in 1.5mL eppendorf tubes at room temperature with nutation, then adding 10ul sera and nutating for two hours. These conjugates were then washed once in 1ml of IPP buffer and three times in 1 ml of NET-2 buffer before the addition of the IP reaction (see below). Antibody conjugates were first chemically crosslinked with dimethyl pimelidate dihydrochloride as described in the protocol of Harlow and Lane before immunoprecipitating for the protein IP samples that were to be western blotted with an antibody that was produced in the same species of animal as the immunoprecipitation antibody. This is necessary because the immunoprecipitating antibody will be stripped off of the Protein A Sepharose beads during the protein extraction steps, along with the proteins recovered from the immunoprecipitation reaction. The IP antibody is typically orders of magnitude more abundant than the experimentally recovered proteins. If these immunoprecipitation antibodies are of the same species as the western blotting antibodies, then the HRP-conjugated secondary antibody will cross react with the constant regions of the abundant immunoprecipitation antibody, overwhelming the visualization of the experimental proteins. Chemically crosslinking the IP antibodies to the Protein A Sepharose beads prevents them from being stripped off by the protein extraction procedure, thus avoiding this visualization problem. Chemically crosslinking the Protein A Sepharose and antisera is not necessary for nucleic acid immunoprecipitation experiments.

Endogenous Transcript Immunoprecipitation Reactions

Extract was prepared by sonication as described previously and approximately $5x10^6$ cell equivalents of extract (100ul) were added to anti-DM2/Protein A Sepharose antibody conjugates in 1.5mL eppendorf tubes. Reactions were rotated for one hour on rotator () at 4'C, with gentle agitation every twenty minutes. The samples were tap spun in a microfuge and 10ul of each reaction was removed, phenol chloroform extracted and the aqueous layer ethanol precipitated. These samples represented the total amounts of RNA present in each of the immunoprecipitation reactions. The IP reactions were washed five times in 1ml aliquots of cold 200mM NET-2 buffer before subsequently phenol chloroform extracting the Protein A Sepharose conjugate linked beads and ethanol precipitating the nucleic acid (2.5 volumes ice cold 100% ethanol, 0.1 volume 3M NaOAc, pH5 and 20ug Glycogen (Roche Diagnostics, Indianapolis, IN)) or boiling for ten minutes in 1xSDS loading buffer for protein isolation. The extracted nucleic acid samples were then analyzed by northern blotting with the radiolabelled *DMPK* antisense probe while the boiled samples were loaded onto SDS gels and analyzed by western blotting.

Exogenous Transcript Immunoprecipitation Reactions

A transcript of the entire *DMPK* 3'UTR was generated by *in vitro* transcription of a XbaI linearized *DMPK* 3'UTR plasmid in a transcription reaction containing: 1ug linearized plasmid DNA, 40u rRNasin (Promega, Madison, WI), 10mM DTT, 2.5mM rATP, .1mM rGTP, 2.5mM rCTP, 2.5mM rUTP, 1xTranscription Buffer (supplied with RNA Polymerase), 25u T3 RNA polymerase (Roche Diagnostics, Indianapolis, IN) and 5 uCi of [alpha-32P]GTP (800 Ci/mmol) (GE Healthcare, Piscataway, NJ) to a volume of 20ul with water, was assembled on ice and incubated at 37'C for one hour to generate

product. RNA loading buffer was added (to a 1x concentration) to the transcription reaction and it was heated for 3 minutes at 65'C before being loaded onto a pre-run 20x20cm 4% polyacrylamide (40:2) 8M Urea gel and run warm (1000V) in ½xTBE buffer until the xylene cyanol dye front reached the bottom of the gel. The gel was then placed on film for 1 minute to visualize the RNA and the RNA band was cut out of the gel. The excised gel slice was incubated in a 42'C water bath overnight in 250ul Crush N'Soak to extract the RNA from the polyacrylamide gel. After phenol chloroform extraction of the Crush N' Soak and ethanol precipitation of the aqueous fraction (2.5 volumes ice cold 100% ethanol, 0.1 volume 3M NaOAc, pH5 and 20ug Glycogen (Roche Diagnostics, Indianapolis, IN)) , the transcript was pelleted then washed in ice cold 100% ethanol and resuspended in 10ul of TE.

Run-off transcripts were created by linearizing the DMPK 3'UTR plasmid with the restriction endonucleases specified in figures 5.5 and 5.10 and performing *in vitro* transcription as above.

The 50ul immunoprecipitation complex reaction was assembled on ice (30ul of frozen C_2C_{12} whole cell extract in 20% glycerol, containing $1.5x10^6$ cell equivalents, 1ul of the freshly made *in vitro* DMPK 3'UTR transcript, 2mM ATP, 7.5ug tRNA, 1mM DTT, 5mM $MgCl_2$, 5ul 10xGlycerol compensation buffer) and incubated for 15 minutes at 30'C in a circulating water bath to facilitate complex formation. Reactions were then added to the prepared antibody conjugates plus 25ul NET-2 buffer and immunoprecipitated as above. The phenol chloroform extracted, ethanol precipitated samples were resuspended in 1xRNA loading buffer, heated at 65'C for 3 minutes and electrophoretically separated on pre-run 3mm thick, 33x40cm 4% polyacrylamide (40:2) 8M urea gels run in ½xTBE buffer at approximately 34mA until the xylene cyanol dye front reached ¾ of the way through the gel. It was then dried for 1 hour at 60'C and

exposed to X-ray film (ISO-MAX film, SciMart Inc., St. Louis, MO) for autoradiographic analysis.

The amounts in the immunoprecipitation reaction can also be halved, reducing the complexing reaction to 25ul rather than 50ul and the immunoprecipitation volume to 37.5ul instead of 75ul with little immunoprecipitation efficiency loss. In this situation, the antibody conjugates are prepared as normal, but only 12.5ul of additional NET-2 is added to the conjugates when the complex reaction is added.

CTG Repeat Competition Immunoprecipitation

The competition immunoprecipitation experiment between the normal, full length *DMPK* 3'UTR transcript and the (CUG)90 containing full length *DMPK* 3'UTR transcript was performed in a very similar manner as the other exogenous transcript immunoprecipitation reactions. The DMPK (CUG)90 3'UTR was created by digesting the DMPK CTG90 plasmid with NruI and performing an *in vitro* transcription reaction containing 1ug linearized plasmid DNA, 40u rRNasin (Promega, Madison, WI), 10mM DTT, 5 mM rATP, 2mM rGTP, 5mM rCTP, 5mM rUTP, 1xTranscription Buffer (supplied with RNA Polymerase), 25u T7 RNA polymerase (New England Biolabs, Beverly, MA) and 7.5 uCi of [alpha-32P]GTP (800 Ci/mmol) (GE Healthcare, Piscataway, NJ) to a volume of 20ul with water, was assembled on ice and incubated at 40'C for one hour to generate product. The transcript was gel purified and prepared for immunoprecipitation as described.

The immunoprecipitation reaction was identical to that described previously for exogenous transcript immunoprecipitation; however, 1ul of wild type *DMPK* 3'UTR and 1ul of (CUG)90 *DMPK* 3'UTR were both added to the complex reaction. The reaction volumes and immunoprecipitation protocol remained unchanged.

PROTECTION IMMUNOSELECTION

Immunoprecipitation reactions were performed as described for exogenous transcripts above except that the reactions are treated with RNase T1 after complex formation and before addition to the antibody conjugates and immunoprecipitation. In this case, the samples were placed on ice after the fifteen minute complex formation incubation and 180 units of RNase T1 (Calbiochem RNase T1, EMD Chemicals, San Diego, CA) were added to each reaction. This was incubated for precisely five minutes on ice, immediately diluted with 100ul NET-2 and added to the antibody conjugates for immunoprecipitation as described previously. The extracted and precipitated samples were separated on pre-run 3mm thick, 33x40cm 15% polyacrylamide (38:2) 8M urea gels run warm in 1/2xTBE buffer (at approximately 34mA) until the xylene cyanol dye front reached 2/3 of the way down the gel. The gel was then dried for 3 hours at 50'C, placed on X-ray film (ISO-MAX film, SciMart Inc., St. Louis, MO) and analyzed by autoradiography.

RNASE H OLIGONUCLEOTIDE MAPPING

Taking advantage of the specific RNA-DNA duplex nuclease activity of RNase H, we designed ten DNA oligonucleotides that corresponded to particular regions of the *DMPK* 3'UTR (see figure 5.7), bound them to *in vitro* transcripts of the DMPK 3'UTR and treated them with RNase H to create a deletion mutant transcript. First, one twentieth of the extracted and precipitated transcript created by a radiolabelled DMPK 3'UTR *in vitro* transcription reaction described previously (1ul) and 10-20ug/mL of DNA oligonucleotide (1ul) were boiled together in a 1.5ml eppendorf tube for five minutes and

allowed to cool on the bench for ten minutes. The RNase H reaction was performed on the cooled potential RNA-DNA duplexes (RNA-DNA duplex, 2.5ul extract buffer (150mM NaCl, 20mM NaPO4, 0.1% NP-40, .2mM EDTA, 1 mM PMSF, 1mM DTT), .5ul 100mM DTT, 20U rRNasin, .5U RNase H) in a circulating 37'C water bath for 20 minutes. This reaction was then supplemented with 15ul of frozen C_2C_{12} extract ($7.5x10^5$ cell equivalents) in 20% glycerol, 2mM ATP, 3.75ug tRNA and allowed to complex for fifteen minutes in a 30'C water bath. Immunoprecipitation was performed and the samples were analyzed on 4% polyacrylamide denaturing PAGE and autoradiography as described for exogenous transcript immunoprecipitation.

Antibody Target	Manufacturer	Working Dilutions Western	C_2C_{12} IF	Tissue IF	IP Amount
Primary antibodies					
BIP (GRP78)	Santa Cruz	n/a	1:1000	1:100	n/a
B-tubulin	Chemicon	1:6000	n/a	1:100	n/a
Calnexin	Santa Cruz	n/a	n/a	1:100	n/a
Calsequestrin	Santa Cruz	n/a	1:1000	1:100	n/a
DM2	Gottlieb Lab	1:2000	1:1000	1:100	15ul
DM2	Novus	1:500	1:1000	1:100	15ul
DMPK	Gottlieb Lab	1:1500	1:1000	1:100	5ul
Dynein	Chemicon	1:15000	n/a	n/a	15ul
HnRNP c1/c2	Santa Cruz	1:500	n/a	n/a	10ul
KDEL	Stressgen	n/a	1:1000	1:100	n/a
Kinesin	Chemicon	1:1000	n/a	n/a	15ul
MBNL 1-3	Santa Cruz	1:2000	1:500	1:50	15ul
PDI	Stressgen	1:500	1:1000	1:100	n/a
SERCA 2	ABR	n/a	1:1000	1:100	n/a
Secondary antibodies					
rabbit anti-goat Rhodamine	ICN Cappell	n/a	1:1500	1:200	n/a
goat anti-mouse Rhodamine	ICN Cappell	n/a	1:1500	1:200	n/a
chicken anti-rabbit Fluorescein	Chemicon	n/a	1:1500	1:200	n/a
sheep anti-mouse Fluorescein	ICN Cappell	n/a	1:1500	1:200	n/a
rabbit anti-goat HRP	ICN Cappell	1:2000	n/a	n/a	n/a
goat anti-mouse HRP	ICN Cappell	1:2000	n/a	n/a	n/a
goat anti-rabbit HRP	ICN Cappell	1:2000	n/a	n/a	n/a
sheep anti-DIG Fluorescein	Roche	n/a	1:200	1:150	n/a
sheep anti-DIG AP	Roche	n/a	1:2500	1:3000	n/a

Table 2.1 Antibodies used in this dissertation

Solution Name	Recipe
Crush N' Soak (50mL)	8.3mL 3M NaOAc 2.5mL 200mM EDTA 500uL 1M Tris pH 7.5 100uL 10% SDS
50 x Denhardt's Solution (1L)	10.0g Ficoll 400 10.0g Polyvinilpyrolidone 10.0g Bovine Serum Albumin
10 x glycerol compensation buffer (200uL)	10.4uL 1M NaPO4 5.2uL 10% NP-40 78uL 1M NaCl
IPP Buffer (1L)	125mL 4M NaCl 10mL 1M Tris pH 8.0 10mL 10% NP-40
10 x Laemmli (1L)	30.0g Tris 10.0g SDS 144.2g Glycine
MOPS Buffer (100mL)	2M MOPS pH7 500mM NaOAc pH 5.2 10mM EDTA pH 8.0
NET-2, pH 7.5 (1L)	6.0g Tris 8.7g NaCl 5mL 10% NP-40
10xPBS, pH 7.4 (1L)	325mL 4M NaCl 200mL KPO4 buffer (pH 7.4)

1 x RNA Loading Buffer (1mL)	0.6g Urea 50ul 10 x TBE 1.25ul 200mM EDTA, pH 8.0 100ul 0.25% Bromophenol Blue 100ul 0.25% Xylene Cyanol
5 x SDS Loading Buffer (10mL)	1.5mL 2M Tris pH 6.8 1.0g SDS 5mL glycerol 1.4mL β-mercapitalethanol 100ul 10% Bromophenol Blue
20xSSC, pH 7.0 (1L)	175.3g of NaCl 88.2g of sodium citrate
10 x TBE, pH 8.3 (1L)	121.1g Tris 61.8g Boric acid 7.44g EDTA
10 x TBS, pH 7.4 (1L)	75.9g of NaCl 48.4g of Tris base
TE (1L)	10ml 1M Tris pH 8.0 400ul 250m M EDTA pH 8.0
20 x Western Transfer Buffer (1L)	30.28g Tris 142.63g Glycine

SDS PAGE Mini-Gels

Stacking (5%)

500uL Acrylamide (30:.8)
625uL 500mM Tris pH 6.7
25uL 10% SDS
1.3mL Autoclaved water

Resolving (14%)

2.4mL Acrylamide (30:.8)
1.95mL 1M Tris pH 8.7
50uL 10% SDS
600uL Autoclaved water

Denaturing Polyacrylamide Gels 33x40cm (half for 20x20cm gels)

4% (100mL)

10mL Acrylamide (40:2)
5mL 10xTBE
125uL 200mM EDTA pH 8.0
48g Urea

15% (100mL)

39.5mL Acrylamide (38:2)
5mL 10xTBE
125uL 200mM EDTA pH 8.0
48g Urea

Table 2.2 Solutions used in this dissertation

CHAPTER 3: DMPK IS SPECIFICALLY LOCALIZED/MISLOCALIZED IN DM1 PATIENT TISSUE

Overview

mRNA localization is a process whereby particular messages are positioned within the cytoplasm to impart distinct cellular or subcellular properties (Bashirullah, 1998; Kloc 2002). This post-transcriptional process is fundamentally important in development (flies, frogs, yeast) and differentiation (epithelia, nerves, muscle). Disrupted localization of key developmental mRNAs causes severe birth defects and infertility; analogously, disruption of specific mRNA's localization in differentiated tissues could result in human disorders, such as those of nerves and/or muscles. Since typical DM patients carry their $(CTG)_n$ expansion in the *DM kinase* gene's 3'UTR, we hypothesized that the corresponding $(CUG)_n$ repeat in the RNA may either disrupt an RNA localization signal or create a new one, resulting in altered *DMPK* mRNA cytoplasmic distribution.

Here we demonstrate that in muscle from multiple DM patients *DM-kinase* mRNA is mislocalized sub-cytoplasmically to sarcoplasmic masses characteristic of DM pathology. Mislocalization is specific to *DMPK* mRNA and appears limited to type I muscle fibers, which are predominantly affected in these patients. Our data redirect attention to the cytoplasm as a primary site of DM pathogenesis and suggest a simple situation whereby the DM 3'UTR mutation directly alters a step in *DMPK* mRNA post-transcriptional gene expression, namely cytoplasmic mRNA localization. As a result, DMPK protein is synthesized at a new location where it can potentially phosphorylate abnormal substrates leading to disease pathology. This scenario explains why the $(CTG)_n$ expansion causing DM occurs exclusively in the *DMPK* gene of afflicted patients,

specifically incorporates a function for the encoded *DMPK* protein and provides a rational framework to understand several recent observations in the literature. DM is, to our knowledge, the first human disease where a naturally occurring 3'UTR mutation in the disease gene is documented to cause cytoplasmic mRNA mistargeting.

Results

DM KINASE mRNA IS SUBCELLULARLY LOCALIZED WITHIN MUSCLE CYTOPLASM AND LOCALLY TRANSLATED

To determine whether *DM kinase* mRNA is normally localized subcellularly, human muscle biopsies were subjected to *in situ* hybridization (Figure 3.1). *DMPK* mRNA was indeed localized exhibiting a striated (sarcomeric) pattern in longitudinal sections (Figure. 3.1L). Signal was specific since it was detected with the *DMPK* antisense but not the sense riboprobe. Moreover, there was a direct correlation between *DMPK* signal and the presence of intact RNA within biopsied tissue as revealed by propidium iodine staining (data not shown).

The major reason to localize an RNA is to spatially control its translation. Anti-DMPK immunofluorescence revealed DMPK protein was localized likewise in striations (Figure 3.1L). Superposition of digitally colorized *DMPK* mRNA and protein profiles from doubly labeled sections indicated *DMPK* mRNA and protein reside in identical striations, consistent with a localized mRNA being translationally deployed to produce spatially restricted protein. Moreover, DMPK protein does not possess any known protein trafficking motifs to provide an alternative explanation for its localization. In contrast, no

discrete localization was apparent in normal transversely sectioned muscle (Figure 3.1T) where *DMPK* mRNA and protein exhibited uniform stippling, consistent with bisection of the RNA and protein striations seen in longitudinal sections.

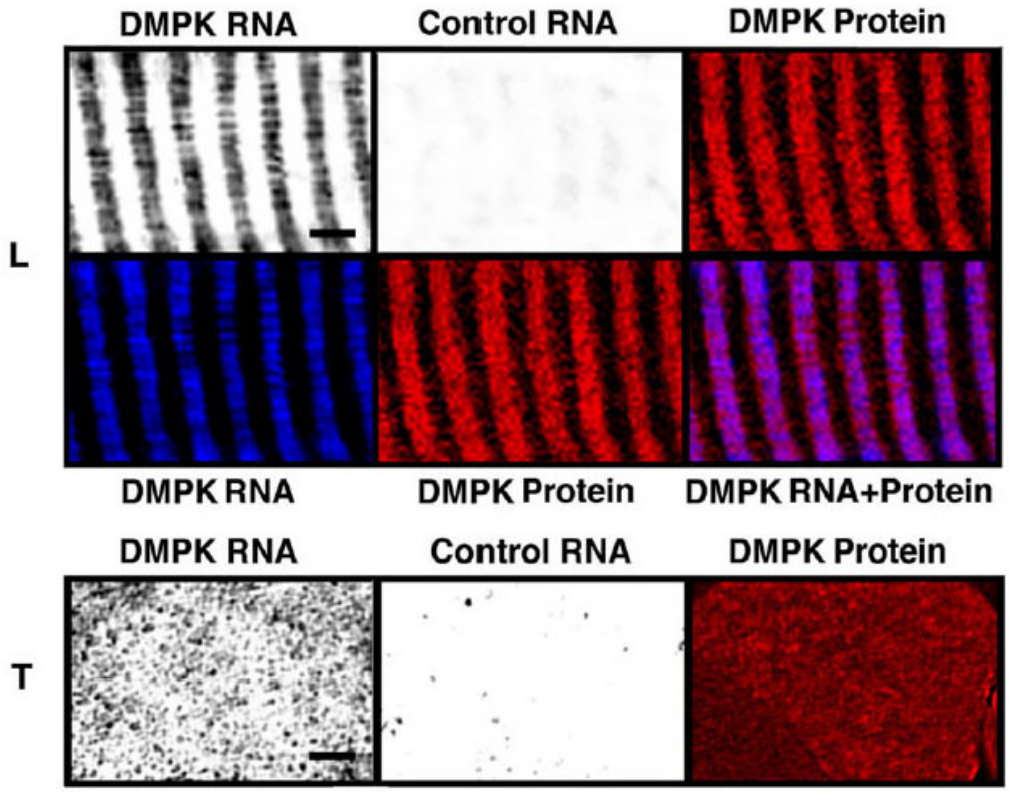

Figure 3.1 DM-kinase mRNA is localized subcellularly in normal human muscle.

Confocal microscopy revealed *DMPK* RNA is localized within normal muscle. L (longitudinal sections), Upper panel: *DMPK* RNA and protein in a double-labeled section. A consecutive section was probed with *DMPK* sense RNA (control); Black bar (2 um). Lower panel: The RNA profile (above) digitally colorized allowed electronic merging of RNA (blue) and protein (red) patterns. T (transverse sections), consecutive sections were either labeled with DMPK antisense or sense (control) riboprobes and anti-DMPK-1 antibody; Black bar (10 um).

To test whether this protein localization pattern reflected DMPK protein, we obtained three independent anti-DMPK antibodies; all recognized DMPK protein in western blots (Figure 3.2A) and detected DMPK protein striations following immunofluorescence on normal longitudinal muscle sections (Figure 3.2B). Further, DMPK protein colocalized with, SERCA II ATPase, a sarcoplasmic reticulum marker (Figure 3.2B, DMPK+SR) by coimmunofluorescence, suggesting *DMPK* mRNA and protein both reside at or near the sarcoplasmic reticulum.

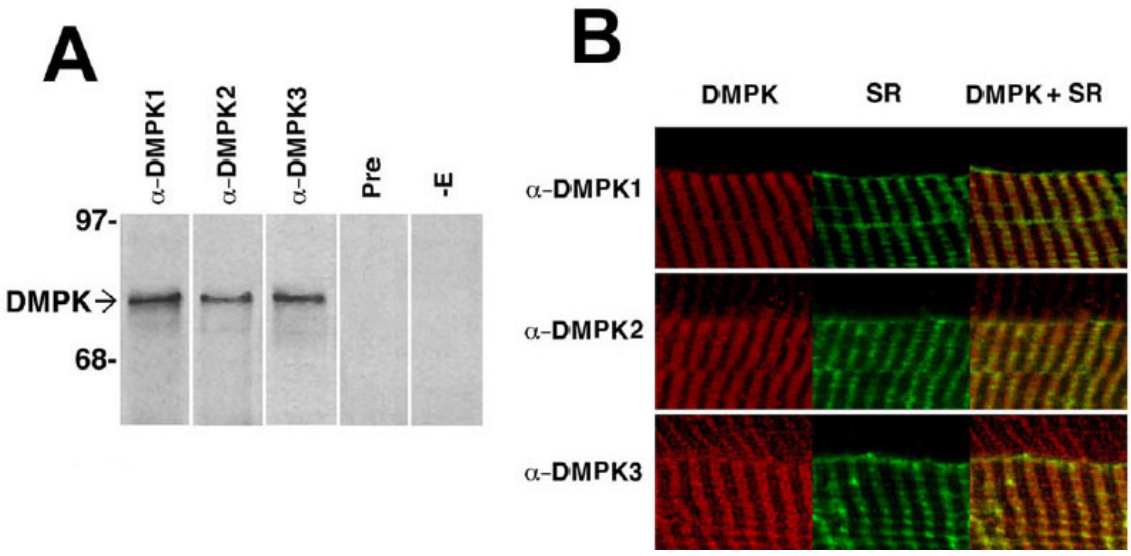

Figure 3.2 DMPK protein is subcellularly localized to the SR of human muscle tissue.

(A) All three anti-DMPK antibodies specifically recognize an ~80 kD DMPK protein in biceps extract via western blot which is undetected with pre-immune serum (pre) or without extract (E-). This size agrees with reports that muscle DMPK protein is modified and has a higher molecular weight than predicted from its amino acid sequence (Lam et al, 2000). (B) DMPK protein overlaps a sarcoplasmic reticulum (SR) marker. Longitudinal muscle sections were independently double-labeled with three anti-DMPK (red) and SR marker (green) antibodies. Digitally merged images revealed colocalization (DMPK+SR; yellow) for all antibodies (anti-DMPK 1,2,3).

DM KINASE MRNA IS MISLOCALIZED IN DM1 MUSCLE TO AREAS OF DISEASE PATHOLOGY

To see if *DMPK* RNA is mislocalized in diseased tissue, *in situ* hybridization was performed on DM1 patient muscle. Strikingly, this revealed an altered *DMPK* RNA localization pattern with high local *DMPK* mRNA concentrations in transverse sections (Figure 3.3A, *DMPK* RNA). This contrasts with the uniform *DMPK* RNA stippling in normal biopsies. *DMPK* mRNA mislocalization is an intrinsic property of DM1 patient muscle: mislocalized *DMPK* mRNA was observed in multiple sections from a single patient (not shown) and in three separate DM1 patients (Figure 3.3A). In every patient, mislocalized *DMPK* mRNA was translated generating DMPK protein at new subcellular positions, coincident with *DMPK* mRNA (Figure 3.3A, DMPK protein). RNase (Figure 3.3B) or protease (Figure 3.3C) treatment of tissue prior to *in situ* hybridization or immunofluorescence confirmed that the observed signals do in fact reflect detection of endogenous *DMPK* RNA and protein, respectively.

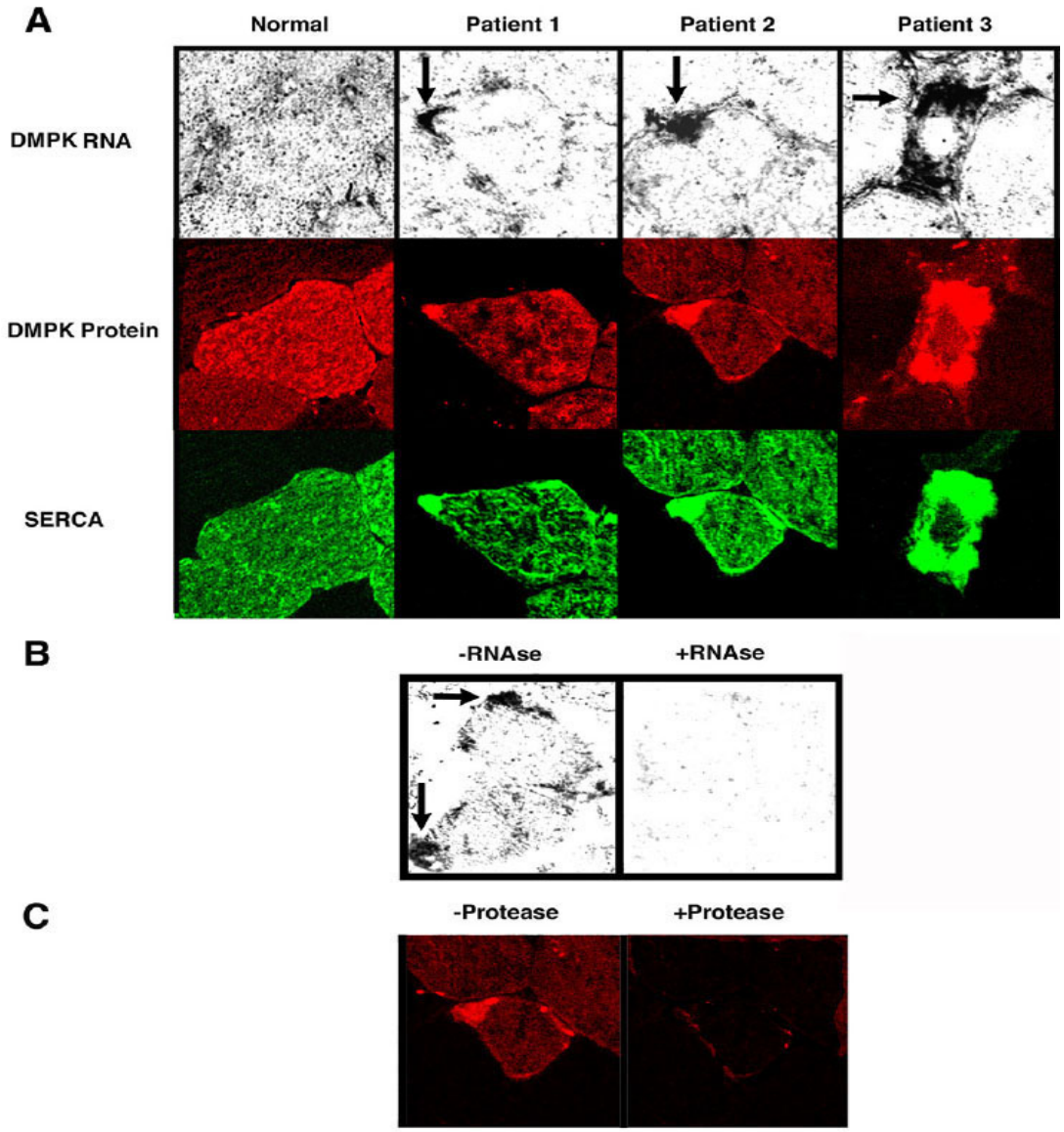

Figure 3.3 DMPK mRNA is selectively mislocalized in DM1 patient muscle.

(A) *DMPK* mRNA mislocalization (DMPK RNA) in transverse sections from DM1 patients (patient 1,2,3); no mislocalization occurred in normal individuals (normal). Consecutive sections double-labeled with anti-DMPK (DMPK protein; red) and anti-sarcoplasmic mass marker (SERCA; green) antibodies revealed mislocalized RNA (black) is expressed and occurs in sarcoplasmic masses, characteristic of DM pathology. (B) Tissue treatment with RNase A prior to *in situ* hybridization eliminated *DMPK* RNA staining. (C) Tissue pretreatment with protease (proteinase K) eliminated anti-DMPK antibody staining.

In DM1 muscle, DMPK protein colocalized with SERCA II ATPase (Figure 3.3A SERCA), a marker for the sarcoplasmic masses (Jorgensen 1988) characteristic of myotonic dystrophy pathology. These masses are one of the best morphological markers of DM (Cassanova 1979), they are specific for myotonic dystrophy (Cassanova 1979; Mussini 1970; Samaha 1967). DMPK protein was visualized in sarcoplasmic masses with all three anti-DMPK antibodies (Figure 3.4). Sarcoplasmic masses are enriched in ribosomes as indicated by 28S rRNA *in situ* hybridization (TR and EG, not shown) and by electron microscopy (Mussini 1970) so that they could support localized *DMPK* mRNA translation into protein. DMPK protein detection in sarcoplasmic masses is consistent with two previous reports (Dunne 1996; Ueda 1999). Our observations on both the RNA and protein levels in multiple patients indicate that *DMPK* mRNA is, indeed, mislocalized and provides a source for subsequent DMPK protein expression in areas of pathological significance within DM1 tissue.

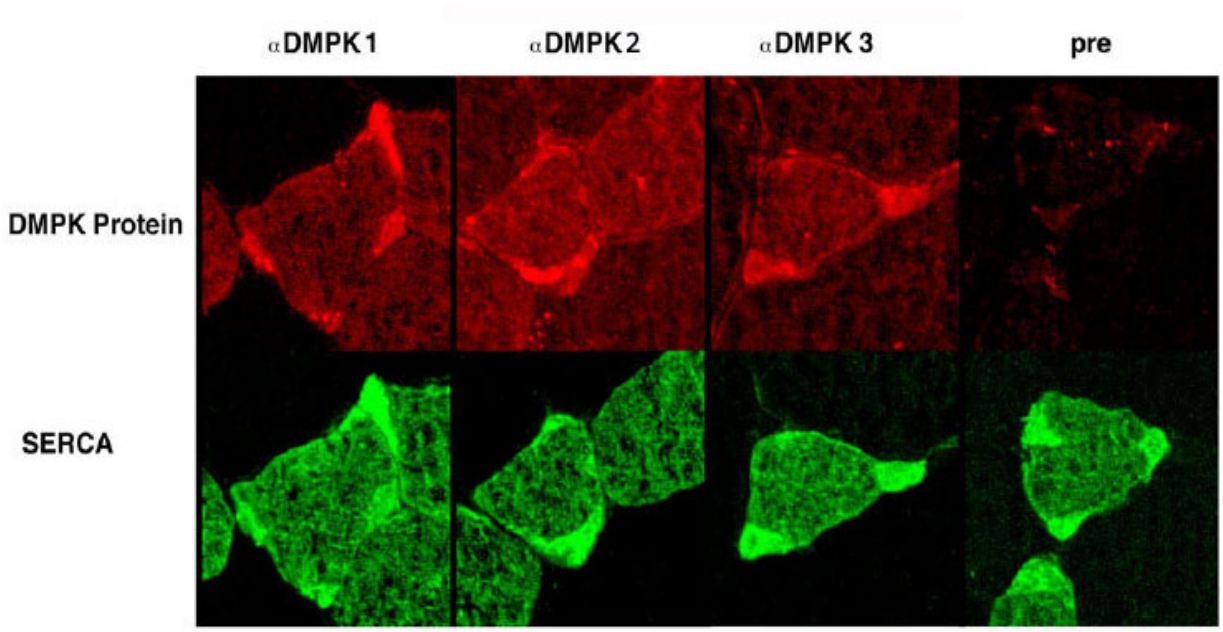

Figure 3.4 DMPK protein product is selectively mislocalized in DM1 patient muscle.

Anti-DMPK antibodies (anti-DMPK 1,2,3) but not pre-immune (pre) serum show DMPK protein in sarcoplasmic masses. Anti-SERCA immunostaining (green; identical sections) marked sarcoplasmic masses. Transverse sections were from normal or DM1 patients.

SPECIFICITY OF DMPK MISLOCALIZATION IN DM1 TISSUE

Several abundant muscle constituents display a cellular distribution distinct from DMPK underscoring the specificity of *DMPK* mRNA localization to sarcoplasmic masses (Figure 3.5A, RNA). Specifically, *actin* mRNA which is localized in several systems and *utropin* mRNA which is up-regulated in DM patients, were not concentrated in sarcoplasmic masses; nor were actin and utropin proteins (Figure 3.5A, protein).

Similarly, the muscle specific *Myosin heavy chain* RNA and the SR localized *Desmin* RNA failed to concentrate in sarcoplasmic masses, with the antisense probe exhibiting uniform fiber staining (data not shown). Sarcoplasmic masses are not merely a massive sarcoplasmic reticulum reorganization with passive *DMPK* RNA association. They contain components such as mitochondria that are missing from the sarcoplasmic reticulum. Several resident sarcoplasmic reticulum proteins colocalized with DMPK protein in normal muscle but did not mislocalize to sarcoplasmic masses in patient muscle (Table 3.1). Our results are not an artifact of some sarcoplasmic mass-related phosphatase activity since NBT/BCIP development following RNA hybridization without a riboprobe failed to exhibit any sarcoplasmic mass staining over background (Figure 3.5B). Likewise, they do not reflect sarcoplasmic mass stickiness: DMPK signal by immunofluorescence required our primary antibody (Figure 3.5C; -α) and immunofluorescence using anti-myosin heavy chain antibodies revealed that this major muscle constituent was totally absent from sarcoplasmic masses while residing in muscle fibers outside the area (Figure 3.5C; MHC). Together, these findings highlight the specific nature of *DM kinase* mRNA mislocalization to sarcoplasmic masses.

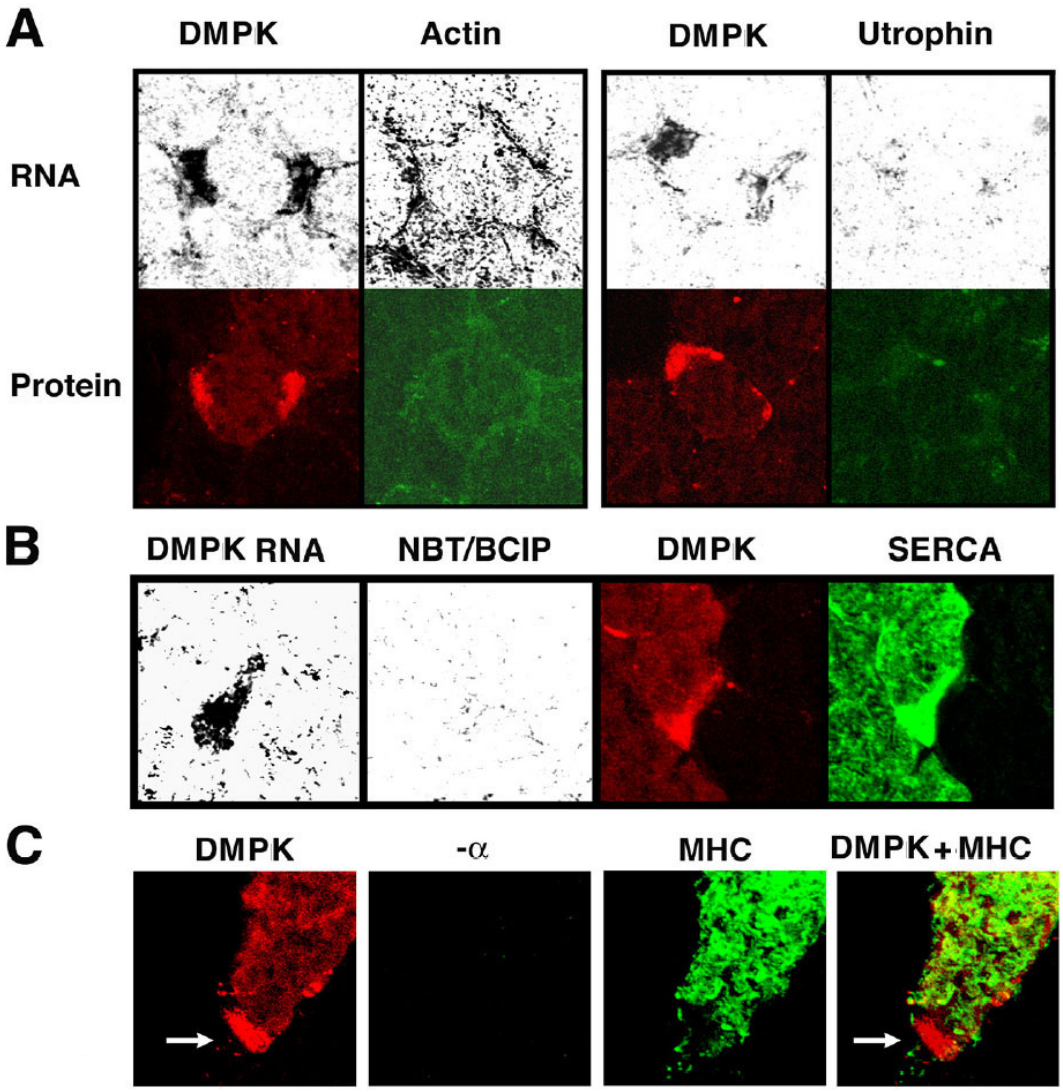

Figure 3.5 DMPK (mis)localization to sarcoplasmic masses in DM patient tissue is specific.

(A) In contrast to *DMPK*, two muscle-specific RNAs (alpha-actin; utropin) are not mislocalized to sarcoplasmic masses (RNA) or expressed there (protein). Sense probes of all mRNAs revealed no specific staining here or elsewhere in the paper. (B) The *in situ* hybridization *DMPK* signal found in sarcoplasmic masses (DMPK RNA) requires anti-sense *DMPK* RNA probe for visualization since *in situ* hybridization without probe does not stain sarcoplasmic masses. (C) Sarcoplasmic masses are not sites of general antibody stickiness: absence of the primary antibody (-α) eliminates anti-DMPK staining and anti-MHC immunofluorescence reveals this major muscle constituent is specifically excluded from sarcoplasmic masses. White arrow (sarcoplasmic masses).

Protein Name		SR Location		Sarcoplasmic Masses?
Grp78 (Bip)		longitudinal and terminal cisternae		No
Calsequestrin		terminal cisternae		No
PDI (Protein Disulfide Isomerase)		SR total lumen		No
KDEL		SR total lumen		No
Calnexin		longitudinal and terminal cisternae membrane		Yes

Table 3.1 DMPK protein localization to SMs is specific.

The presence of several classic SR resident proteins in sarcoplasmic masses was tested via immunofluorescence. Of these tested only one, calnexin, was seen in sarcoplasmic masses with the DMPK protein.

$(CUG)_N$-CONTAINING RNA BUT NOT CUG-BINDING PROTEIN IS ENRICHED IN SARCOPLASMIC MASSES

Three additional findings are pertinent to our hypothesis for myotonic dystrophy's etiology (Figure 3.6). First, DM patients contain wild type and $(CUG)_n$ expansion-harboring *DMPK* mRNAs from their wild type and mutant *DMPK* alleles, respectively; it seems logical that mutant *DMPK* mRNA is preferentially mislocalized to sarcoplasmic masses. Indeed, our *in situ* hybridization analyses of DM1 tissue samples revealed concentrated areas of *DMPK* mRNA that was accompanied by stippling outside the sarcoplasmic masses (Figure 3.3A). Such stippling occurred at a density significantly less than the stippling in normal patient material (Figure 3.1T and 3.3A). This is consistent

with repositioning of a subset of *DMPK* mRNA in patient muscle. Since all the sequences contained in the wild-type message occur in the mutant message as well, no probe for the wild-type mRNA exists that will detect the wild-type RNA over the mutant *DMPK* message. However, a probe complementary to the CUG repeat expansion has been employed to preferentially detect mutant *DMPK* transcripts (Taneja 1995). *In situ* hybridization of transverse DM1 muscle with such an anti-$(CUG)_n$ probe showed CUG-containing mRNA heavily concentrated in sarcoplasmic masses (Figure 3.6A, CUG RNA, DM patient). Since the same probe revealed background staining in normal tissue (Figure 3.6A, CUG, normal) and only background staining occurred with the anti-CAG probe on tissue samples from both DM1 and control patients, this suggests that the mutant *DMPK* message is highly localized to sarcoplasmic masses in DM1 tissue. Second, combined *in situ* hybridization and immunofluorescence showed that while *DMPK* mRNA is expressed in all muscle fibers, it is visibly mislocalized to sarcoplasmic masses exclusively in type I but not in type II skeletal muscle fibers (Figure 3.6B). This is consistent with the observation that type I fibers are preferentially affected in DM patients. Third, it has been postulated that DM etiology might be due partly to *DMPK* mRNA's (CUG) expansion titrating CUG-BP (Timchenko 1996a; Caskey 1996; Timchenko, 1999). If so, *DMPK* mRNA's cytoplasmic mislocalization might cause CUG-BP's subcellular redistribution. Inconsistent with this idea, CUP-BP was not notably redistributed to the cytoplasmic site of mutated *DMPK* mRNA, sarcoplasmic masses (Figure 3.6C). This observation is in line with suggestions that CUG-BP may mediate a downstream effect in DM (Michalowski 1999) while having a significant effect in some DM patients (Savkur 2001).

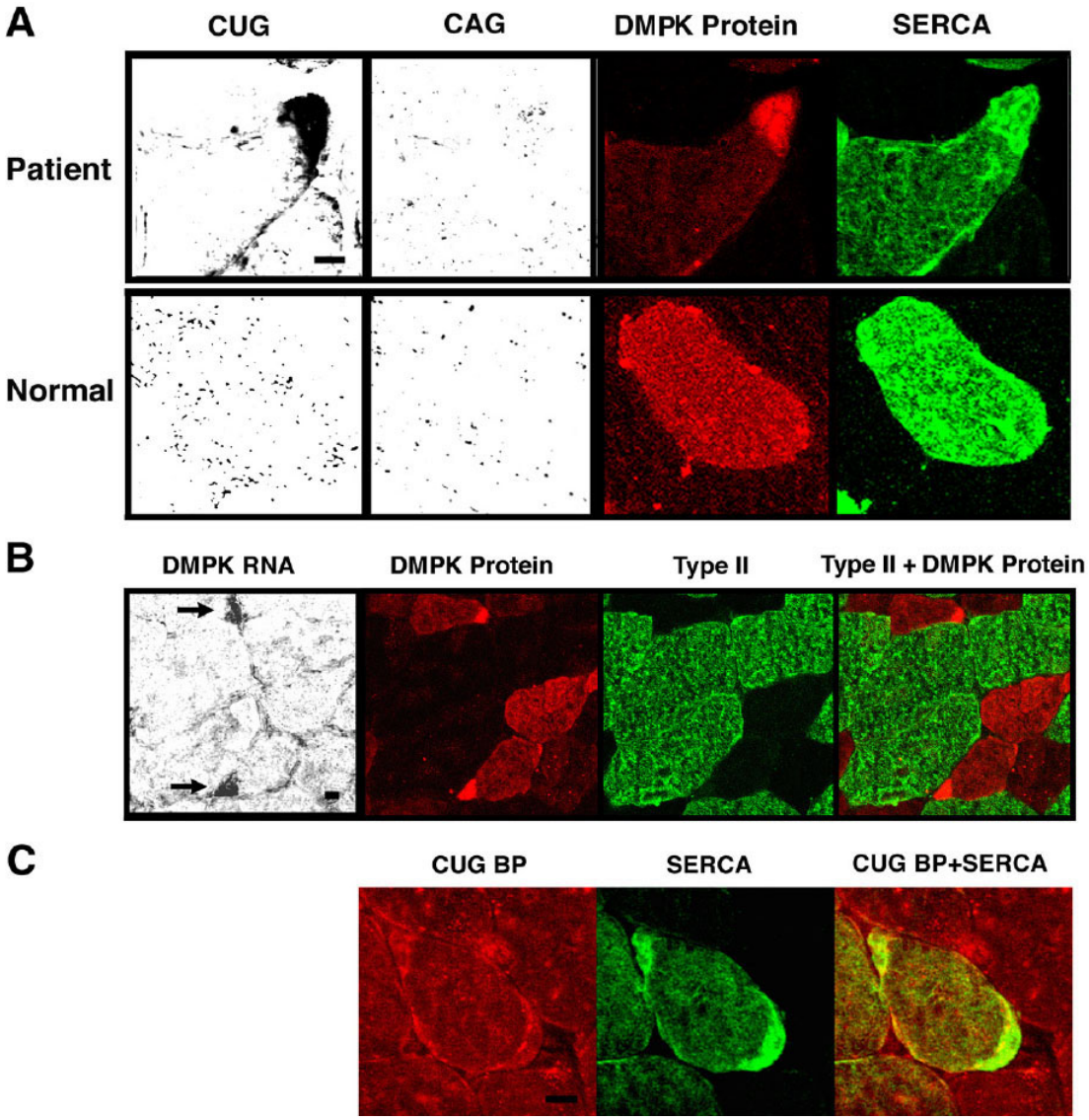

Figure 3.6 Additional sarcoplasmic mass properties.

(A) $(CUG)_n$-containing RNA specifically concentrates in sarcoplasmic masses. Consecutive patient (top) and normal (bottom) muscle sections labeled with a riboprobe to detect CUG-containing mRNA (CUG), the corresponding sense riboprobe (CAG), anti-DMPK and anti-SERCA antibodies, respectively. (B) *DMPK* RNA is visibly mislocalized only in type I and not type II muscle fibers. Consecutive patient sections probed for *DMPK* RNA (black; first panel) or double-labeled with anti-DMPK 1 (red) or anti-type II fiber (green) antibodies. (C) CUG-BP resides in but is not strongly enriched in sarcoplasmic masses. Patients' tissue sections were double-labeled with anti-CUG-BP (red) and anti-SERCA (green).

NOVEL PHOSPHOPROTEINS APPEAR IN DM MUSCLE HARBORING MISLOCALIZED DMPK

Since DMPK's protein coding region is unmutated in DM, mislocalized *DMPK* mRNA would yield functional DM kinase at its new cellular locale(s). This serine-threonine kinase (Timchenko 1995) could encounter and phosphorylate novel substrates. In agreement with this prediction, we detected novel phosphorylated proteins in DM1 biopsies containing mislocalized *DM kinase* mRNA (Figure 3.7; arrows). One is hyperphosphorylated (*) with phosphoserine and at least three each appear newly phosphorylated (**) with phosphoserine (Figure 3.7A) or with phosphothreonine (Figure 3.7B) in all three patients. Although biopsy amounts are insufficient to identify these substrates, these data indicate newly phosphorylated substrates appear in DM1 tissue containing cytoplasmically mislocalized *DMPK* mRNA. Clearly, phosphorylation of a subset of these molecules could modify their activities triggering an altered signal transduction cascade(s) and the resulting constellation of muscle symptoms characteristic of myotonic dystrophy.

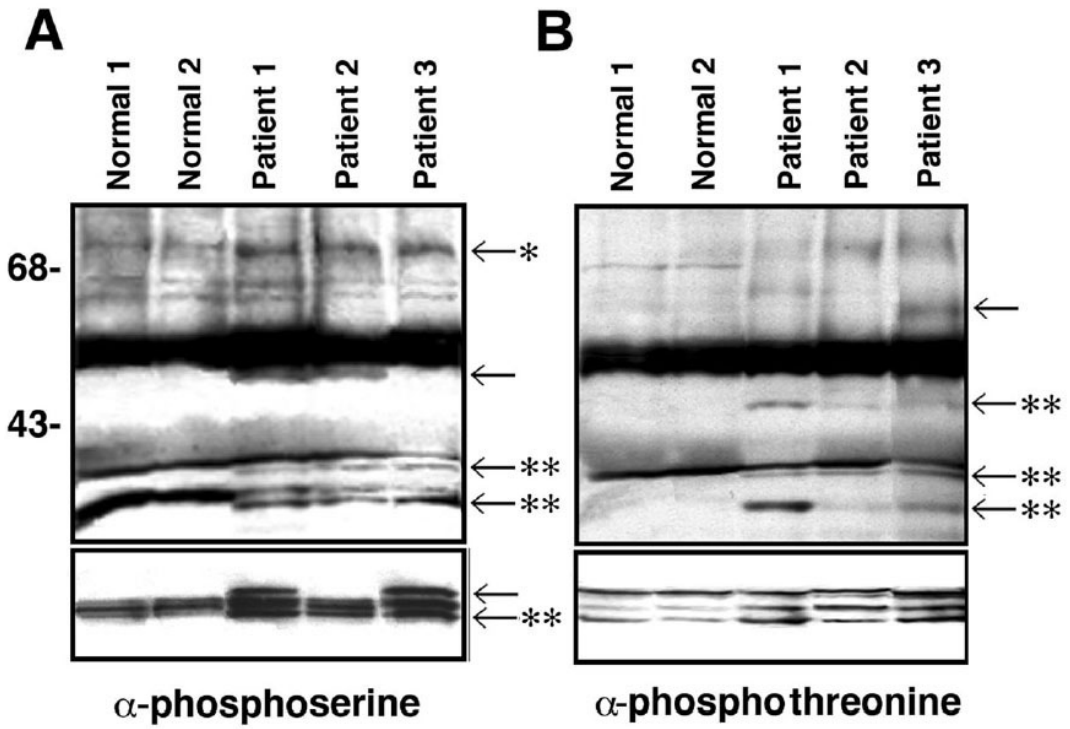

Figure 3.7. New phosphoproteins appear in DM patient muscle containing mislocalized DMPK mRNA and protein.

Extracts from three DM1 patients vs. two normal individuals were western blotted using anti-phosphoserine (A) or anti-phosphothreonine (B) antibodies. (Arrows): newly phosphorylated or hyperphosphorylated proteins detected in at least one patient vs. normal individuals; newly phosphorylated (**) or hyperphosphorylated (*) proteins detected in all three patients vs. normal individuals. Phosphoproteins were electrophoretically separated on SDS polyacrylamide gels; Upper panels: 10% gels; Lower panels: lighter exposures of strongly staining phosphoproteins in mid-section of 12.5% gel.

Conclusions

Here we provide key observations, which indicate that the $(CTG)_n$ expansion in the 3'UTR of the *DMPK* gene creates a new cytoplasmic RNA localization signal. We show that in normal muscle *DMPK* mRNA is subcellularly localized and deployed, whereas in DM1 patient muscle, *DMPK* mRNA containing the 3'UTR mutation is mislocalized to a new cytoplasmic locale. We link *DMPK* mRNA mislocalization to the diseases' 1) molecular genetics, by showing mislocalization occurs with the *DMPK* mRNA harboring DM1's causative triplet repeat mutation, 2) cytopathology, by demonstrating the mutated mRNA is mislocalized to the established site of disease pathology (sarcoplasmic masses) and 3) cell biology, by finding mislocalization occurs exclusively in type I muscle fibers which are the predominantly affected muscle fibers in DM. We show further that this mislocalized *DMPK* mRNA results in mislocalized DMPK protein, a kinase that can potentially encounter novel substrates at its new subcellular position. Notably, we detect at least seven newly phosphorylated proteins in DM1 patient muscle, which contains mislocalized *DMPK* RNA and protein. These results lead to the model shown in Illustration 3.1. Our data are the first implicating the 3'UTR-dictated process of cytoplasmic RNA (mis)localization in human disease.

Our findings in DM1 tissue suggest how *DMPK*'s 3'UTR $(CTG)_n$ expansion could directly generate a diseased state. Parallel to other triplet repeat disorders where coding region repeats cause a "gain of function" by giving the disease gene's protein product the new ability to interact with novel cellular components, *DMPK*'s triplet repeat expansion can cause a "gain of function" because the gene's RNA product has acquired a

new property, namely the ability to localize to new cytoplasmic regions, where its encoded protein can likewise interact with novel cellular components. Conceivably altered cytoplasmic *DMPK* RNA localization may be the causative mechanistic event in disease initiation. Since the wild-type *DMPK* mRNA is itself localized to a different sub-cytoplasmic region in normal muscle (Figure 2A), future experiments will have to determine if *DMPK* RNA's 3'UTR triplet repeat expansion creates a new autonomous cytoplasmic localization signal or does so in conjunction with existing 3'UTR localization information and whether existing localization information is simultaneously disrupted.

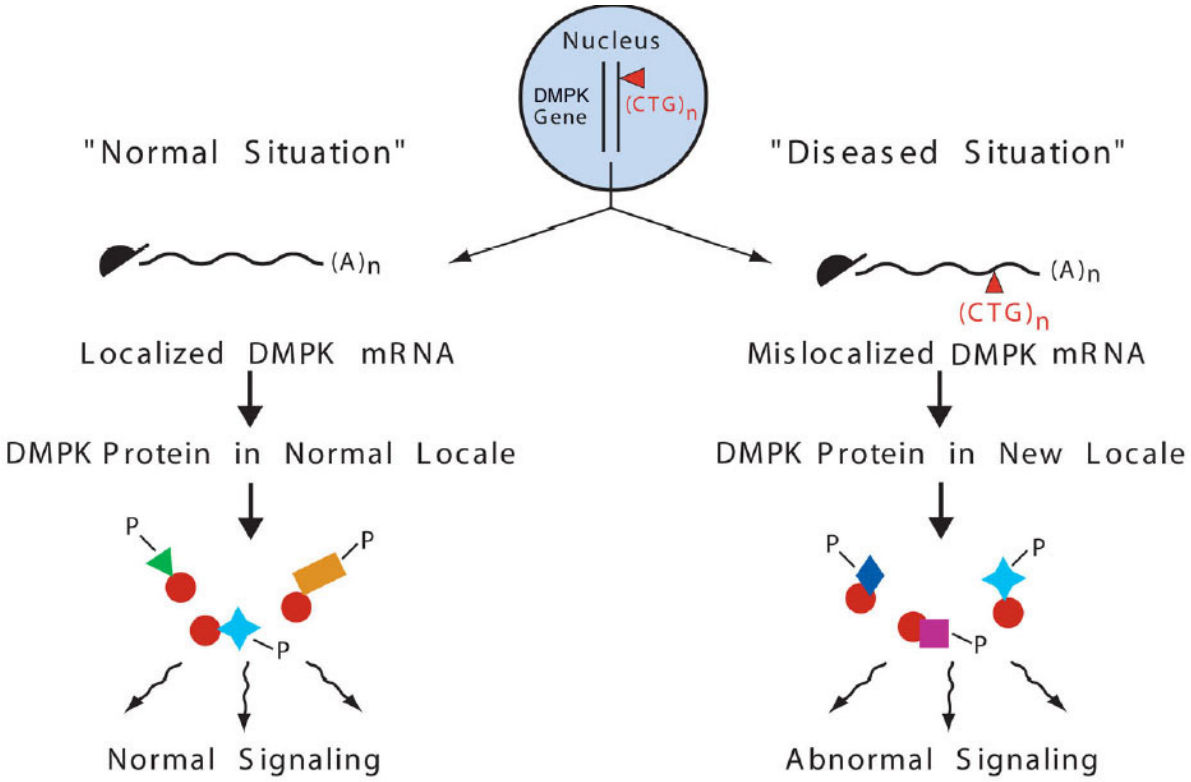

Illustration 3.1 A model for DMPK mRNA (mis)localization and its molecular consequences.

Information flow within the myoplasm represents the pathways for RNA from the normal (left) and mutated (right) *DMPK* alleles. While muscle cells are multinucleated, one nucleus is shown for simplicity. The triplet repeat expansion in *DMPK*'s mutated allele and its encoded mRNA are denoted (CTG) for ease of understanding. (Note that the CTG in the gene would be CUG in the RNA). In the normal situation, localized *DMPK* mRNA translation produces DMPK protein, which would carry out its normal phosphorylation function. In the diseased situation, mislocalized *DMPK* mRNA translation would likewise generate DMPK protein, which would be a fully functional kinase since the *DMPK* coding region is not mutated in this disease. However, since this kinase now occurs at a new locale it can encounter and phosphorylate novel (inappropriate) substrates, potentially resulting in abnormal signaling. Cytoplasmic mislocalization of only small mRNA amounts from *DMPK*'s mutant allele would suffice to contact and phosphorylate novel substrates ultimately causing disease. This situation would not be compensated for by the presence of properly localized *DMPK* mRNA from the normal allele. Hence, the autosomal dominance of DM.

CHAPTER 4: MRNA LOCALIZATION FUNCTIONALLY UNDERLIES BOTH FORMS OF MYOTONIC DYSTROPHY

Overview

Two diverse mutations, DM1 and DM2 can independently cause Myotonic Dystrophy; each resulting in the same large constellation of diverse symptoms. This led us to consider that DM1 and DM2 gene products likely function in the same molecular pathway. Then mutation of either would result in disruption of the pathway, ultimately leading to the same symptoms. Unfortunately, little work has been done on the DM2 gene product as it relates to myotonic dystrophy. Notably, highly conserved homologs for the DM2 protein (pDM2) exist in a variety of organisms (Armas 2008). The protein is spatially and temporally regulated (Shimuzu 2003, Chen 2003, De Dominicis 2000) and is crucial for development in a variety of model systems, including several stages of *Xenopus laevis* embryogenesis (Flink 1998), *Chaunus arenarum* oogenesis and early embryogenesis (Calcaterra 1999, Armas 2001), forebrain formation in mice and chickens (Chen 2003, Abe 2006) and neural crest development in *Xenopus laevis* (Armas 2008). Although the exact mechanism of action in these pathways is unknown, pDM2 is required because loss of its function results in severe defects (Chen 2003, Abe 2006, Weiner 2007).

Proteins which have severe effects on development in model systems cause disease when mutated in humans. The strong evolutionary conservation of pDM2, its severe developmental consequences and its mapping as a locus causing DM, led us to consider that DM2 provides vital clues to DM etiology that were previously neglected. Hence, we considered that pDM2 examination could open a new window into understanding DM etiology/pathology.

The data presented in this chapter 1) establish a disease relevant tissue culture system using C_2C_{12} muscle cells for the analysis of *DMPK* mRNA localization and 2) provide evidence that the *DMPK* 3'UTR is positioned subcellularly to the ER/SR via the post-transcriptional processes of RNA localization. Our results provide a mechanism for the DMPK results in human muscle (Chapter 3). Further, detailed examination indicates 1) sequence information required for ER localization resides within the *DMPK* 3'UTR 2) RNA localization is the apparent cause of DMPK protein localization 3) the disease causing CUGn mutation seen in the *DMPK* 3'UTR of DM patients compromises this proper wild type localization and d) C_2C_{12} myoblasts have sufficient machinery to localize endogenous mouse *DMPK* mRNA as well as exogenous mRNA harboring the human *DMPK* 3'UTR. Together with the data in human muscle, these results suggest RNA localization underlies the human disease Myotonic Dystrophy. This is the first human disease where RNA localization has been shown to function; however, it is not likely to be the last (see Chapter 6).

Results

CREATION OF A DM2 ANTIBODY

For the analysis of the role of the DM2 protein in *DMPK* RNA localization, we required antibodies against the DM2 protein, which were not commercially available. Hence, we generated an anti-DM2 antibody.

Antibody design began with examination of the protein sequence to determine which regions of the protein had hydrophilic stretches of at least ten amino acids, making them more likely to be on the surface of the folded protein and accessible for antibody binding. From the available areas we excluded regions that had highly conserved

domains that would potentially cross-react with other proteins, as confirmed by database examination. Since the predicted protein is small (19 kD) and has eight conserved motifs (seven CCHC zinc fingers and an RGG box), this left us with only one optimal epitope target which was at the carboxy-terminus of the protein (amino acids 158-177). Database analyses revealed this epitope sequence was unique to pDM2, limiting potential cross-reactivities that would have complicated our analyses. A peptide corresponding to this region was synthesized. After peptide purification by HPLC and subsequent KLH conjugation to increase its immunogenicity, the peptide was sent to AnimalPharm (Healdsburg, CA) for injection, by a regimen that we designed for optimal antibody production. Animals were prescreened to ensure they possessed no initial cross-reactivity to pDM2.

We screened successive bleeds via western blot using C_2C_{12} myoblast cell extract. As indicated (Fig 4.1), 2 independent animals made high titer antibodies to a protein of 19 kD, the size of the DM2 protein. The sera from both animals were highly specific and recognized no other entities on our blots. Moreover, pre-immune sera failed to cross-react with this or any other entity. Taken together, these data indicate that the 19 kD protein is DM2 and that we possess high tier anti-peptide rabbit sera specific for pDM2 for our subsequent analyses.

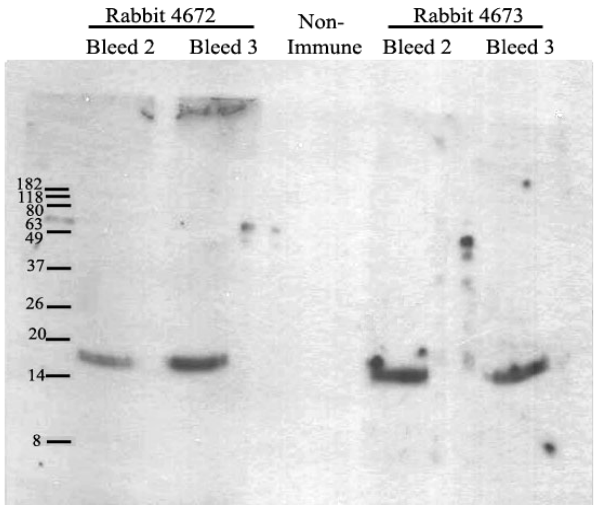

Figure 4.1 Anti-DM2 antibody specifically binds to pDM2

We designed and created an antibody against the DM2 protein that is specific and selective. Western blot analysis of C_2C_{12} extract using anti-DM2 antibodies from two different animals and demonstrate that our antibodies cleanly and specifically bind the 19kD DM2 protein. Paired non-immune sera do not recognize this band, underscoring the specificity of this reaction**.**

LIKE *DMPK* MRNA, DM2 PROTEIN LOCALIZES TO THE SARCOPLASMIC RETICULUM IN HUMAN MUSCLE TISSUE

Our analyses of human muscle tissue demonstrated that the *DMPK* mRNA is specifically localized to the sarcoplasmic reticulum (SR). The hypothesis, put forth here, that DM2 functions in the *DMPK* mRNA localization pathway predicts that at least a subset of DM2 protein should localize to the same subcellular locale as *DMPK* mRNA. To test this idea, we assessed the cellular location of DM2 protein via anti-DM2 immunofluorescence. On longitudinal sections of normal human muscle, anti-DM2 fluorescence revealed a familiar striped pattern (Figure 4.2). Careful examination using

confocal microscopy indicated that these stripes are comprised of stacked discs. This distinctive pattern is characteristics of the SR. Immunofluorescence with antibodies against two independent SR proteins, Serca II ATPase (Dormer 1993) and Protein Disulfide Isomerase (Ohba 1977), revealed the same striped pattern composed of stacked discs. Co-immunofluorescence of each marker independently with anti-DM2 protein followed by electronic merge, revealed co-localization of Serca II ATPase and DM2 as well as co-localization of Protein Disulfide Isomerase and DM2. Hence, DM2 protein, like DMPK mRNA co-localized to the SR.

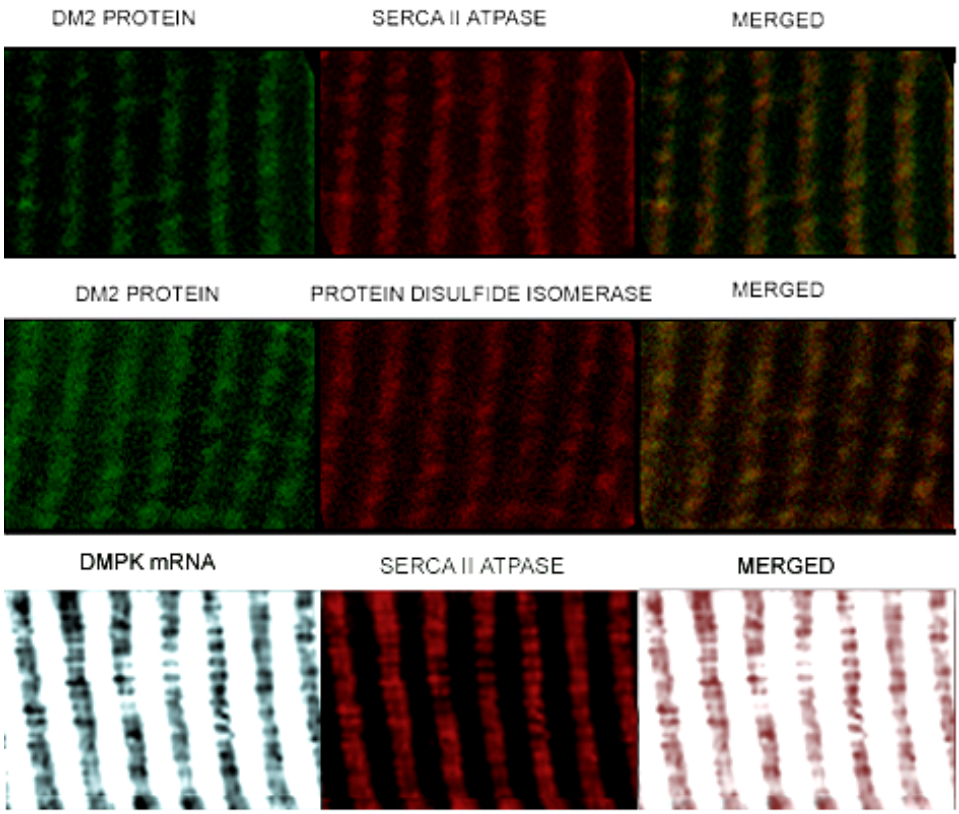

Figure 4.2 The DM2 protein localizes to the SR in muscle with DMPK mRNA

Analyses of human longitudinal muscle tissue demonstrate that the DM2 protein localizes to the SR along with the *DMPK* mRNA. Co-immunofluorescence of human longitudinal muscle tissue with anti-DM2 and the SR markers SERCA II ATPase or protein disulfide isomerase demonstrate that the DM2 protein localized to the SR. This is similar to the pattern seen by *in situ* hybridization using an antisense DIG-labelled *DMPK* probe followed by anti-SERCA II ATPase immunofluorescence. Thus, the DM2 protein and the *DMPK* mRNA both localize to the SR in human muscle cells.

ESTABLISHMENT OF A DISEASE RELEVANT CELL CULTURE SYSTEM

In Chapter 3, we provided a body of data indicating that *DMPK* mRNA localization/mislocalization underlies Myotonic Dystrophy etiology. These data established this phenomenon in actual muscle biopsies from multiple DM patients. While it was imperative to establish these observations in patient tissue and, while we were fortunate to have access to this material, human tissue is not a system that lends itself to experimental manipulation.

For our analyses we required an experimental system to examine the localization/mislocalization mechanism that appeared to underlie Myotonic Dystrophy. There were two possible approaches; a mouse model system for DM or a tissue culture system. The former are costly and slow. Moreover, no single mouse model for DM1 exists. This may be due to the fact that humans are the only organism known to have diseases caused by triplet repeat insertions (RH Singer, personal communication). This situation may arise from the fact that other organisms have detailed mechanisms for reducing or eliminating triplet repeat expansions that do not exist in humans. Despite numerous attempts reported in the literature of creating several mouse models for DM1 (Chapter 1., Appendix 1), each recapitulates only a small subset of DM's diverse symptoms and none recapitulate the multi-systemic nature of this disease. The second approach, and the one we employed, was to turn to cells in culture. Cultured cells have been the system of choice to study most post-transcriptional processes and the vast majority of information about these processes has come from the study of tissue culture cells. Hence, it was the better choice for our studies.

Myotonic dystrophy is a disease of muscles, our localization/mislocalization observations were made in actual skeletal muscle and we desired a muscle related culture

system. Our initial examination of the literature revealed greater than 90% of muscle-related studies *in vitro* focused on the C_2C_{12} system, suggesting it was the preferred research tool. C_2C_{12} cells have many advantages including: they are hardy, have a rapid doubling time and behave in a well-defined control manner. They can be easily and reproducibly differentiated in culture over a ten day period by the addition of Dulbecco's Modified Eagle Medium + 2% horse serum. This allows one to study the undifferentiated state (myoblasts), the differentiated state (myotubes) and the transition between the two states. Our analyses primarily focused on undifferentiated myoblasts since diseased DM tissue expresses undifferentiated characteristics (Timchenko 2001). Whether this is due to the fact that DM muscle tissue is undifferentiated or whether it becomes de-differentiated is a matter of active debate (Amack 2004) that has not been resolved.

Our first order of business was to determine whether these cells possessed ample amount of *DMPK* mRNA and DMPK protein. Both northern blots and RT-PCR analyses confirmed that these cells contained ample amount of *DMPK* mRNA (Figure 4.3). Parallel western blots indicated that these cells also contained experimentally accessible amount of the DM Kinase protein (Figure 4.1). Taken together, we concluded that this was a desirable system to examine the *DMPK* localization process that we had detected in muscle tissue.

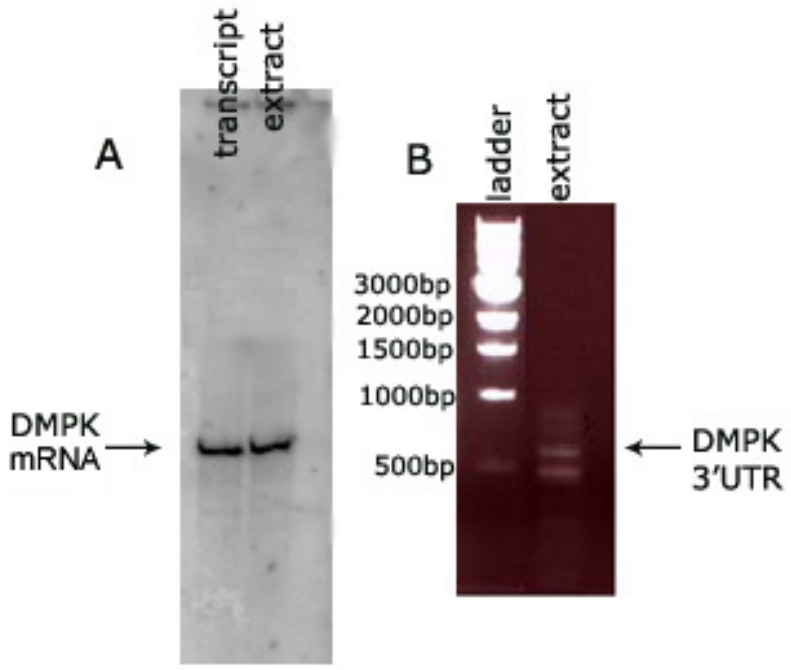

Figure 4.3 C2C12 cells Contain Substantial Amounts of DMPK mRNA

For C_2C_{12} mouse myoblasts to be an appropriate cell culture model to examine DM etiology, these cells must contain a reasonable amount of DMPK mRNA, so we checked this level by two means. A) Northern blot analysis of DMPK mRNA levels in C_2C_{12} extract. *In vitro* transcribed DMPK mRNA serves as a positive control to ensure that the signal seen reflects DMPK message and not a cross-reaction with another message. B) Reverse Transcriptase-PCR analysis of C_2C_{12} extract with primers specific to the DMPK 3'UTR demonstrate that there is a detectable amount of DMPK mRNA by another means.

ENDOGENOUS DMPK MRNA IS LIKEWISE LOCALIZED IN C_2C_{12} MYOBLASTS

From the data we present in Chapter 3, we infer that *DMPK* mRNA is localized to the muscle sarcoplasmic reticulum and is subsequently translated into protein at this subcellular locale. In order to use the C_2C_{12} system to examine this presumptive localization, endogenous *DMPK* mRNA would also have to be subcellularly localized in these cultured cells.

In situ hybridization using an anti-sense *DMPK* riboprobe on C_2C_{12} myoblasts resulted in a bibbed pattern surrounding the cell nucleus which was often asymmetric (Figure 4.4A). Signal was attributable to hybridization of the antisense *DMPK* riboprobe as the corresponding *DMPK* sense riboprobe gave no apparent signal (Figure 4.4). Specificity of the anti-*DMPK* riboprobe is underscored further by the fact that this pattern is not seen using an antisense riboprobe to an abundant muscle protein, *Myosin Heavy Chain* (Figure 4.4A).The highly specific pattern was detected with the antisense DMPK mRNA probe is characteristic of the endoplasmic reticulum (ER), which is often seen as an asymmetric bib around the cell nucleus. To ask whether the *DMPK* mRNA was localized to the ER, we performed co-immunofluorescence with antibodies against several ER markers, Protein Disulfide Isomerase (PDI), KDEL (Munro 1987) and Calsequestrin (MacLennan 1971), followed by an electronic merge. These two signals (DMPK mRNA *in situ* hybridization signal and ER marker immunofluorescence) co-localized (Figure 4.4B), indicating that the *DMPK* mRNA is indeed localized to the ER.

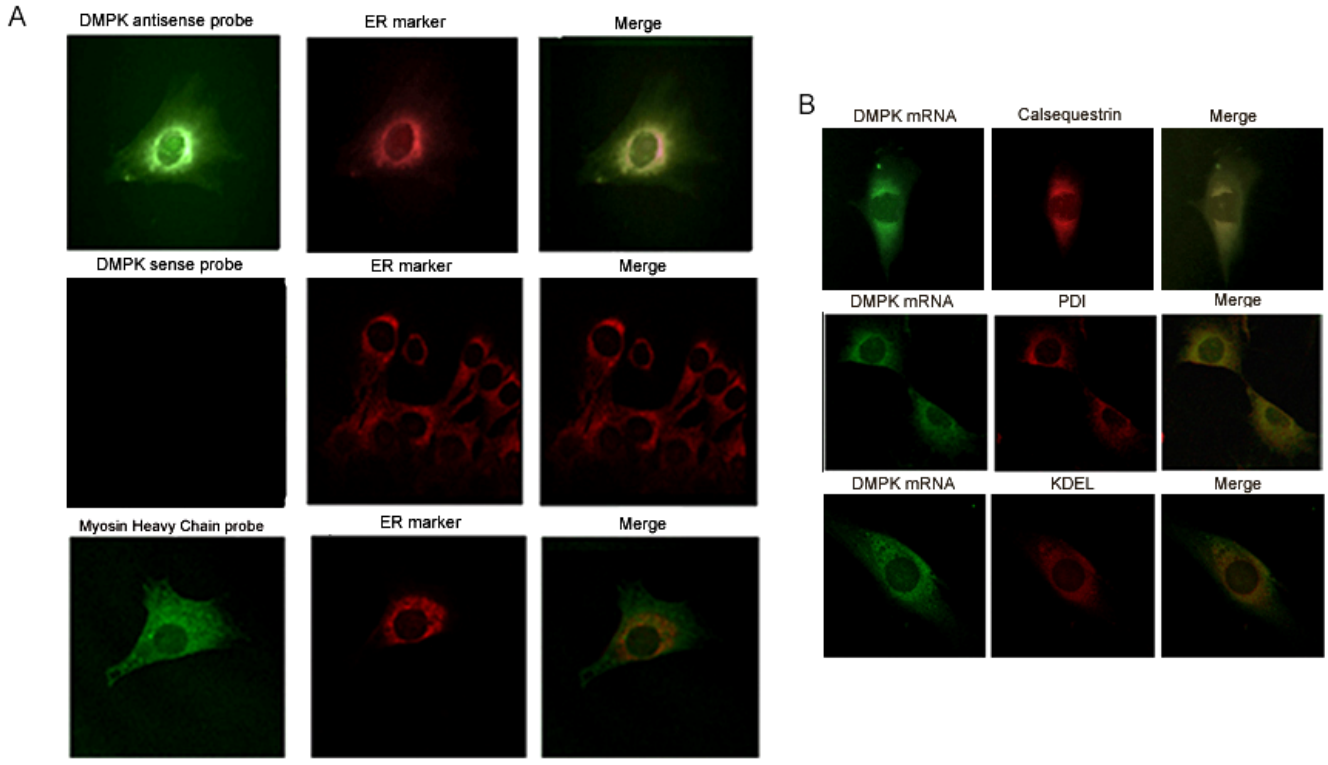

Figure 4.4 DMPK mRNA specifically localizes to the ER in C2C12 myoblasts

In situ hybridization analysis of DMPK mRNA followed with immunofluorescence with an ER marker reveals that the DMPK mRNA specifically localizes to the ER in C2C12 mouse myoblasts. A) The specificity of this localization is demonstrated because in situ hybridization using a DMPK mRNA sense probe reveals no significant fluorescent signal and in situ hybridization to an abundant message, myosin heavy chain, does not show an ER specific pattern. B) Verification of the ER pattern of the DMPK mRNA was achieved by immunofluorescence with several common ER markers, PDI, KDEL and Calsequestrin. The DMPK mRNA pattern colocalized with all three of these ER-specific protein markers.

One of the major reasons to localize an mRNA is to translate it in a spatially and/or temporally controlled manner. To assess whether the endogenous localized *DMPK* mRNA is translated at this locale, we conducted immunofluorescence with anti-DMPK antibodies. As indicated (Figure 4.5), DMPK protein is found encircling the nucleus with a punctuate pattern emanating from that. Co-immunofluorescence with an ER marker (anti-PDI) and electronic merge demonstrated that the bulk of endogenous DMPK protein signal co-localizes with the ER and a small portion abuts the ER (Figure 4.5). This is consistent with DMPK protein being locally translated from the ER localized *DMPK* mRNA, followed by some limited diffusion from that site. This is often the case with localized mRNA translation and the mechanism by which developmental gradients, which are crucial to establishing developmental patterning, are formed (ex: *bicoid* message in *Drosophila* ((Driever 1988 a,b)). Indeed, disruption or alterations in these gradients are due to mislocalized RNAs and result in birth defects.

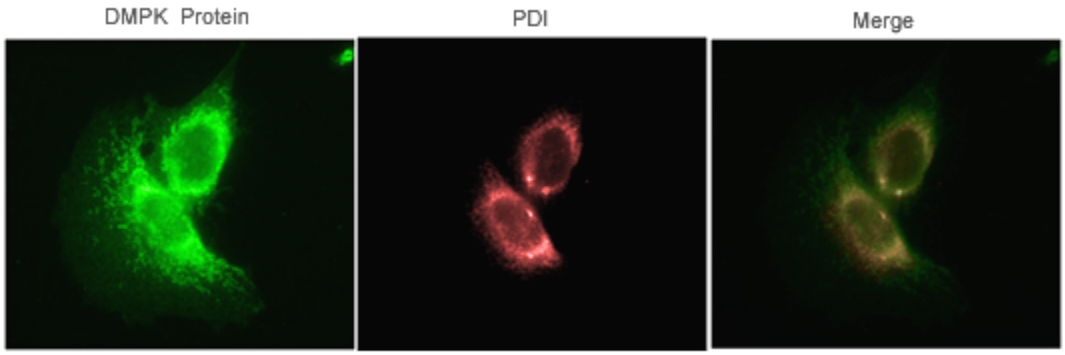

Figure 4.5 DMPK protein localizes the ER in C2C12 cells

Co-immunofluorescence analysis reveals that the DMPK protein localizes to the ER in C_2C_{12} mouse myoblast cells, corresponding to the ER specific localization of the *DMPK* message. This ER localization in mouse myoblasts concurs with the SR specific localization of both of these components in human muscle tissue (Chapter 3).

The *DMPK* mRNA localization pattern observed in these C_2C_{12} myoblasts is particularly informative in that the endoplasmic reticulum, the site of *DMPK* mRNA localization in cultured myoblasts, is the cellular equivalent of the sarcoplasmic reticulum in muscle tissue, the site of *DMPK* mRNA localization in human muscle biopsies. Therefore, even though the actual architecture of these two organelles are dissimilar, the mRNA localization is the same, truly underscoring the specificity of the *in situ* hybridization signals and, hence, the actual localization. Taken together, these data demonstrate endogenous *DMPK* mRNA is subcellularly localized and translated in cultured cells analogous to its situation in muscle tissue. Thus, C_2C_{12} cells are an ideal system to study the *DMPK* localization process.

DM2 PROTEIN RESIDES IN THE ER OF C_2C_{12} CELLS WITH *DMPK* MRNA, MIRRORING THE MUSCLE TISSUE SITUATION

If DM2 protein functions in the proper wild-type localization of *DMPK* mRNA in C_2C_{12} myoblasts at least a subset of DM2 protein should reside in the ER of these cells, mirroring the finding that *DMPK* mRNA and pDM2 exhibit overlapping locations in human muscle (in SR, Figure 4.2). As in Figure 4.4, *in situ* hybridization exhibited an ER localization pattern in C_2C_{12} myoblasts. This was shown to be ER localization by overlap with ER markers previously in this chapter (Figure 4.4B). Immunofluorescence with anti-DM2 antibody showed the bulk of DM2 protein co-localized with the ER marker (Figure 4.6), thus the DM2 protein also resided in the ER. (a minor portion of pDM2 was detected in discrete speckles elsewhere in the cytoplasm with this antibody). Finally, performing an electronic overlap of cells subjected to *in situ* hybridization with an antisense *DMPK* mRNA probe and immunofluorescence with anti-DM2 sera indicated

that these two cellular components at least partially overlapped in the same cellular locale, the ER. Therefore, pDM2 is in the right place to function in *DMPK* mRNA localization.

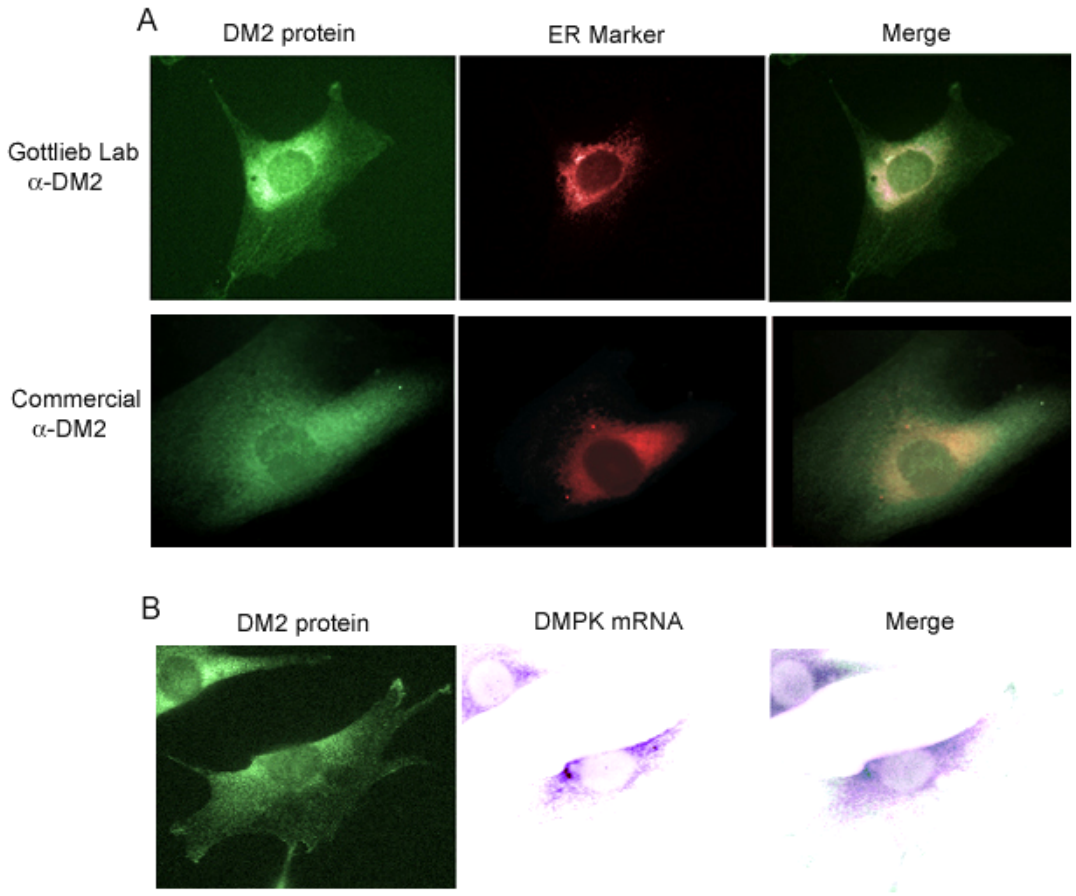

Figure 4.6 The DM2 Protein Co-localizes to the ER with the DMPK mRNA

A) Co-immunofluorescence analyses of the DM2 protein were conducted using the anti-DM2 antibody that we made (Gottlieb Lab α-DM2) or a commercially available anti-DM2 antibody (Commercial α-DM2) and an antibody against an ER marker. A subset of the DM2 protein co-localized with the ER marker in both samples; however, the signal was more specific using our antibody that is made against only a small portion of the DM2 protein chosen to reduce cross-reactivity, not the entire protein as the commercially available antibody was prepared. B) A portion of the DM2 protein pool co-localized with

the *DMPK* mRNA message. Both showed a characteristic asymmetrical bib-like pattern around the nucleus, the common presentation of the ER.

THE DMPK 3'UTR DICTATES ER LOCALIZATION

All of our data in human muscle cells and C_2C_{12} cultured myoblasts points to the conclusion that *DMPK* mRNA is subcellularly targeted to the SR/ER by the post-transcriptional processes of localization. To further test whether that *DMPK* mRNA subcellular localization arises via RNA trafficking, we focused on the fact that signals dictate RNA localization reside within the 3'UTRs of virtually every localized mRNA examined (reviewed in Chabanon 2004). We turned to a reporter construct system which has been used to successfully demonstrate that *c-MYC* (Veyrune 1996) and *beta-Actin* (Kislankis 1993) are localized post-transcriptionally within subsets of mammalian cells. Such systems have likewise been used to demonstrate that classically localized mRNAs (eg, *bicoid* in *Drosophila*, *Vg1* in *Xenopus*) are subcellularly partitioned via a post-transcriptional mRNA localization process.

For these experiments we used an EGFP reporter system (Clonetech, EGFP-C1). Two parallel constructs were created, one with a normal SV-40 3'UTR and a second with the human *DMPK* 3'UTR. Mouse cells and mouse and human *DMPK* coding regions are 90% homologous, but the (CUG)n disease-causing expansion and associated properties are specific to human *DMPK* 3'UTR. Our data from C_2C_{12} mouse cells (Section 4.4) indicated that machinery is in place to localize endogenous mouse *DMPK* mRNA.

The EGFP reporter constructs were separately transiently transfected into parallel C_2C_{12} myoblast cultures. Following overnight incubation to allow ample transgene expression, cells were fixed and microscopically examined. EGFP signal was used as a readout and indicator of the transgenic RNA and encoded protein. The basic control constuct, which encodes the EGFP coding region attached to the SV-40 3'UTR, EGFP-SV40, is evenly distributed throughout the cell cytoplasm and nucleus. This distribution

is as one would predict (Healy 1999, Munster 2002, Adereth 2005) since the SV-40 3'UTR harbors no localization information. In contrast, cells transfected with the parallel construct containing the EGFP coding region attached to the *DMPK* 3'UTR, EGFP-3'UTR, signal is dramatically restricted. Indeed, the EGFP signal now co-localizes with an ER marker. From these data, we conclude that 1) signals sufficient for ER subcellular localization reside in the *DMPK* 3'UTR and that 2) *DMPK* mRNA is positioned to the ER/SR via a post-transcriptional mRNA localization process.

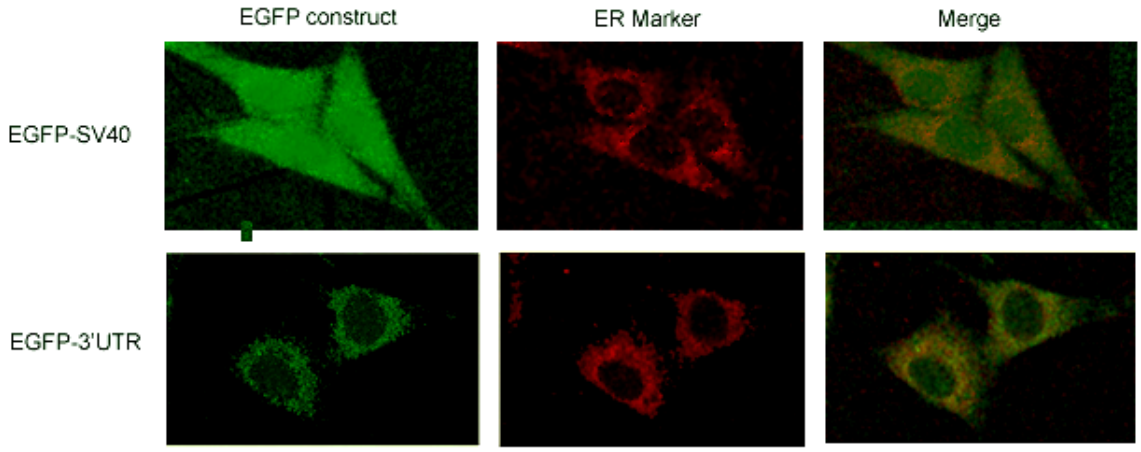

Figure 4.7 The DMPK 3'UTR is sufficient to specifically localize a reporter construct

An EGFP reporter construct with an SV40 3'UTR transiently transfected into C_2C_{12} cells did not show any specific localization, as would be expected because this 3'UTR is not know to contain any localization signals. However, when the SV40 3'UTR was replaced with the *DMPK* 3'UTR the reporter construct was specifically localized to the ER, mirroring the endogenous *DMPK* localization pattern. Therefore, the *DMPK* 3'UTR contains sufficient localization signals to independently dictate ER localization to a reporter, solidifying, once again, that *DMPK* mRNA is a localized message.

THE $(CUG)_N$ DISEASE-CAUSING TRIPLET REPEAT EXPANSION DISRUPTS PROPER LOCALIZATION

Cis-acting sequences dictating mRNA localization reside within the 3'UTR of localized mRNAs. Our data thus far show that the wild type *DMPK* 3'UTR contains information sufficient to localize a reporter construct to the ER. We postulated that the (CUG)n disease causing expansion might disrupt or impair wild type localization. Localization information could be dictated by one signal or multiple signals in a combinatorial manner. Therefore, depending upon the effect of the (CUG)n expansion on the 3'UTR, logistically we could disrupt part or all of the localization process.

To assess this situation, we examined the localization of EGFP expressed from a reporter construct containing a (CUG)n expansion embedded within the normal human *DMPK* 3'UTR. For these experiments, we used a repeat with 90 CTG triplets (called EGFP-(CTG)90). This is the maximal number of CUG repeats that can be maintained stably in cells and we wanted a uniform, consistent population of RNA molecules for this analysis. Moreover, this number of repeats is sufficient to inhibit differentiation and elicit misregulation of calcium channel expression (Quintero-Mora 2002, Andrade 2007) in cell culture. Immunoflourescence with an antibody probe to the ER was used as an internal signpost, since the ER is the site of normal *DMPK* mRNA localization. Following transgene expression, cells were fixed and examined microscopically. Some of the reporter is still found in the ER; however, it also diffuses from that area and it can be found throughout the extent of the cytoplasm. Notably, it is still excluded from the nucleus. Therefore, the (CUG)90 repeat contained within the context of the DMPK 3'UTR is sufficient to disrupt wild type DMPK mRNA ER localization.

It may be pathologically significant that the DMPK mRNA is localized to the SR/ER and that the (CUG)n repeat expansion compromises that situation. The SR is the

site of calcium regulation within the muscle, a process that functions to control muscle contraction. The DM kinase may participate in that process; thus, the (CTG)n repeat would disrupt the full complement of DMPK protein at that locale which could contribute to altered calcium currents and the myotonia characteristic of DM patients.

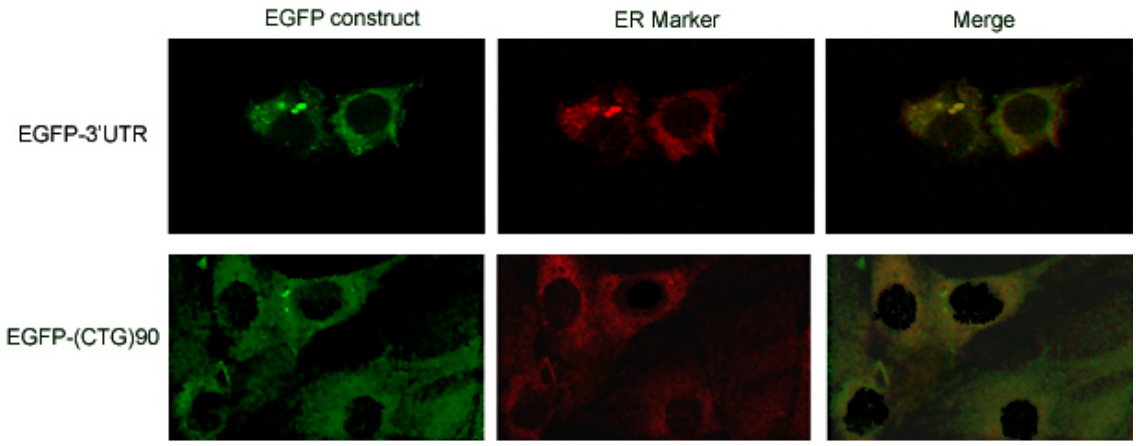

Figure 4.8 The DM1 causative mutation disrupts proper reporter localization

A reporter construct containing the EGFP coding region attached to the wild type *DMPK* 3'UTR was transiently transected into C_2C_{12} cells. As seen previously, this 3'UTR is sufficient to localize a reporter construct to the ER. Intriguingly, insertion of 90 CUG repeats into that *DMPK* 3'UTR is sufficient to disrupt that ER localization, resulting in a more diffuse pattern of signal. Thus, the DM1 causative mutation specifically results in loss *DMPK* 3'UTR dictated ER localization.

RNAi Technology is Used to Biochemically Generate pDM2 Mutant Cells

Our finding that the *DMPK* mRNA and the pDM2 protein are both found within overlapping areas of myoblast ERs meet an important prerequisite for the DM2 protein to function in *DMPK* mRNA localization: namely, to reside at the same place at the same time. Additionally, since pDM2 contains one known RNA binding domain and seven nucleic acid binding domains while residing outside the nucleus, this raises the distinct possibility that pDM2 could bind to RNA and be part of the *DMPK* mRNP (DMPK messenger RNP). Recall that no mRNA exists as naked RNA in eukaryotic cells. Rather, they exist as mRNPs and a subset of particle proteins can support the biogenesis of that mRNA.

Does pDM2 function in the process of localizing the *DMPK* mRNA? To experimentally address this question directly, we required cells with decreased amounts of DM2 protein. To derive such cells, we turned to RNA interference (RNAi) knockdown technology. RNAi has become one of the most cost efficient and effective technologies for creating a knockdown mutant in cultured mammalian cells (reviewed in Shi 2003, Scherer 2004).This can be accomplished via a variety of means including introducing chemically synthesized RNA oligonucleotides complementary to the target RNA into the cell cytoplasm or alternatively, putting a vector into the cell where RNA complementary to the target RNA is encoded and can be transcribed by the cellular machinery. Depending on the properties of the vector, this RNA can remain in the nucleus or be transported to the cell cytoplasm. There, it can encounter its target and form a double stranded RNA stem with the target mRNA. This double stranded RNA is subsequently a target for RNA degradation by the RISC complex (RNA induced silencing complex)

(reviewed in Fire 1999, Sharp 2001, Hannon 2002, Zamore 2002). The result is a decrease in the overall amount of specific target message.

For our experiments targeting *DM2* mRNA via RNAi, we selected the pcDNA3-H1 vector system (Invitrogen, Carlsbad, CA). This system has the useful property of using an H1 RNA Polymerase III promoter, which produces a targeting RNA that is transported to the cytoplasm where it can interact with its target transcript (Myslinksi 2001). To tailor this vector system to our specific purpose, we generated three DNA constructs, each containing oligonucleotides complementary to three distinct areas of the DM2 mRNA (Construct 1 nucleotides 239-258, Construct 2 nucleotides 473-492, Construct 3 nucleotides 645-665). In our design we selected predicted DM2 RNA regions that were a) 21 nucleotides in length, b) would hybridize under physiological conditions to the DM2 mRNA and c) did not cross react with any other mammalian RNA species in the database. We designed probes complementary to three different regions of the *DM2* mRNA coding regions in hopes that at least one of these might be accessible for hybridization dependent on the packaging of the mRNA in mRNPs and its structure.

As a preamble to our experiment, we transfected all three DNA constructs independently into parallel C_2C_{12} myoblast cultures using TransIT-LT1 (Mirus Biotechnology). Using this transfection reagent, we were able to consistently achieve 80-85% transfection efficiency in C_2C_{12} cells. Following thirty six hours of incubation of these transfected cultures (a time that was empirically determined to produce efficient knockdown on the DM2 protein), we harvested the cells, made a total protein extract and assayed for the relative level of DM2 protein via western blot analysis. Using beta-Tubulin as a loading control, we discovered that two specific DM2 constructs resulted in dramatic knockdown of the DM2 protein. Specifically, construct 2 caused knockdown up to 85%, while construct 3 resulted in 60-65% reduction in DM2 protein level. In contrast,

construct 1 did not result in a significant DM2 protein level depletion and the vector without a DM2 specific insert did not have an appreciable affect on the cells. Therefore, we had a system for specific analysis of DM2 function by effectively reducing the amount of DM2 protein in cell culture.

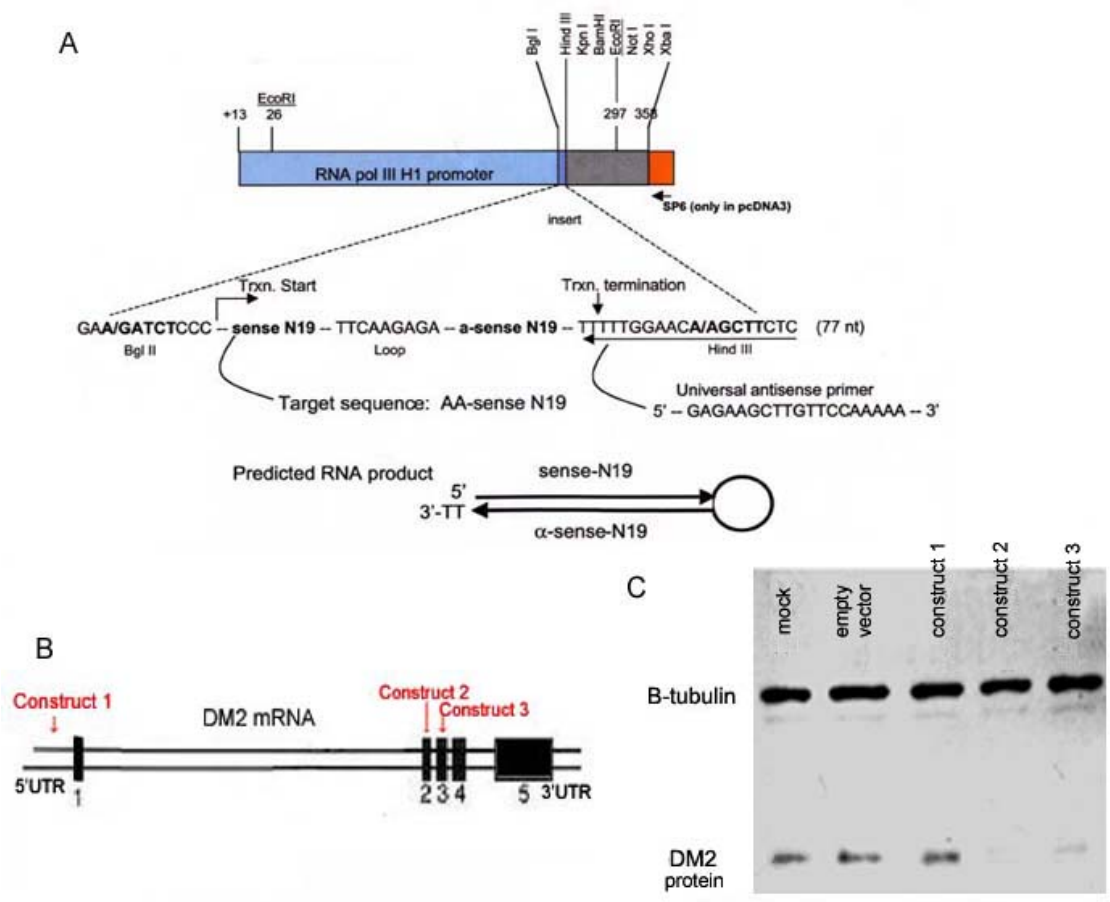

Figure 4.9 RNAi constructs effectively result in pDM2 reduction

A) RNAi constructs were designed using shRNAs in the pcDNA3-H1 vector from Invitrogen. This vector contains the H1 RNA Polymerase III promoter, which transcribes small RNAs that are transported into the cytoplasm and interact with the RNAi pathway. B) Three constructs were created which target different regions of the *DM2* message, increasing the chance that one of the constructs will be effective. C) Western blot analysis of C_2C_{12} cells that were transiently transfected with no construct, an empty vector or one of the three DM2 constructs, demonstrates that two of the constructs did

result in a dramatic loss of DM2 protein. Beta-tubulin protein was probed for as a loading control and to verify the specificity of the DM2 knockdowns.

KNOCKDOWN OF PDM2 SPECIFICALLY CAUSES *DMPK* MRNA MISLOCALIZATION

mRNA localization requires many proteins which all work coordinately to complete subcellular message localization. Loss of any of these protein factors may affect the ultimate localization. Therefore, if pDM2 is a localization factor for *DMPK* mRNA, reduction of this protein by RNAi knockdown may disrupt normal *DMPK* transcript localization. To visualize the potential impact of this loss on *DMPK* mRNA, DM2 protein knockdown in C_2C_{12} cells was followed by *in situ* hybridization to visualize the resulting localization of the *DMPK* message. C_2C_{12} cells were transiently transfected with the pDM2 RNAi constructs described previously followed by an empirically determined incubation time to allow the RNAi degradation of the *DM2* mRNA to occur and additional time for its effects to be seen. The cells were then fixed and hybridized with a DIG labeled antisense riboprobe to the *DMPK* message and mRNA localization was visualized via anti-DIG immunofluorescence. Parallel processing of a DIG-labelled antisense probe to *Desmin* mRNA functioned as a specificity control.

Visualization of the endogenous *DMPK* mRNA profile within pDM2 knockdown cells revealed that *DMPK* mRNA localization was, in fact, dramatically altered. Using immunofluorescence with an ER marker as a cellular signpost, mock transfected cells contained normal *DMPK* mRNA compact ER localization. However, the cells with reduced DM2 protein levels, due to transfection with an anti-DM2 RNAi construct, exhibited a diffuse, punctuate pattern throughout the cell cytoplasm. The extent of the *DMPK* mRNA localization redistribution varied with the efficiency of pDM2 knockdown, with the most efficient construct, Construct 2, showing a more diffuse *DMPK* mRNA pattern than a less effective RNAi construct, Construct 3. Disruption of the *DMPK* mRNA localization was specific to DM2 protein knockdown constructs since

it was seen with both pDM2 knockdown constructs (Figure 4.10, Constructs 2 and 3), but not the cells transfected with an empty vector (Figure 4.10, mock) or those treated with the RNAi construct that did not decrease pDM2 levels (data not shown).

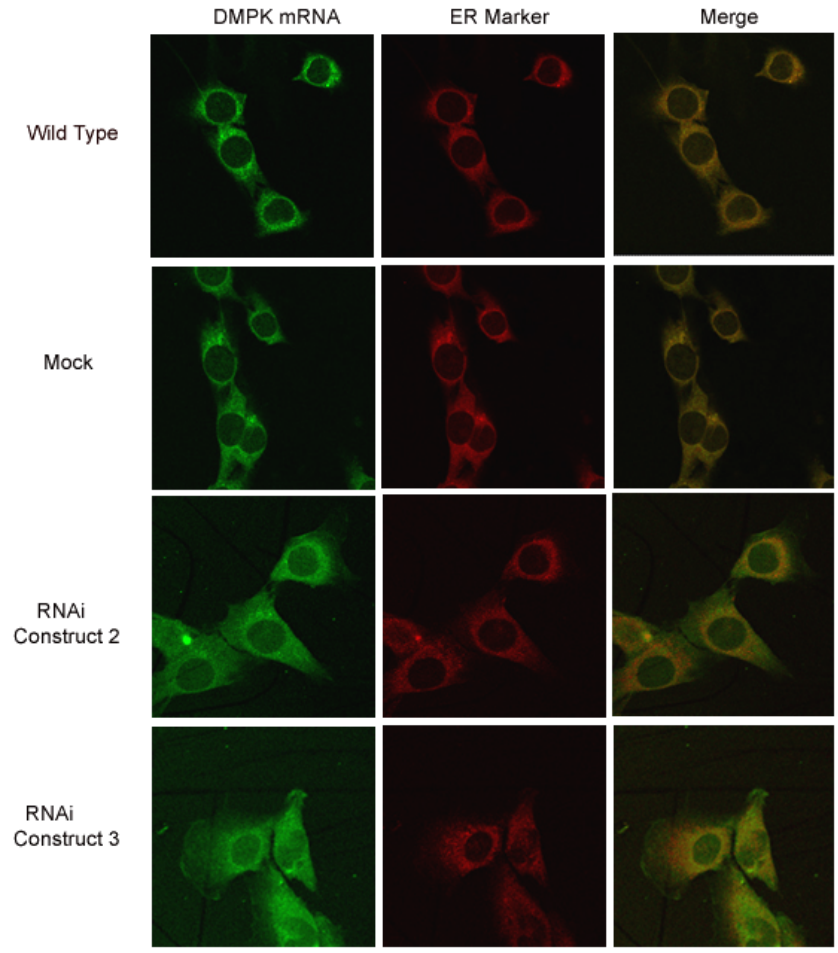

Figure 4.10 RNAi knockdown of pDM2 causes mislocalization of DMPK mRNA

C2C12 cells were subjected to RNAi knockdown and the resulting DMPK mRNA localization was visualized using DMPK antisense probe in situ hybridization. The cells that were transfected with no DNA, wild type, or empty vector, mock, displayed the previously seen ER mRNA pattern when compared to an ER marker. However, the DMPK mRNA localization in the cells that were transfected with DM2 construct 2 or 3 was noticeably diffuse when compared to an ER marker. Therefore, the reduction of the DM2 protein in C2C12 cells resulted in mislocalization of the DMPK mRNA.

Results were specific to *DMPK* mRNA distribution and did not reflect a massive, general redistribution of messages since the normally ER localized *Desmin* mRNA retained its ER localization in mock and DM2 knockdown cells alike (Figure 4.11). Finally, the change in *DMPK* mRNA cellular localization observed upon pDM2 knockdown did not result indirectly from an overall disruption of the ER and its resident contents. In addition to the proper unaltered ER *Desmin* mRNA localization, anti-KDEL immunofluorescence demonstrated that the localization pattern of the protein components of the ER were unaffected by the DM2 protein knockdown. These experiments demonstrate that pDM2 functions in *DMPK* mRNA localization; they indicate its importance in the localization process, as loss of the DM2 protein specifically disrupts the wild type DMPK message distribution in cells.

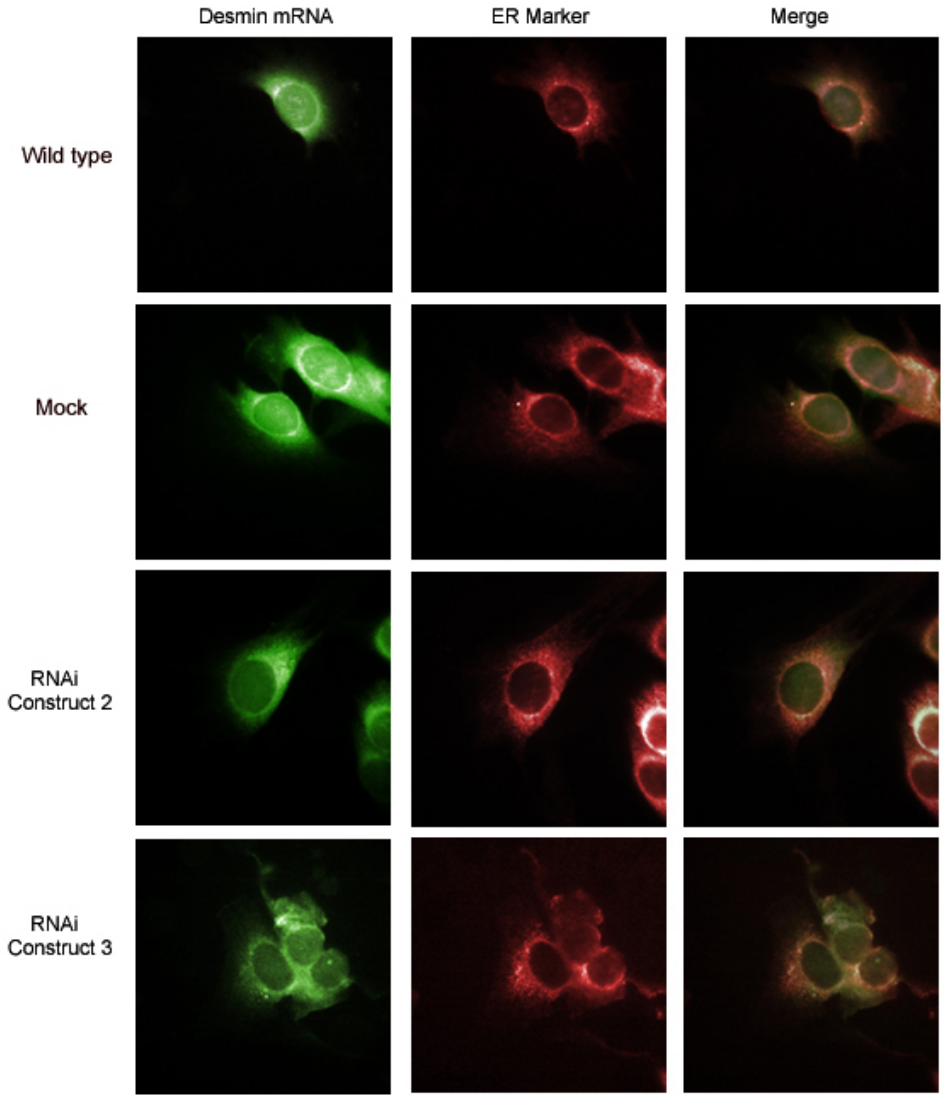

Figure 4.11 Mislocalization from RNAi reduction of pDM2 is specific

Desmin mRNA is an ER localized message, so *in situ* hybridization with *Desmin* antisense probe was performed in parallel to *DMPK in situ* hybridization analysis as a specificity control. The *Desmin* mRNA localization pattern was unchanged in all samples, regardless of the protein level of pDM2, showing that the role of pDM2 in *DMPK* mRNA localization is specific and that the mislocalization of *DMPK* mRNA is not a general phenomenon of all ER localized messages in these cells.

KNOCKDOWN OF PDM2 IS ALSO SUFFICIENT TO MISLOCALIZE AN EGFP REPORTER, UNDERSOCIRNG ITS ROLE IN LOCALIZATION

Our data demonstrated that the *DMPK* 3'UTR is sufficient to localize an EGFP reporter (Figure 4.7) and the data above indicate that reduction of the DM2 protein is sufficient to mislocalize endogenous *DMPK* transcripts. The consequence of pDM2 knockdown on *DMPK* 3'UTR directed localization was examined on the reporter construct to determine if the *DMPK* 3'UTR, which confers specific mRNA localization, is sufficient to transmit the pDM2 effect observed on endogenous message. RNAi constructs were co-transfected into C_2C_{12} cells with an EGFP-DMPK 3'UTR construct, incubated to allow the RNAi to take affect, fixed and the EGFP protein signal was examined as a read out for the EGFP reporter. Indeed, depressed cellular amounts of the DM2 protein caused altered localization of the EGFP reporter in these cells as compared to mock construct. Mirroring the situation with endogenous *DMPK* mRNA, the reporter was seen extending throughout the cell cytoplasm, rather than being ER confined (Figure 4.12). The fact that pDM2 knockdown's effect on altered cellular distribution is recapitulated on a reporter construct that is localized due to signals within the *DMPK* 3'UTR, underscores the conclusion that the DM2 protein functions directly in *DMPK* mRNA localization.

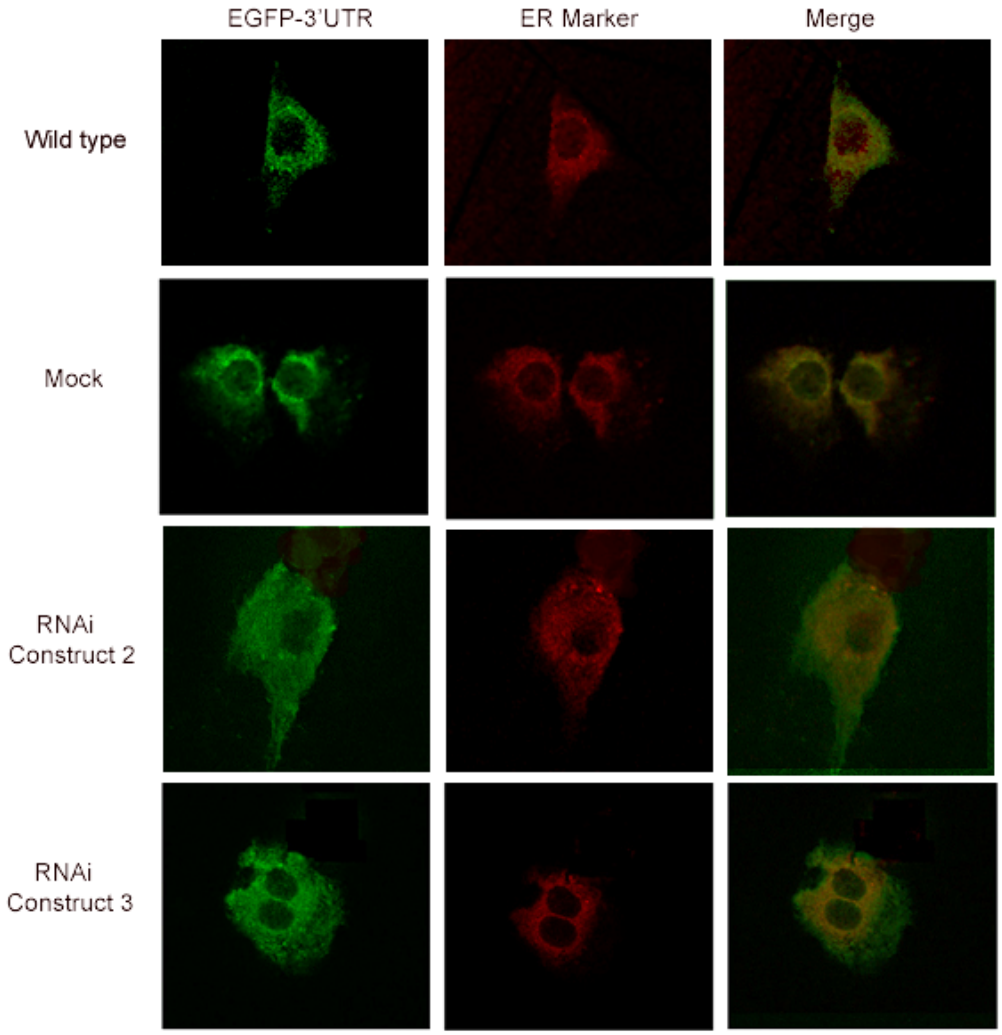

Figure 4.12 Reduction of pDM2 levels alters reporter localization

C_2C_{12} cells were co-transfected with the EGFP-3'UTR reporter (a construct shown to specifically be ER localized, Figure 4.7) and RNAi constructs and the RNAi protocol was performed. Visualization of the reporter construct revealed that the cells that were transfected with no DNA, wild type, or an empty vector, mock, showed the ER localized pattern seen previously. Those cells transfected with either effective DM2 RNAi construct, RNAi construct 2 or 3, displayed a marked change in the localization pattern of the reporter, demonstrating that the mislocalization effect of pDM2 knockdown functions completely through the DMPK 3'UTR and is independent of the rest of the DMPK message.

LOCALIZATION CHANGE FOR DMK PROTEIN AFTER PDM2 KNOCKDOWN

A fundamental purpose of mRNA localization is to control the translational location and timing of the localized message; therefore, localizing transcripts are held translationally silent until the proper signals have been received to release this repression. There are two potential translational consequences of a message whose subcellular localization is incorrect: the message is never translated or it is translated at the incorrect location. It is possible that the message will not be translated because the signals to release the translational repression have not been received and the message will never be made translationally active. The other possibility is that the protein is made at the new, incorrect location because either 1) the aberration that caused the mislocalization also compromises its translational repression by kicking off the repressor or altering the RNA structure into a productive one or 2) a new factor is encountered at the new location that can relieve the repression. In DM patient tissue, the mislocalized *DMPK* message was translated since the DMPK protein was observed in sarcoplasmic masses, the site of RNA mislocalization and disease pathology.

To determine if the *DMPK* mRNA mislocalization resulting from the loss of pDM2 also results in the mislocalization of the DMPK protein, immunofluorescence of the DMPK protein was performed after the RNAi knockdown of pDM2 in C_2C_{12} cells. Like the *DMPK* mRNA, in cells that were transiently transfected with effective DM2 knockdown constructs, the DMPK protein distribution shifted away from concentrated endoplasmic reticulum staining to a more diffuse cytoplasmic pattern. Thus, the DMPK protein is translated at the mislocalized site, recapitulating the disruption of DMPK protein distribution upon *DMPK* mRNA mislocalization that was observed in muscle tissue.

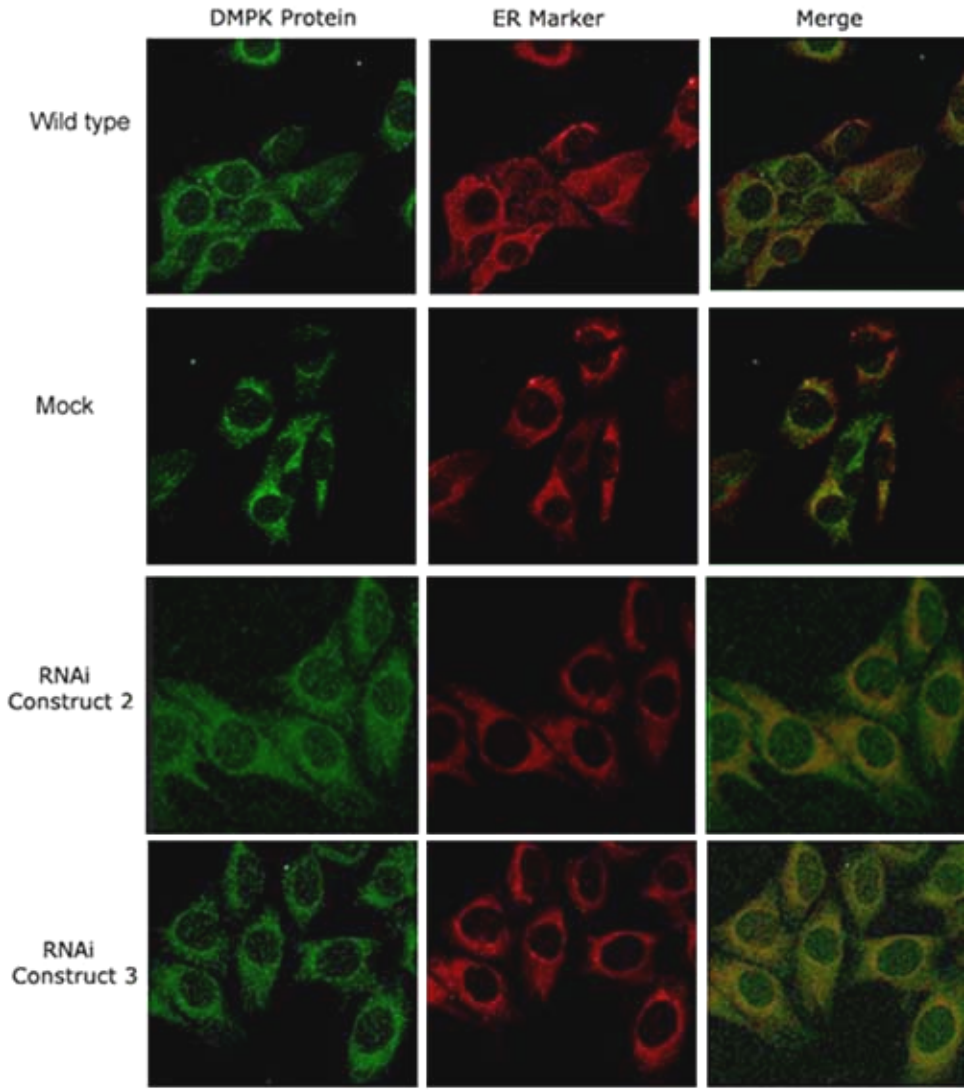

Figure 4.13 DMPK protein localization is altered in pDM2 knockdown cells

C_2C_{12} cells that were treated with the DM2 RNAi procedure were subsequently probed via immunofluorescence for the localization of the DMPK protein. In wild type, no DNA was transfected, and mock, an empty vector was transfected, cells the DMPK protein pattern is restricted to the ER, as seen by co-localization with an ER marker. Upon DM2 protein knockdown, the DMPK protein pattern extends beyond the ER into the cytoplasm (RNAi Constructs 2 and 3), thus loss of the DM2 protein alters DMPK protein localization.

Conclusions

mRNA localization is crucial for proper embryonic development in model systems and in the nerves and muscles of humans. While disruption of localization in models systems in well established to cause birth defects and other severe abnormalities, no analogous mislocalization disease has been identified in humans. Our work examining *DMPK* mRNA localization in human muscle tissue from normal and DM afflicted individuals (Chapter 3) implicated mRNA (mis)localization as a cause of Myotonic Dystrophy. These data, along with my data in the disease relevant muscle culture system, provides proof that mRNA localization does indeed underlie the human neurodegenerative disease DM (this chapter). Reporter constructs reveal information necessary for proper *DMPK* mRNA localization resides in the *DMPK* mRNA 3'UTR, the site of localization information in most messages. Moreover, we show that DM1's causative mutation disrupts *DMPK* localization when harbored within the *DMPK* 3'UTR (this chapter).These data solidify a role for RNA (mis)localization in DM pathology and provide a molecular mechanism whereby DM1's disease mutation acts *in cis* to function in DM etiology/pathology.

To date, the role of DM2 in DM etiology has received comparatively little attention. DM2 was characterized by a large (up to 49kb) insertion within the first intron of the *ZNF9* gene. Initial speculation suggested that here was a common pathology of DM1 and DM2 with the DM repeat expansion and the ZNF9 insertion coalescing into nuclear foci and chelating the same RNA binding protein, thus removing them from their appropriate tasks (Liquori 2001). From our perspective these two large sequences (CUG vs. CCUG) could not be expected to form the same RNA structure and, therefore, would

not be able to specifically bind the same RNA binding proteins. Additionally, the specificity of the nuclear foci and their relationship to DM has recently been called into question (Ho 2005, Houseley 2005, Holt 2007).

While DM2 is relatively rare (<2% of DM cases), we considered that this might provide vital clues to the process underlying DM pathology. Given that DM1 and DM2 produce the same diverse constellation of symptoms, we postulated that DM1 and DM2 gene products function in the same molecular pathway. The RNAi experiments delineated here suggest that this is indeed the case. They show that the DM2 protein is essential for the proper cellular localization of *DMPK* mRNA and its disruption alters *DMPK* mRNA localization. Therefore, these data establish that the gene products identified by the two forms of DM are functionally linked.

CHAPTER 5: DM2 PROTEIN BINDS A SUBSET OF THE *DMPK* MRNA 3' UNTRANSLATED REGION

Overview

Based on the *DMPK* mRNA mislocalization resulting from pDM2 knockdown seen in Chapter 4, we hypothesize that the DM2 protein performs an essential role in the normal subcellular localization of the *DMPK* mRNA. The domain composition of the DM2 protein gives further insight into its potential function in this pathway. This small protein possesses eight RNA binding domains: seven CCHC type Zinc fingers and an RGG box (Armas 2008). Zinc fingers are a class of common protein motifs that fold into finger-like structures that make tandem contacts with the target molecule, whether the target is a protein, RNA or DNA depends on the amino acid composition affecting the fold of the domain (reviewed in Laity 2001). The CCHC type of zinc finger, as seen in the DM2 protein, is an unusual zinc finger that is found in a small subset of zinc finger containing RNA binding proteins (Hall 2005). The other domain in the DM2 protein, the RGG box, is an arginine-glycine-glycine rich region highly similar to the RNA binding domain found in hnRNP proteins and proposed to be a predictor of RNA binding activity (Kiledjian 1992).

The overwhelming presence of known RNA binding domains strongly predicts that pDM2 has RNA binding ability. I hypothesize that pDM2 is an RNA binding protein that interacts with the localization element within the 3'UTR of the DMPK mRNA. A series of experiments intended to biochemically characterize pDM2's hypothesized interaction with the DMPK mRNA were conducted. These data 1) establish a molecular association between the DMPK mRNA and the DM2 protein.2) map the interaction to a very small subset of the DMPK 3'UTR and demonstrate this region acts as an mRNA

localization element and 3) provide insight into a potential simple molecular switch underlying the disease state.

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Results

DMPK mRNA and pDM2 Biochemically Cofractionate

We have demonstrated the importance of pDM2 in *DMPK* mRNA localization (Chapter 4) and established C_2C_{12} mouse myoblasts as a disease relevant tissue culture system for studying this process. This system provides a means of biochemically analyzing the hypothesized role of pDM2 as a *trans*-acting factor in the *DMPK* RNA localization process. An interaction between the DM2 protein and the *DMPK* mRNA would absolutely require that the two at least partially exhibited overlapping subcellular localization. Although I have shown previously that these two components co-localize in both human muscle tissue and myoblast cell culture and that they exhibit a functional overlap, these data have not determined if pDM2's function in this pathway is through direct association with the localized mRNP or more tangentially.

To elucidate whether the DM2 protein and the *DMPK* mRNA reside within the same molecular particle, I employed cofractionate analyses using sucrose gradients. This technique separates cellular extract components based on the density of the naturally occurring complexes found within a cellular extract. Thus, if two factors reside within the same complex, at least a subset of each component will be found in the same gradient fraction. A 10-40% sucrose gradient was layered with C_2C_{12} cell extract and separated via centrifugation. Resulting fractions were analyzed for the presence of the DM2 protein via western blot and the *DMPK* mRNA via northern blot.

Since the DM2 protein itself is 19KD, free protein will reside at the top of the gradient. Its sedimentation elsewhere in the gradient should reflect its stable association in a molecular complex with other cellular components. Notably, gradient analysis revealed no significant free DM2 protein exists in my extracts; rather it is found sedimenting further into the gradient (Figure 5.1). This implies it exists as a component of larger molecular complex(es). This finding has direct implications for our subsequent experiments (see below). Moreover, the DM2 protein and the DMPK mRNA cofractionate into two separate peaks, of approximately (35s) and (50s). This sedimentation implies the DM2 protein is found in larger particulates which likely have multiple other components (RNAs and/or proteins). This is consistent with the large number of interaction domains (7 Zinc fingers + RGG box) within the 19 kD DM2 protein. Indeed, DM2 could act as a potential scaffold or adaptor where it simultaneously interacts with a variety of components.

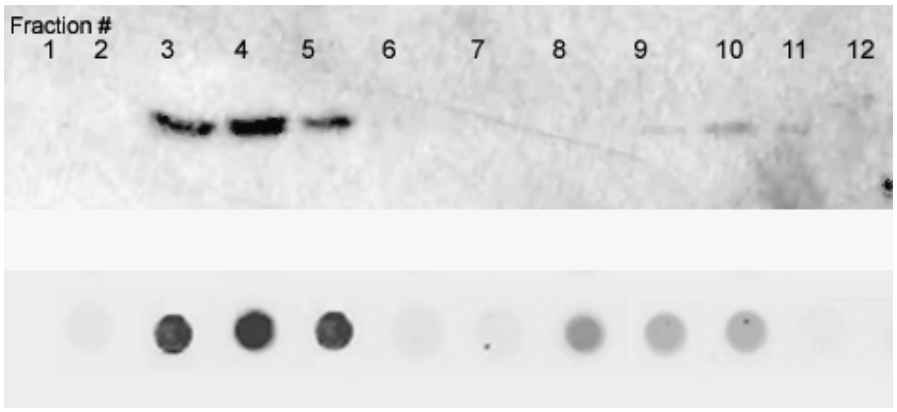

Figure 5.1 DMPK mRNA and DM2 protein Cofractionate in Sucrose Gradients

C_2C_{12} myoblast extract was fractionated through a 10-40% sucrose gradient and the resultant gradient fractions were analyzed for the presence of *DMPK* mRNA and DM2 protein. Top panel: Half of the total RNA from each gradient fraction was dotted onto a nylon membrane and probed for the presence of *DMPK* mRNA using an *in vitro* transcribed, radiolabelled antisense mouse *DMPK* mRNA riboprobe. Bottom panel: DM2 protein distribution was determined by anti-DM2 antibody western blot analysis on a quarter of the total protein from each gradient fraction.

pDM2 Associates with Endogenous *DMPK* mRNA

mRNAs do not exist in the cell as naked RNAs; rather, they are part of an mRNP. A subset of cellular proteins instrumental in mRNA localization should be part of the mRNP harboring the localized mRNA. Cosedimentation of pDM2 and the *DMPK* mRNA in the previous gradient analyses in two complexes, each with a similar density, does not directly demonstrate these two molecular components reside in the same molecular complex/particle.

To probe for potential association of pDM2 and *DMPK* mRNA, C_2C_{12} whole cell extract was subjected to anti-DM2 immunoprecipitation. As a control, immunoprecipitation with non-immune sera was performed in parallel. Following immunoselection, conjugates were eluted and nucleic acids in each eluate were extracted. Samples were fractionated via denaturing gel electrophoresis, and subjected to northern blot analysis. Probing the blot with a internally radiolabelled antisense *DMPK* riboprobe revealed pDM2 and *DMPK* mRNA do indeed interact (Figure 5.2 lane 2). Controls indicate that this molecular association was highly specific. Therefore, pDM2 and *DMPK* mRNA reside in the same molecular particle(s).

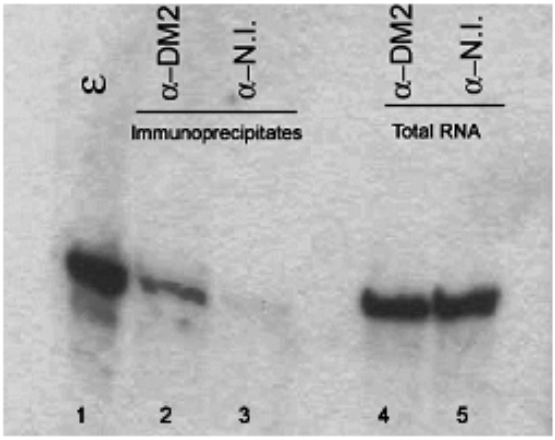

Figure 5.2 pDM2 specifically associates with endogenous DMPK mRNA

C_2C_{12} whole cell extract was phenol extracted (lane 1, ε) or subjected to immunoselection with anti-DM2 (lanes 2,4) or non-immune (lanes 3,5) sera. Antibody conjugated Protein-A Sepharose beads (lanes 2,3) or supernatants (lanes 4,5) were extracted. Samples were fractionated via 8M Urea 4% polyacrylamide gel electrophoresis, electophoretically transferred to Nitrocellulose and subjected to northern blot analysis using a radiolabelled riboprobe complementary to the *DMPK* 3'UTR. Lane 1 indicates the mobility of *DMPK* mRNA and lanes 4, 5 indicate that equal amounts of intact *DMPK* mRNA were present for both immunoprecipitation reactions.

DM2 PROTEIN CAN ASSOCIATE WITH EXOGENOUS *DMPK* MRNA

Further biochemical analyses of pDM2 binding to *DMPK* mRNA required an *in vitro* binding assay. For these analyses, I chose to use a whole cell C_2C_{12} extract since this has components for *DMPK* mRNA and pDM2 association and an *in vitro* transcribed, radiolabelled *DMPK* 3'UTR transcript since localization information for the vast majority of localized messages reside there (reviewed by Chabanon 2004, Jambhekhar 2007). The *DMPK* 3'UTR and extract were mixed and incubated under complex forming conditions.

This should allow any factors which associate with the *DMPK* 3'UTR to bind/assemble. This procedure has been used successfully to map known Drosphila localization factors with the 3'UTR of the localized Drosphila *bicoid* mRNA (exuperantia, staufen) in the Gottlieb lab. Taking into account the numerous zinc fingers within the DM2 protein and their requirement for the presence of zinc to properly bind their target, 1mM $ZnCl_2$ was included in the binding reactions. This amount was determined based on the conditions for Muscleblind RNA binding (Miller 2000), another zinc finger containing RNA binding proteins involved in DM biogenesis, as well as other zinc finger containing proteins (Zang 1995, Rakitina 2006). After complex formation, this reaction was probed via immunoprecipitation as before using anti-DM2 or non-immune serum. Nucleic acids in the resulting samples were extracted, fractionated via denaturing polyacrylamide gel electrophoresis and visualized by autoradiography. Mirroring the endogenous immunoprecipitation experiments, the DM2 protein successfully and selectively interacts with the exogenous *DMPK* 3'UTR transcript (Figure 5.2). This demonstrates that endogenous DM2 protein in a cell extract is able to bind to an *in vitro* transcript of the DMPK message, thus creating a system for the biochemical examination of this interaction.

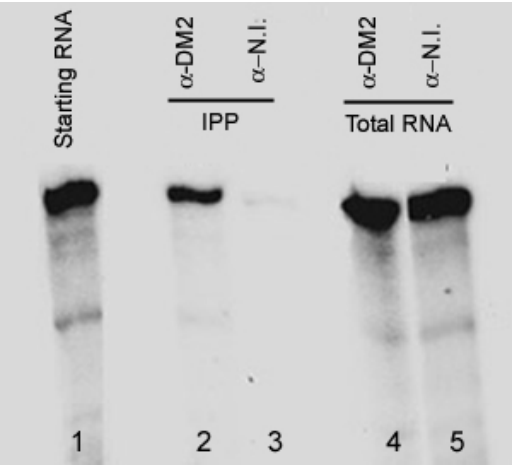

Figure 5.3 The DM2 protein recognizes the DMPK 3'UTR in vitro.

An internally radiolabelled *DMPK* 3'UTR riboprobe (lane 1) was mixed with C_2C_{12} whole cell extract and incubated under complex formation conditions containing zinc. Reactions were subjected to immunoselection with DM2 (lanes 2,4) or nonimmune (lanes 3,5) antibodies. Nucleic acids in precipitates (lanes 2,3) and supernatants (lanes 4,5) were extracted, electophoretically fractionated on an 8M Urea 4% polyacrylamide gel and transcripts were visualized via autoradiography. Supernatants (lanes 4,5) reveal equal amounts of full length mRNA were present in both immunoprecipitation reactions (lanes 2,3) and that the binding in immunoprecipitation procedures do not lead to transcript degradation.

pDM2 Specifically Recognizes a Subset of the *DMPK* mRNA

For the first time, these data establish a physical interaction between the DM2 protein and the *DMPK* mRNA and, additionally, they reveal that association occurs via the *DMPK* 3'UTR. However, the *DMPK* 3'UTR is almost 800 nucleotides long, so further study is desirable to define the regions of the *DMPK* mRNA that are necessary for pDM2 recognition. A smaller, more defined mRNA region is preferable for application of a battery of analytical RNA-protein interaction techniques (usually 500 nts or less).

To achieve this goal, a DNA plasmid carrying the *DMPK* 3'UTR cloned downstream of a T3 promoter were digested independently with 4 specific restriction enzymes, SmaI, PstI, BamHI and XbaI. Parallel *in vitro* transcription reactions using T3 RNA polymerase created a nested set of internally radiolabelled run-off transcripts of increasing length (Figure 5.4a). Each has the same 5'end with increasing length 3' ends such that the final transcript (XbaI) encompasses the entirety of the 3'UTR.

As a source of DM2 protein for these experiments, we considered using purified material (synthesized off a molecular clone). Instead, we turned to a whole cell extract known to possess large amounts of DM2 protein functional in the localization process (C_2C_{12} myoblast cells). This was done for several reasons; 1) there was no appreciable free pool of DM2 protein in our gradient analyses (Figure 5.1), 2) the large number of zinc fingers in DM2 suggested that this protein is capable of molecular interactions and it is possible that one or more factors facilitates or stabilizes any *DMPK* mRNA-DM2 interaction and 3) to date, no *in vitro* RNA localization assay is available to confirm that DM2 protein synthesized off a clone is in the proper form (with regard to folding, modification, etc) to function in localization. It should be noted that whole cell extracts mixed with synthetic riboprobes have been employed extensively to characterize RNA-protein and RNA-RNA interactions at the heart of seminal post-transcriptional RNA processes, including mRNA splicing, mRNA polyadenylation, histone 3' end processing, and mRNA stability. Moreover, this type of extract along with synthetic riboclones have been highly fruitful in identifying *trans*-acting factors and their associated *cis*-acting RNA sequences in many of the aforementioned processes. More specifically, it has been successful in linking RNA factors and *cis*-acting signals instrumental in the mRNA localization process in Drosophila (Linn, L and Gottlieb, E unpublished data, Tsalkova,T and Gottlieb, E unpublished data).

Experimentally, the transcripts described above were combined with C_2C_{12} myoblast cell extract, incubated under complex formation conditions, and subjected to immunoselection with anti-DM2 and non-immune antibodies as indicated previously. Once again, nucleic acid in immunoprecipitates and supernatants were extracted, purified, and fractionated via denaturing polyacrylamide gel electrophoresis. Autoradiographic analysis revealed *DMPK* 3'UTR mRNA sequences essential for DM2 protein recognition are localized between the 5' end of the 3' UTR and the PstI site (335 nucleotides) (Figure 5.4B). This conclusion arises from the fact that transcripts independently ending at the PstI (335 nt), BamHI (537 nt) and XbaI (745 nt) sites are each selectively precipitated with anti-DM2 antibodies while the shortest transcript ending at the SmaI site (5' most 83 nt) failed to precipitate. This indicates that sequences essential for DM2 association exist in the 252 nucleotide region between the SmaI and Pst I sites.

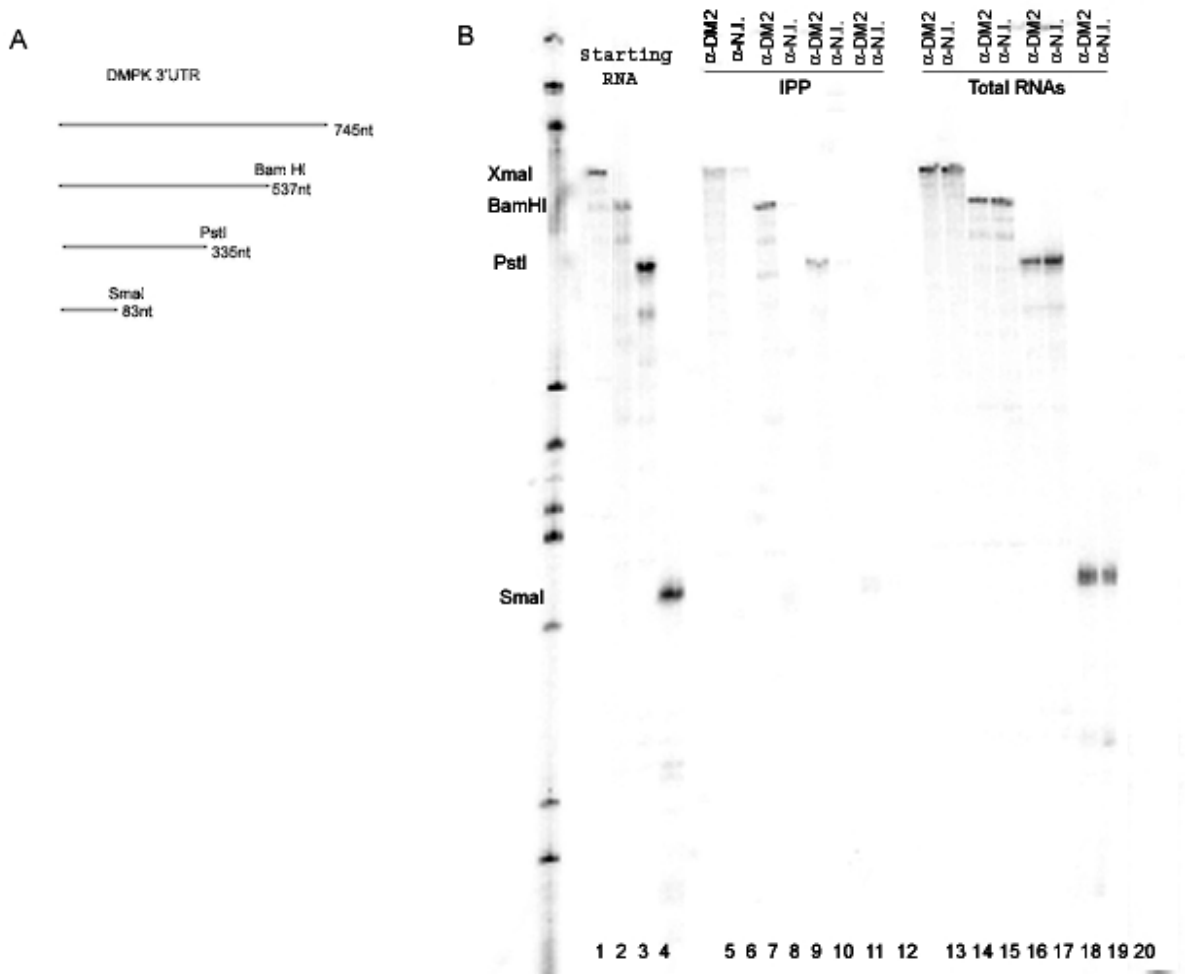

Figure 5.5 Only a subset of the DMPK mRNA 3'UTR is necessary for pDM2 binding

A) A collection of in vitro transcripts, radiolabelled run-off transcripts of the *DMPK* mRNA 3'UTR were created, each successively shorter. B) The run-off transcripts were complexed with whole cell C_2C_{12} extract and immunoprecipitated with anti-DM2 (lanes 6,8,10,12) or non-immune (lanes 7,9,11,13) sera. Nucleic acids in precipitates (lanes 6-13) and supernatants (lanes 14-21) were extracted, electophoretically fractionated on an 8M Urea 6% polyacrylamide gel and transcripts were visualized via autoradiography. Supernatants (lanes 14-21) reveal equal amounts of mRNA were present in both anti-DM2 and non-immune) immunoprecipitation reactions for each transcript and that the binding in immunoprecipitation procedures do not lead to transcript degradation.

pDM2 Interaction with the *DMPK* Message is Sensitive to RNA Structure

Our initial analyses indicated that pDM2 interacts with the *DMPK* mRNA via element(s) contained within the first 335nt of the *DMPK* 3'UTR, particularly the 252 nucleotides between the SmaI and PstI sites. Among other things, this segment of the 3'UTR contains a region predicted to form a G-quartet and the site where the disease-causing (CTG)n expansion would reside in the corresponding mutant *DMPK* 3'UTR found in DM1 patients. We wished to further define the DM2 protein-*DMPK* 3'UTR interaction and determine whether any of the aforementioned features were influential in binding. These analyses could lead to further insights into the molecular basis underlying DM as well.

We showed that the 3'UTR was necessary and sufficient for wild type ER localization of a reporter and the literature indicates that localization signals often contain structural components. Therefore, we wanted to take an RNA approach to this problem, rather than use a series of small 3'UTR fragments that would likely not tell the entire story. Fortunately, the newly defined 3'UTR region fell within the range (<500nt) that is applicable to a wide variety of RNA-protein interaction techniques.

To more precisely map the pDM2 binding site on this 335nt portion of the *DMPK* 3'UTR, we chose to employ a protection-immunoselection experiment (Figure 5.6A). Protection-immunoselection has been successfully used to investigate factor-mRNA interactions for several regulated steps of RNA biogenesis, including mRNA splicing (Black 1985, Black 1986), histone 3'end formation (Mowry 1987) and mRNA localization (Lin, Tsalkova), in order to map the exact binding site of a specific protein or RNP cofactor on a corresponding specific mRNA target. This extremely powerful tool has two huge advantages: 1) a binding site can be determined whether it is a linear stretch

of RNA, different parts of the RNA brought together in space by RNA folding or a binding site influenced by global RNA structure and 2) interaction sites can be determined whether the candidate *trans*-acting factor cellular component binds the mRNA directly (such as the polypyrimidine binding protein involved in mRNA splicing), indirectly through association with another protein or RNA cofactor (ex: U1 snRNP in 5' splice site recognition) or in association with another factor which stabilizes RNA binding (ex: the exuperantia mRNA localization factor).

For this experiment, we generated an internally radiolabelled T7 transcript encompassing nucleotides 1 to 335 of the *DMPK* 3'UTR. This was allowed to fold and was then mixed with a while cell extract from C_2C_{12} myoblasts which possesses amounts of the DM2 protein in a form competent for proper wild type *DMPK* mRNA localization (Chapter 4). Following incubation under complex formation conditions to allow any factors that normally associate to assemble, reactions were treated with T1 RNase, an RNase that normally cuts after G residues. It will cleave the target DMPK RNA transcript with the exception of G residues that are protected by RNA structure and/or binding of associated factors (proteins and/or RNPs). T1 treated reactions are then immunoselected with anti-DM2 antibodies and the radiolabelled transcript portions bound to pDM2 are then isolated, purified and electrophoretically separated on a denaturing polyacrylamide gel. Fragments which are selected due to DM2 association are then sized and their subsequent composition is determined unambiguously by nuclease mapping. Parallel non-immune precipitates and nuclease treated transcript without extract exposure facilitate this analysis.

This experiment involved titration of a variety of components (T1 concentration, RNA competitor, extract concentration, salt concentration, etc). While these experiments revealed that DM2 protein did indeed specifically and selectively recognize a subset of

the *DMPK* 335nt 3'UTR region, the pattern did not lead to a clean mapping which contrasted with two Drosophila RNA localization factors (exuperantia and staufen) that the Gottlieb lab previously mapped. For DM2, I consistently observed a protected linear band with migration between roughly 35 and 45 nucleotides. Additionally, there was a dominant set of apparently nested bands that moved around in size with different binding conditions. Many were found independently of extract, suggesting that they may be RNA structures. Most telling was the change in nested selected bands seen upon Mg^{2+} titration, a divalent metal ion which often influences RNA structure (reviewed in Draper 2004). Taken together, we concluded the DM2 protein "binding site" likely included a core 35-45 nucleotide RNA element which was highly influenced by RNA structure, as is the case for a substantial subset of localization factors. I concluded that I needed to visualize DM2 protein binding of *DMPK* RNA by an alternative method that took into account, but was not confounded by, RNA structure.

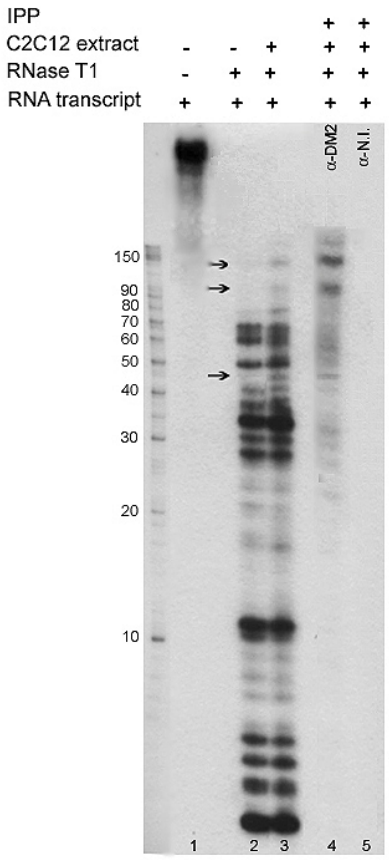

Figure 5.6 A small portion of the DMPK 3'UTR is protected and immunoprecipitated by anti-DM2

An in vitro transcribed, internally radiolabelled transcript of the first 335 nucleotides of the *DMPK* 3'UTR (lane 1) was treated with RNase T1 either as is (lane 2) or after incubation with C_2C_{12} whole cell extract (lanes 3-5). The banding pattern in lane 2 represents the areas of the transcript that are naturally protected by internal structure. Several new bands were visible after incubation with extract (lane 3), indicating that portions of the transcript were protected from RNase degradation by factors in the extract. Only a subset of these new bands (black arrows) was immunoprecipitated by anti-DM2 antibodies; they represent the area of the transcript that was protected by DM2 protein containing complexes in the extract (lane 4). The immunoprecipitation of these bands by anti-DM2 was specific, as non-immune sera did not precipitate RNA (lane 5). Despite consistently seeing a discrete set of bands in the DM2 immunoprecipitated lane, the background bands prevented secondary analysis to decipher their sequences.

DISCRETE *DMPK* 3'UTR REGIONS ARE NECESSARY FOR PDM2 BINDING

The protection-immunoselection experiment indicated that a small region of the DMPK mRNA is consistently protected by DM2 or a DM2 containing complex, but the intensive natural structure within the message prevented a clear mapping of the DM2 protein interaction site. These data revealed the importance of preserving the structure that mapping this RNA-protein interaction required an experimental design which retains structures within the transcript during the binding analyses. A technique was designed exploiting the function of RNase H, which precisely destroys RNA-DNA duplexes, to selectively degrade 20-25 nucleotide sections of the *DMPK* 3'UTR mRNA. In this experiment, an oligonucleotide was bound in a sequence specific manner to one site on an in vitro synthesized *DMPK* 3'UTR transcript, boiled and allowed to slowly cool to room temperature to open any RNA structure and create a DNA-RNA duplex. These potential duplexes were then treated with RNase H to degrade the RNA in the duplex region, biochemically creating a mutant transcript lacking the targeted region of RNA. The 'mutant' transcript was then complexed with C_2C_{12} extract and immunoprecipitated with anti-DM2 or non-immune antibody. This strategy was undertaken to determine which sequence of the *DMPK* 3'UTR was required for pDM2 binding. The analysis focused on the region of the *DMPK* 3'UTR shown to accommodate pDM2 binding in the run-off immunoprecipitation experiment (Figure 5.5).

Transcripts individually bound by one of the nine designed oligonucleotides against the DMPK message or no oligonucleotide were treated with RNase H and separated by denaturing gel electrophoresis to confirm that only the predicted sizes of fragments exist after treatment. Indeed, the products of the predicted sizes were seen, indicating that the nine oligonucleotides specifically targeted the desired site of the

transcript and did not bind multiple places. The cleanliness of the RNA verified that the RNA was not non-specifically degraded by the procedure.

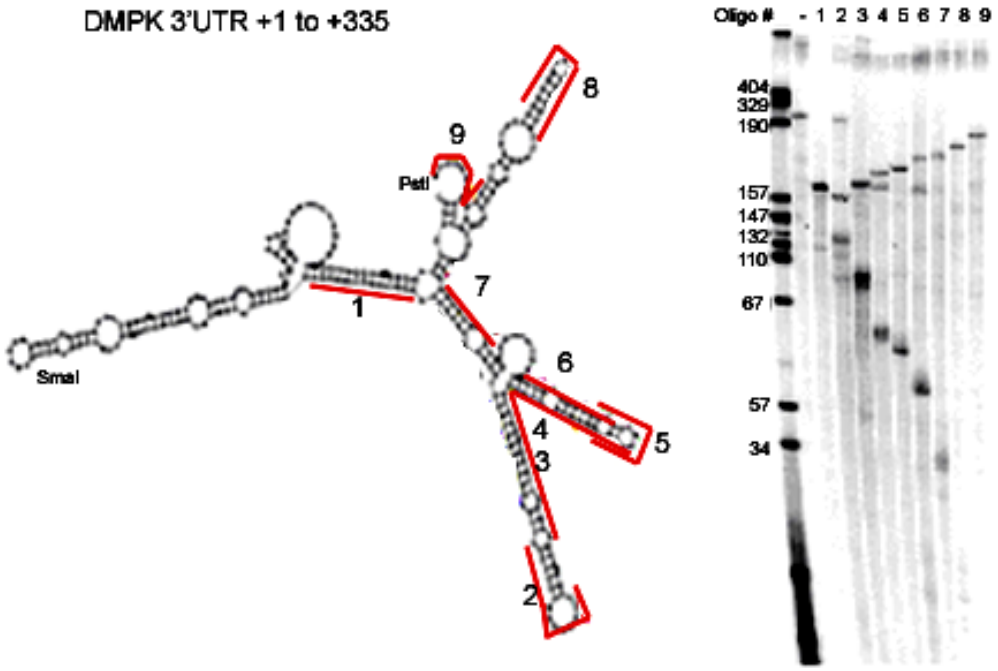

Figure 5.7 Nine oligonucleotides specifically bound the DMPK 3'UTR and were cut by RNase H

Nine oligonucleotides were designed to sequence specifically bind selected segments of the *DMPK* 3'UTR. This area corresponded to the region between the SmaI and the PstI sites, the area we showed to be required for pDM2 binding. The predicted *DMPK* PstI cut 3'UTR structure displayed was created with MFOLD software (Jacobson 1993). RNase H treatment of in vitro transcribed, internally radiolabelled PstI cut *DMPK* 3'UTR transcript individually complexed with each oligonucleotides resulted in RNA fragments of predicted size (oligos 1-9). Transcript that was not complexed with oligonucleotides was subjected to RNase H treatment (lane -) demonstrated that the procedure does result in non-specific cutting or RNA degradation.

These freshly cut transcripts were then incubated with whole cell C_2C_{12} extract, immunoprecipitated with anti-DM2 or non-immune antisera and run on denaturing polyacrylimide gels. During the titration of the binding conditions, varying magnesium concentrations in the reaction were tested because magnesium is a divalent cation known to be required for proper RNA structure formation and stability (reviewed in Draper 2004). Interestingly, the efficiency of the immunoprecipitation was dramatically affected by the amount of magnesium, reinforcing the importance of RNA structure in this interaction. The condition with the optimum binding was 5mM magnesium which corresponds to the physiological concentration of magnesium in cells, as well as the required magnesium conditions for splicing, another essential post-transcriptional RNA process. The magnesium sensitivity of the association indicates that the structure of the message at suboptimal magnesium conditions reduces the binding efficiency of the DM2 protein.

At optimized conditions, the deletion of one region of the transcript, corresponding to RNase H treatment with oligos 2, 3, 4 and 6, was found to eliminate pDM2 binding (Figure 5.8). The predicted structure of this region of the *DMPK* 3'UTR contains two strong hairpins with oligos 2 and 3 targeting one hairpin and oligos 4 through 6 targeting the other (Figure 5.7). Therefore, the presence of both of these hairpins influences the ability of the DM2 protein to associate with the *DMPK* 3'UTR mRNA. Intriguingly, this subset of the transcript is in very close proximity to the site of repeat expansion in the diseased transcript, the second hairpin is only six nucleotides 5' of the first CUG triplet, suggesting that the predicted double stranded structure formed by the (CUG)n repeat expansion may have potential structural consequences on the pDM2 binding site.

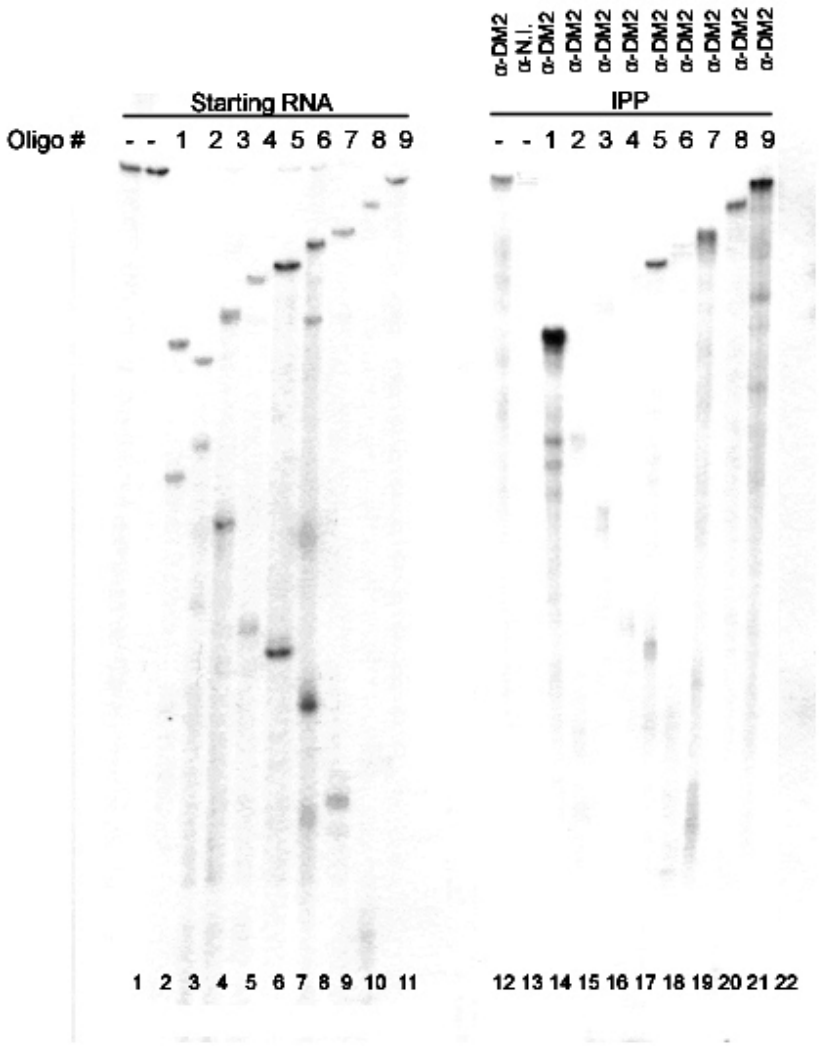

Figure 5.8 The DM2 protein binding requires only a subset of the DMPK 3'UTR

A PstI cut *DMPK* 3'UTR internally radiolabelled in vitro transcript was incubated with one of the nine oligonucleotides designed to bind this region of the *DMPK* 3'UTR, or none at all, and treated with RNase H which degrades RNA in RNA-DNA duplexes (lanes 1-11). The RNA was then incubated with C_2C_{12} extract in complex forming conditions and immunoprecipitated with anti-DM2 antibody (lanes 12. 14-22) or non-immune sera (lane 13) and fractionated by denaturing PAGE. Four regions were required for DM2 binding (lanes 15-17, 19) as RNase H deletion of these regions prevented DM2 from binding the transcript. Non-immune sera did not precipitate the transcript (lane 13), showing the specificity of the precipitation reaction.

HnRNP C1, an abundant heterogeneous nuclear RNP protein is known to preferentially bind many mRNAs via a U rich sequence (Koloteva-Levine 2002, Irimura 2008). Tiscornia et al demonstrate that HnRNPC1 can bind to the *DMPK* 3'UTR and mapped this interaction to the sequence between the CUG repeat expansion and the PstI site (Tiscornia 2000). Therefore, the sequence required for HnRNPC1 protein binding should reside within the subset of the *DMPK* 3'UTR that I have targeted by RNase H analysis. This procedure was performed and interpreted with the same conditions used for pDM2 binding RNase H analysis except that the immunoprecipitation reactions were carried out with anti-HnRNPC1 rather than anti-pDM2 antisera. This experimentation confirmed that the HnRNPC1 protein interacts with the *DMPK* 3'UTR and demonstrated that the interaction strongly requires only one small region of the transcript. The necessary site is within the confines of the region identified by Tiscornia et al and contains a large proportion of U nucleotides, coinciding with the known characteristics of HnRNPC1 binding and its interaction with the *DMPK* message. This data confirms and expands our knowledge about the pHnRNPC1-*DMPK* mRNA association site while simultaneously demonstrating the specificity and selectivity of the RNase H oligo mapping method as a technique for probing RNA-protein interaction.

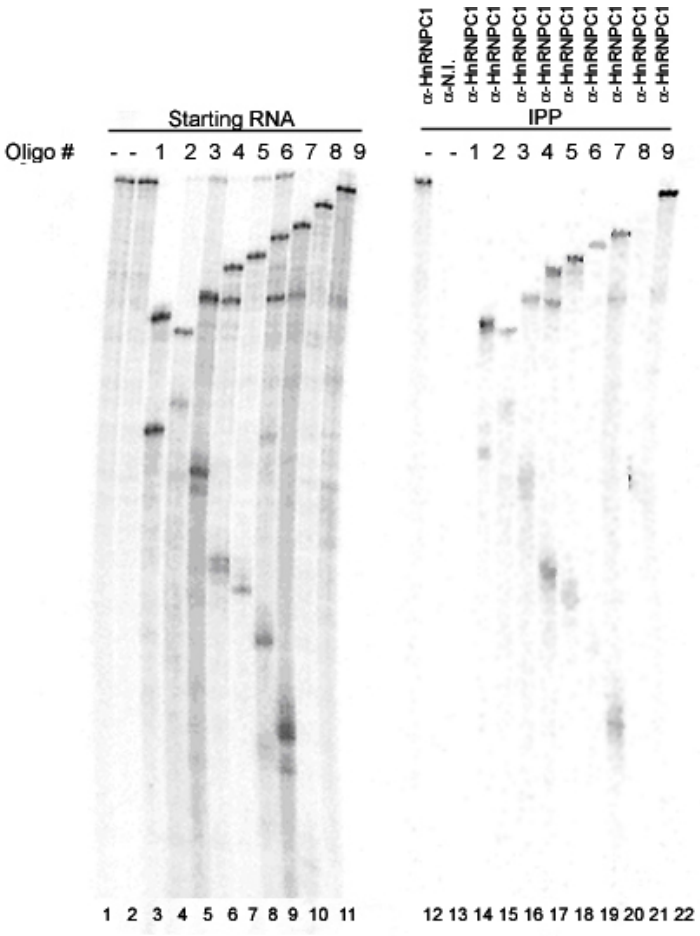

Figure 5.9 HnRNPC1 binding specifically maps to a different region of the DMPK 3'UTR than the DM2 protein

A PstI cut *DMPK* 3'UTR internally radiolabelled in vitro transcript was incubated with one of the nine oligonucleotides designed to bind this region of the *DMPK* 3'UTR, or none at all, and treated with RNase H which degrades RNA in RNA-DNA duplexes (lanes 1-11). The RNA was then incubated with C_2C_{12} extract in complex forming conditions and immunoprecipitated with anti-hnRNPC1 antibody (lanes 12. 14-22) or non-immune sera (lane 13) and fractionated by denaturing PAGE. Only one small region (lane 21) was required for HnRNPC1 binding to this segment of the *DMPK* 3'UTR, as the rest of the deleted transcripts were precipitated by anti-HnRNPC1 antibody. Non-immune sera did not precipitate the transcript (lane 13), showing the specificity of the precipitation reaction. Therefore, HnRNPC1 binding specifically maps to a different region of this transcript than the DM2 protein, demonstrating the selectivity of this procedure.

A 122 NUCLEOTIDE SEGMENT OF THE DMPK 3'UTR IS SUFFICIENT FOR PDM2 BINDING

RNase H oligonucleotide mapping analyses reveal that only a small subset of the *DMPK* 3'UTR is required to support pDM2 binding (Figure 5.8); however, these data only determine which sequences are required to lose interaction, not which sequences are sufficient for DM2 protein binding. Therefore, a set of transcripts were constructed to examine the sequences necessary for pDM2 binding, composed of the DMPK 3'UTR region required for DM2 binding that was identified by the RNase H mapping experiment.

The smallest transcript, referred to as SmaI, represents the 5'end of the DMPK 3'UTR, from the NarI cut site at the 5' end to the SmaI site. This transcript was shown not to contain the sequences necessary for pDM2 binding in the run-off transcription immunoprecipitation experiment (Figure 5.5) and serves as the negative control. The second transcript, called SacII, contains the region from the NarI cut site to the SacII site. The SacII site resides approximately halfway through the possible pDM2 binding site (corresponding to RNase H mapping oligos 1-3). The third transcript, SacII-PstI, contains the region from the SacII cut site to the PstI site, the other half of the possible pDM2 binding site (corresponding to RNase H mapping oligos 4-9. The final transcript is the PstI cut *DMPK* 3'UTR which was shown previously to support pDM2 interaction (Figure 5.5) and serves to confirm that the experimental conditions are conducive to pDM2-*DMPK* mRNA association. The transcripts were in vitro transcribed and radiolabelled and complexed with C_2C_{12} whole cell extract. These complexes were then immunoprecipitated against anti-pDM2 or non-immune sera, extracted from the beads and analyzed by denaturing PAGE.

The binding site suggested by the RNase H experiment was refined by the discovery that the SacII-PstI transcript is sufficient to support robust pDM2 binding, while the region just upstream of this site (represented by the SacII transcript) does not contain the required sequences. As expected, the PstI transcript did support pDM2 binding and the SmaI did not. The specificity of the binding was verified by parallel immunoprecipitation with non-immune sera and the stability of the transcripts was checked by loading total RNA as was described in the run-off transcript immunoprecipitation experiment above. These data map the pDM2 binding site to within a small, 122 nucleotide area of the *DMPK* 3'UTR transcript. They verify and extend the data from the RNase H oligonucleotide mapping experiment by demonstrating that only a subset of the potential pDM2 binding site contains the sequences necessary to facilitate the pDM2-*DMPK* mRNA interaction. The larger pDM2 binding requirement in the RNase H experiment may be due to structural stability. It is conceivable that the required structure was constrained in the longer transcript and required the presence of the upstream sequence to stabilize the local structure of the binding site. Thus, removal of that region in the RNase H experiment compromised the structure of the actual binding location which immediately abuts this area. However, the shortened transcripts did not have the structural strain placed upon them by the rest of the DMPK 3'UTR sequence, so SacII-PstI could fold into and maintain the proper structure without the additional aide of the upstream sequence.

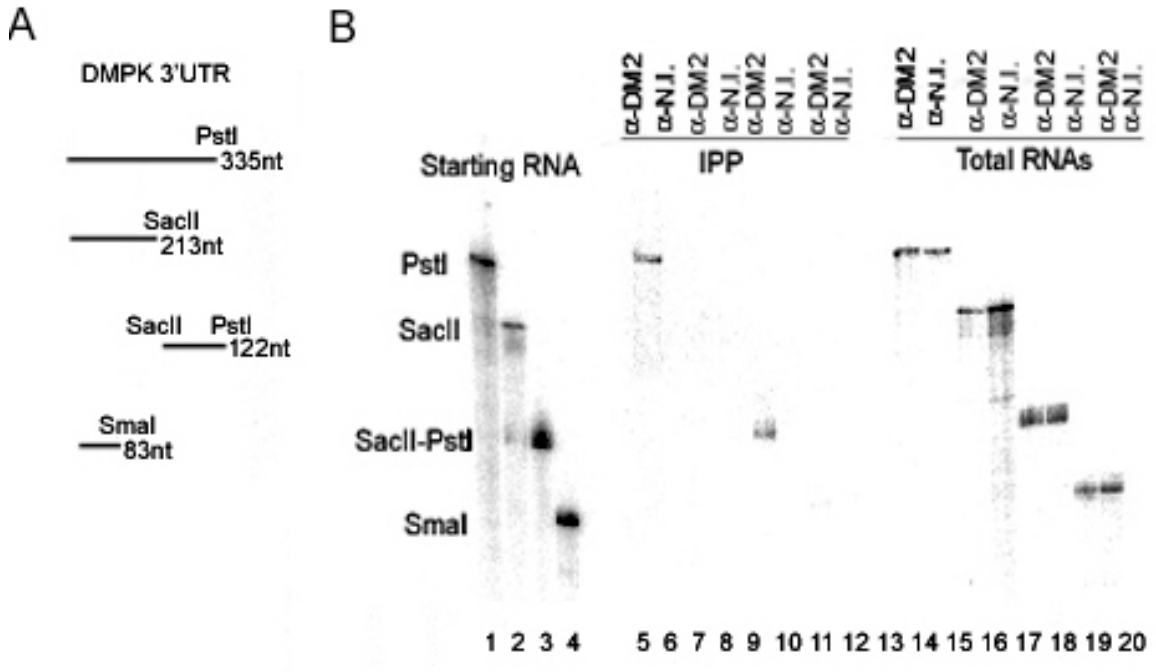

Figure 5.10 pDM2 specifically binds to a 122nt subset of the DMPK 3'UTR

A) Constructs of the DMPK 3'UTR were designed to analyze the region required for DM2 binding as defined by the RNase H mapping experiment (Figure 5.8). The area of particular interest in halved by the SacII restriction site, so constructs SacII and SacII-PstI were made to split the region in order to determine which sequences are required for pDM2 binding. PstI and SmaI transcripts have been shown previously to bind and not bind the DM2 protein respectively (Figure 5.5) and served as controls. B) Transcripts were incubated with C_2C_{12} extract under complex forming conditions, immunoprecipitated and resulting RNA separated by denaturing PAGE (lanes 5-12). This study revealed that only two constructs, PstI and SacII-PstI, contain the sequences necessary to facilitate pDM2 binding (lanes 5,9); therefore, the 122nt region between the SacII and PstI sites is sufficient for pDM2 binding. Non-immune sera did not precipitate the transcripts (lanes 6,8,10,12), showing the specificity of the precipitation reaction. Total RNAs verify that the transcripts were not degraded during the procedure (lanes 13-20).

THE *DMPK* 3'UTR REGION REQUIRED FOR PDM2 BINDING IS NECESSARY AND SUFFICIENT TO LOCALIZE AN EGFP REPORTER.

In considering my data in the context of a localization model for Myotonic Dystrophy, pDM2 represents a candidate RNA localization factor. Therefore, the 122nt region of the *DMPK* mRNA 3'UTR that is required for pDM2 association as defined by my RNase H protection experiments *in vitro*, should be required for function (i.e. proper wild type *DMPK* mRNA localization) *in vivo*. From the functional data presented in Chapter 4, the *DMPK* 3'UTR is sufficient to properly localize an EGFP reporter construct to the endoplasmic reticulum in cultured myoblast C_2C_{12} cells (Figure 4.7). In contrast, we discovered that an EGFP construct containing the *DMPK* 3'UTR devoid of this 122nt region was unable to exhibit proper wild type subcellular localization in this C_2C_{12} system (EGFP-ΔHairpin, Figure 5.11), proving that the region of the *DMPK* 3'UTR required for pDM2 association is necessary for proper wild type *DMPK* mRNA localization to the ER.

Given what we know about RNA localization in general, this 122 nucleotide region of the *DMPK* 3'UTR may also be sufficient for localization. Alternatively, it is possible that additional portions of this 3'UTR are required to direct proper wild type ER localization in a combinatorial manner. To test these possibilities, the localization of an EGFP reporter construct containing only the 122 nucleotide segment of the *DMPK* 3'UTR was assessed (EGFP-Hairpin, Figure 5.11). Significantly, this 122nt 3'UTR region which is required for pDM2 binding to the *DMPK* mRNA, is definitively sufficient to direct proper wild type localization. Thus, we conclude that the DM2 protein represents an mRNA localization factor, and that this 122nt subsection of the *DMPK* 3'UTR harbors a *cis*-acting mRNA localization signal.

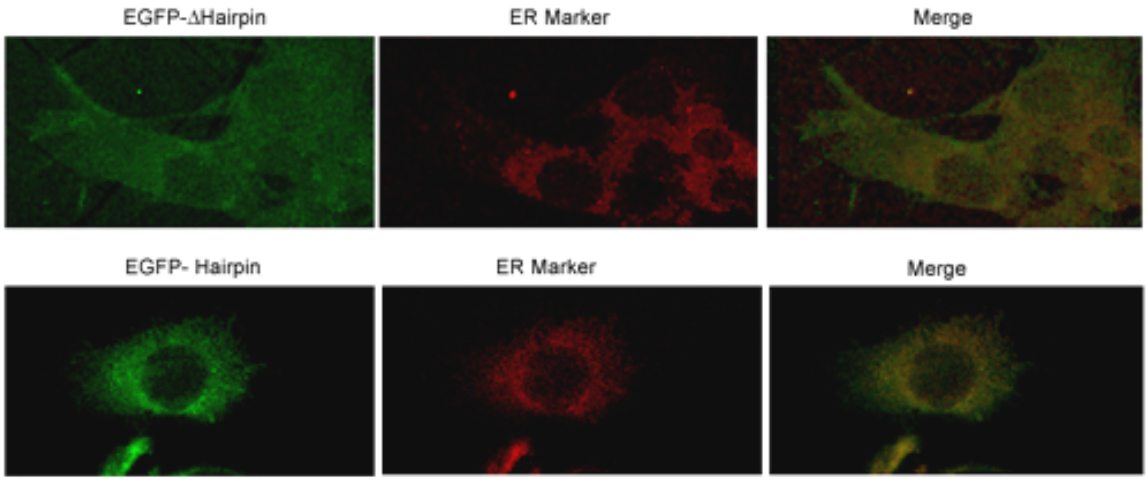

Figure 5.11 The 122nt pDM2 binding site is necessary and sufficient to localize a reporter

The localization of an EGFP reporter construct containing the entire *DMPK* 3'UTR except for the 122nt region between the SacII and PstI sites (the area predicted to contain the pDM2 binding site), referred to as EGFP-ΔHairpin, was analyzed. Notably, the contained ER localization pattern seen previously was markedly disrupted, showing that this section of the *DMPK* 3'UTR is necessary for proper *DMPK* mRNA localization. The localization of an EGFP reporter construct containing just this 122nt region (EGFP-Hairpin) was analyzed and found to be sufficient to induce ER specific localization.

DM2 PROTEIN FAILS TO BIND THE DMPK 3'UTR HARBORING THE (CUG) N DISEASE CAUSING REPEAT EXPANSION

A combination of my *in vivo* localization experiments and complementary binding analyses provide insight into the wild type interaction between the DM2 protein and the normal, wild type *DMPK* transcripts. For the first time, they molecularly link the two cellular components which operate in the human disease Myotonic Dystrophy. Additionally, they provide a wild type function for the DM2 protein in *DMPK* mRNA

expression. However, so far, they do not directly provide information on the diseased state.

It is well established that the majority of Myotonic Dystrophy cases are caused by a (CUG)n expansion in the 3'UTR of the *DMPK* mRNA (DM1). Upon examination of this data, we realized that the (CUG)n disease causing expansion occurs just 6nt downstream of the 3' end of the *DMPK* 3'UTR region required for pDM2 recognition. This situation provided a means to test the role of (CUG)n expansion on pDM2 interaction. Due to the proximity of these two elements, we hypothesized that the presence of the repeat expansion could completely occlude pDM2 binding. Alternatively, since the (CUG)n repeat expansion is known via crystallography and EM analysis to form a large double stranded RNA stem (Napierala 1997, Michalowski 1999), the formation of this (CUG)n repeat structural element could cause a rearrangement in the *DMPK* 3'UTR structure. (Note, the number of CUGs in the wild type *DMPK* 3'UTR is insufficient to induce a double stranded stem [5 versus 11 for minimal number to induce a double stranded stem]). Our data already reveals that structure is a dominant feature of the *DMPK* 3'UTR and that structure is a key element underlying pDM2 binding to the wild type *DMPK* 3'UTR (protection-immunoselection). Therefore, formation of the (CUG)n repeat expansion double stranded stem could compromise the integrity of the pDM2 binding site, destroying specific pDM2-*DMPK* 3'UTR association.

To test this idea, we examined the ability of pDM2 binding to the wild type *DMPK* 3'UTR versus the *DMPK* 3'UTR harboring 90 CUG repeats (DMPK 3'UTR (CUG)90). Internally radiolabelled *in vitro* transcripts of the two *DMPK* 3'UTR species were subjected to anti-DM2 immunoselection as before. While DM2 bound the normal *DMPK* 3'UTR as above, it failed to associate with the repeat-harboring, disease causing *DMPK* 3'UTR (Figure 5.12). These data are consistent with and provide an underlying

molecular means for why the *DMPK* 3'UTR harboring the (CUG)n expansion exhibits disrupted normal localization (Chapter 4).

In these experiments, we also simultaneously assessed the ability of the Muscleblind proteins to bind these to *DMPK* transcript species. Mouse Muscleblind proteins are known to selectively bind the (CUG)n repeat expansion within the context of the *DMPK* 3'UTR (Miller 2000), causing this family of proteins to be implicated in DM pathology. Subsequent analyses demonstrated Muscleblind mutant mice exhibited a subset of DM symptoms (Kanadia 2003, Hao 2008). Probing my complexes via anti-Muscleblind immunoselection revealed that the *DMPK* 3'UTR$(CUG)^{90}$ is bound by Muscleblind as expected, while the wild type *DMPK* 3'UTR is not. These findings validate this experimental approach and underscore the specificity of the immunoprecipitation analyses.

Significantly, these data reveal that two molecular components, both implicated in DM pathology, each bind the *DMPK* 3'UTR; however, perhaps surprisingly, they do not associate simultaneously. pDM2 exclusively binds the normal *DMPK* message while Muscleblind exclusively binds the (CUG)n expansion harboring *DMPK* message. We hypothesize that the occlusion of pDM2 binding and the alternative binding of the Muscleblind proteins, resulting from (CUG)n expansion, forms a simple molecular switch to transition from proper wild type localization to the disrupted localization-mislocalization of the *DMPK* mRNA that underlies disease development. Notably, these data also define two discrete windows into the molecular events underlying DM etiology; one fairly unexplored and defined by pDM2-*DMPK* mRNA interaction and the other much more understood and defined by pMuscleblind- *DMPK* mRNA binding.

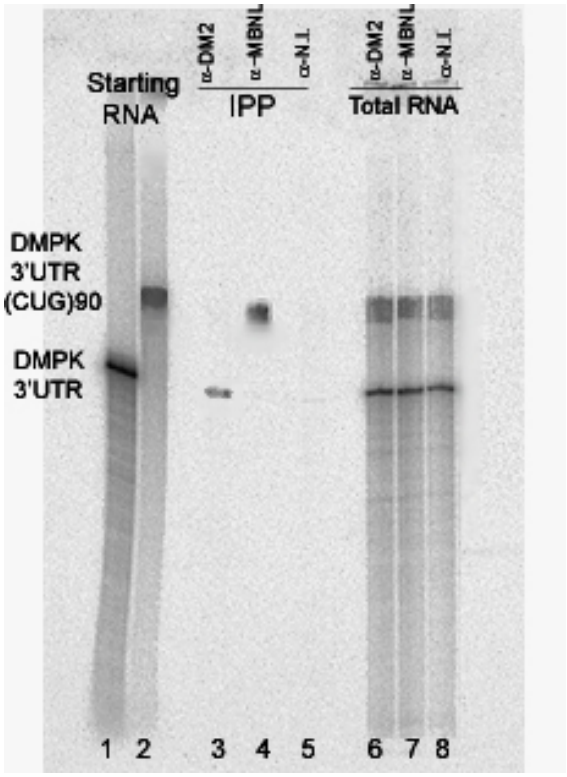

Figure 5.12 (CTG)n expansion in the DMPK 3'UTR disrupts pDM2 binding and facilitates Muscleblind protein binding

Immunoprecipitation was conducted on reactions containing both wild type and (CTG)90 expanded *DMPK* 3'UTRs that had been complexed with C_2C_{12} extract. These reactions were precipitated with anti-DM2 (lane 3), anti-MBNL (lane 4) and non-immune sera (lane 5). DM2 precipitates the wild type *DMPK* 3'UTR, but this association is disrupted by the (CUG)n repeat expansion. The exact reverse is true for the Muscleblind proteins. The specificity of the precipitations was shown by the non-immune sera, which did not precipitate either *DMPK* 3'UTR species. Total RNA lanes (6-8) show the relative levels of the two transcripts in each reaction.

Conclusions

Myotonic Dystrophy is a debilitating neuromuscular disease that affects hundreds of thousands of adults worldwide. Until now, the vast majority of research conducted has been focused solely on the most prominent form of the disease, DM1, while completely neglecting a secondary and nearly clinically identical form, DM2. The two forms share a diverse set of symptoms despite being independently caused by different mutations in separate genes on different chromosomes. The similar clinical presentation due to unrelated causative mutations implies a molecular overlap in the gene products of these two alleles; my previous data establishes that the overlapping pathway is the localization mechanism of the *DMPK* message (See chapter 4). The intent of this series of experimentation was to biochemically investigate the hypothesized interaction between these two gene products, the DM2 protein and the *DMPK* message, an association which has not been explored previously. My analyses address this potential role by exploring pDM2 binding of the *DMPK* mRNA 3'UTR, a fundamental prediction of our hypothesized RNA localization model of DM etiology. These data show that the DM2 protein is, in fact, an RNA binding protein and does interact with the *DMPK* transcript. Moreover, these data map the binding site of the DM2 protein down to 122 nucleotides from the starting 745 nucleotides of the entire *DMPK* 3'UTR and define this region as the localization element for the *DMPK* message.

CHAPTER 6: CONCLUSIONS, DISCUSSION AND IMPLICATIONS

Overview

RNA localization is a key regulated step of gene expression in embryogenesis of several model systems whose disruption results in severe birth defects. By analogy, the fact that this process is crucial in the function of nerves and muscles in humans lead us to consider that disruption of RNA localization could underlie certain neuromuscular diseases. Therefore, we initiated a hunt to find a human disease caused by RNA mislocalization. Here, using a multi-disciplinary approach, our efforts have come to fruition, identifying Myotonic Dystrophy (DM) as the first human disease where RNA localization is shown to be the basis of disease etiology/pathology.

This experimental study was guided by parameters/properties of the disease itself. Consequently, our results provide insights into DM's molecular genetics, cell biology and cytopathology, as well as providing several major conclusions that reveal new understanding of DM pathology. First, we demonstrate that RNA localization underlies DM. The *DMPK* mRNA is shown to be localized via 3'UTR signals to the SR/ER and translated into DMPK protein at this locale. This is relevant since the SR controls calcium currents, a system that is altered in DM patients (for further discussion see below). Second, the causative triplet repeat in the *DMPK* 3'UTR acts as a mislocalization signal and causes the mutated *DMPK* mRNA to be mislocalized to the site of ultimate disease pathology, sarcoplasmic masses. The message is translated, resulting in a fully functional serine-threonine kinase in an inappropriate location. At this new locale, this kinase will be exposed to novel substrates, which it can phosphorylate, altering signal transduction cascades and leading to DM's diverse symptoms. We provide data from

patient tissue to support this novel model of DM etiology. Third, using *in vivo* and in vitro techniques we show for the first time that the gene products of the two loci that can independently cause Myotonic Dystrophy physically and functionally interact. Specifically, we show that the gene product encoded by the second locus capable of causing DM, pDM2, acts as a localization factor for *DMPK* mRNA. Fourth, we demonstrate that the disease causing DM1 mutation specifically disrupts *DMPK* mRNA's association with pDM2, resulting in the disruption of *DMPK* mRNA localization. Fifth, we provide mechanistic data revealing a simple RNA-protein interaction switch that molecularly distinguishes the wild type situation from the diseased state. This defines two separate windows underlying DM etiology; one involving the DM2 protein and the second involving the Muscleblind proteins (MBNL), factors that have previously been indentified in DM pathology. While previous work in the myotonic field has identified nuclear alternative splicing events that are effected by the *DMPK* mRNA 3'UTR triplet repeat *in trans*, our data open a new, complementary window into the disease due to the action of the *DMPK* mRNA 3'UTR triplet repeat *in cis*, effecting cytoplasmic events stemming from altered mRNA localization. Our findings have seminal importance in understanding the molecular mechanism causing DM and open a variety of novel research avenues, both basic and applied.

Conclusions and Discussion

THE *DMPK* MRNA IS A LOCALIZED MESSAGE

Our *in situ* hybridization data presented here reveal a SR/ER localization pattern for the *DMPK* mRNA, which results in the SR/ER localization of the DMPK protein and corresponding to the location of DM2 protein. This agrees with a few previous reports

that the DMPK protein is SR located and provides a mechanism for that position (Mussini 1999, Kaliman 2005).

The fact that this message and these proteins are ER localized is significant. Growing evidence suggests that the ER plays a major role in the localization and transport of many important messages (reviewed in Cohen 2005). One relevant example is the Xenopus *Vg1* mRNA. *Vg1* mRNA association with Vera, an ER localized protein, is required for successful *Vg1* mRNA localization (Deshler 1997), indicating that the ER plays a fundamental role in the localization pathway of this message. In fact, several muscle specific mRNAs are ER localized, including *Desmin*, *Vimentin* (Cripe 1993), *alpha-Actin*, *slow Troponin C* and *slow Troponin I* (Reddy 2005), raising the possibility that the ER plays a vital role in mRNA localization in muscle function. The ER plays an important role in intracellular calcium regulation, crucial to muscle contraction and relaxation. One of the known DMPK protein phosphorylation targets in vitro is an ER localized protein *in vivo*, Phospholamban, and its underphosphorylation due to a lack of pDMPK affects calcium uptake in cardiac muscle (Kaliman 2005). The ER/SR plays a critical role in muscle cell regulation and activity and the specific localization of the *DMPK* message/protein to this region corresponds to the known importance of DMPK in proper calcium regulation (Kaliman 2005).

THE DMPK MRNA LOCALIZATION IS DISRUPTED IN DISEASED TISSUE.

RNA localization is a highly regulated process which specifically controls spatial/temporal expression of a subset of highly important proteins. Disruption of this process has dire consequences in many model systems, including severe morphological abnormalities (i.e. birth defects) and infertility (reviewed in Bashirullah 1998). Prior to

our work, no human disease has been shown to have a mislocalized RNA at the core of its etiology. Taken together, our *in vivo* and in vitro data demonstrate this is the case for Myotonic Dystrophy. In DM1 patient muscle tissue, the normal distribution of the *DMPK* message is disrupted and *DMPK* mRNA is mislocalized to cytoplasmic bodies, Sarcoplamic Masses (SM). These SMs are a diagnostic hallmark of DM and were employed by clinicians to diagnose DM before the molecular mapping of the DM mutations and resulting molecular diagnostic test. Thus the altered localization of *DMPK* mRNA to SMs reflects a disruption of normal location to a site of known DM cytopathology, underscoring the role of this disruption in disease etiology. Moreover, data presented here demonstrate that the DMPK protein is translated at this new location and correlates with the appearance of a small subset of novel phosphorylated and at least one underphosphorylated proteins common to several DM1 patient muscle tissues. These phosphorylation changes highlight a potential model of DM pathology wherein the change in DMPK protein location results in inappropriate regulation of signaling cascades with numerous potential downstream consequences in the cell.

THE PROTEIN PRODUCT FROM THE SECOND DM GENE FUNCTIONS IN THE PROCESS OF DMPK MRNA LOCALIZATION AND ASSOCIATES WITH THE DMPK MESSAGE THROUGH A DISCRETE REGION OF THE DMPK 3'UTR

Causative mutations in two separate genes have been mapped for Myotonic Dystrophy which can independently cause this disease. The first, a (CUG)n expansion in the *DMPK* 3'UTR, has been shown here to result in the mislocalization of the *DMPK* message in patient tissue. The second mutation, a large insert in the first intron of the *Znf9* gene, has been virtually ignored by the DM community and little research has been

conducted on the effects of this mutation. Our data demonstrate, for the first time, that gene products encoded by the two affected DM genes molecularly associate. Moreover, we demonstrate that this interaction is required for the proper localization and subsequent expression of the *DMPK* message in a disease relevant cell culture system.

Reduction of DM2 protein amounts substantially altered the localization of the *DMPK* mRNA. We demonstrate that the functional interaction observed is a result of a physical interaction of the DM2 protein with the *DMPK* message. These data map the association to a small area of the *DMPK* 3'UTR. This region harbors the ER localization element of the *DMPK* message; reporter constructs demonstrate that this region is necessary and sufficient for reporter localization. The structure of this region appears to be crucial for the pDM2- *DMPK* mRNA interaction as demonstrated by its sensitivity to magnesium. Although no known consensus localization element has been identified, two key characteristics emerge from analysis of the zip codes that have been identified: they reside in the 3'UTR of the localized message and they have both a structural and sequence requirement (Jambhekar 2007). Thus, the characteristics of the *DMPK* localization element defined here mirrors the two key features of localization signals in other messages.

A NEW, COMPLEMENTARY WINDOW INTO DM ETIOLOGY

Prior to this study 'nuclear sequestration' was the primary RNA based theory for DM pathogenesis. This theory arose from the findings of Taneja et al (1995) who originally demonstrated *DMPK* RNA harboring a (CUG)n expansion was retained in nuclear foci in cultured DM fibroblasts. A substantial body of data lead to the idea that these (CUG)n nuclear foci act in trans to sequester RNA binding protein such as

Muscleblind (Taneja 1995, Davis 1997, Liquori 2001). This then removes these RNA binding proteins from their normal function in RNA splicing, indirectly resulting in altered RNA splicing and DM symptoms (Timchenko 1996b, Philips 1998, Charlet-B 2002). While this body of data is substantial, studies in transgenic mice indicate that this cannot account for the entirety of DM symptoms and that something is missing from the puzzle. Therefore, using the DM2 gene product to provide several clues, we generated data that is complementary to this theory and open a new window on DM etiology. We present an RNA mislocalization mechanism whereby normal *DMPK* mRNA is localized to the SR and translated there. In the diseases state, the presence of the 3'UTR triplet repeat expansion in DM1 or loss of wild type DM2 protein in DM2 disrupts the existing localization.

The second window that we've opened into DM etiology/pathology differs from the nuclear sequestration situation in two important ways. First, it deals with consequences of the 3'UTR (CUG)n expansion *in cis* (rather than *in trans* as the previous model suggests) and explains why DM symptoms arise when the (CUG)n expansion occurs in the context of the *DMPK* mRNA, but not when it occurs in other mRNAs. For example, a (CUG)n repeat in the 3'UTR of *Junctophilin*-3 mRNA results in Huntington-like symptoms rather than DM symptoms. Second, it shifts the focus from altered nuclear events to cytoplasmic post-transcriptional processing. It is relevant that the original isolation of the Muscleblind proteins as factors that bind the *DMPK* 3'UTR with a (CUG)n repeat expansion, Miller et al (2000) found that the bulk of the factors were cytoplasmic. Also, a second RNA binding protein hypothesized to be nuclear sequestered, CUG-BP1, has a role in altered DM splicing, but its primary role is in RNA stability and translation, two cytoplasmic processes (Philips 1998, Timchenko 1999, Timchenko 2005).

Whether these two windows operate in tandem or exist in parallel and contribute to different DM symptoms is presently open to debate. The fact that DM2 mice (Chen 2007) and DM2 patients show no altered splicing (other than the insulin receptor in patients, Savkur 2004) while the Muscleblind 1 mouse (Kanadia 2003) and nuclear sequestration effects in vitro (recently reviewed in Orengo 2007, Dick 2006) both generated alternate splicing events suggests that the two mechanism exist side by side and each contribute different elements to the DM phenotype. However, the unexpected finding that DM2 knockdowns, via RNAi, not only results in *DMPK* mRNA mislocalization, but also causes a large portion of the Muscleblind protein to selectively relocate to cell nuclei (Croft and Gottlieb unpublished data) raises the possibility that *DMPK* mRNA mislocalization could exist upstream of the previously described splicing defects.

Notably, this is not the first time a neurodegenerative disease has been assigned a nuclear mechanism of pathology and subsequently a cytoplasmic trafficking process has been shown to play a complementary role in disease pathology. Huntington's disease is a caused by a coding triplet repeat which results in a polyglutamine expansion within the Huntingtin protein (Ross 2002). The brain-enriched wild type protein is essential for embryogenesis and neurogenesis (Zeitlin 1995, White 1997). A large body of established data supported the idea that the mutant protein formed inclusions in the nucleus that then interfered with gene transcription of neuroprotective proteins indirectly, leading to disease pathology (Ross 2004) However, subsequent work that considers that the protein is predominantly cytoplasmic and several known molecular partners, revealed a complementary mechanism. They discovered that altered vesicle trafficking of brain-derived neurotrophic factor (BDNF) along microtubules in axonal processes played a seminal role in Huntington's pathology (Gauthier 2004, reviewed by Ross 2004).

A SIMPLE MOLECULAR SWITCH UNDERLYING DM

While we started with a complicated situation defined by the characteristics and properties of DM, our multidisciplinary analyses resulted in the delineation of a simple molecular switch underlying Myotonic Dystrophy. One state defines the normal, non-diseased situation where the *DMPK* mRNA possesses the wild type 3'UTR. We show that the normal DM2 protein complexes with a small (122nt) subset of the *DMPK* 3'UTR. This portion of the message is shown here to be necessary and sufficient to direct *DMPK* mRNA localization to the cellular SR/ER to be translated and function. In the disease sate, the *DMPK* mRNA 3'UTR harbors the (CUG)n triple repeat expansion. It is documented by electron microscopy and crystallography that this repeat forms a large double stranded hairpin (Napierala 1997, Michalowski 1999). As such, it significantly alters the structure of the *DMPK* 3'UTR.

The ER localization signal within the *DMPK* 3'UTR defined by the DM2 protein binding site resides only six nucleotides upstream of the (CUG)n disease causing expansion. This suggests that the structural alteration caused by the expansion could likely prevent DM2 protein recognition of the *DMPK* mRNA binding site by changing its crucial secondary structure or by physically occluding DM2 protein accessibility to the interaction site. Experimental evidence for this hypothesized disruption is cleanly revealed by the immunoprecipitation experiment in Chapter 5 (Figure 5.12). Indeed, the wild type *DMPK* mRNA is complexed with the DM2 protein while the *DMPK* mRNA harboring with disease expansion is not. Conversely, the (CUG)n expansion containing *DMPK* 3'UTR of the diseased state, consistent with the literature, is complexed with Muscleblind proteins (Miller 2000, Yuan 2007), while the normal *DMPK* mRNA is not.

Therefore, this defines two mutually exclusive states, one which contains a wild type *DMPK* mRNA-pDM2 complex and a separate state containing a (CUG)n repeat *DMPK* mRNA-MBNL complex.

IMPLICATIONS FOR DM ETIOLOGY AND TREATMENT

Our investigation was guided by properties of DM, so our results directly address several aspects of the disease and its etiology/pathology. First and foremost, our mislocalization scenario provides a simple, elegant explanation for a Myotonic Dystrophy hallmark, autosomal dominance, wherein only one mutated *DMPK* gene is required to exhibit the disease. Regardless of the presence and position of the *DMPK* mRNA from the wild type allele, mutated *DMPK* mRNA would be mislocalized, allowing functional DMPK protein to contact and phosphorylate inappropriate substrates which results in disease symptoms.

Second, as indicated above, the localization of the normal *DMPK* mRNA to the ER and its disruption by either triplet repeat expansion of the *DMPK* 3'UTR or depression of normal levels of DM2 protein, may explain the DM symptom of myotonia. The ER controls cellular calcium signaling and several ER localized proteins that are involved in this process are proposed DM kinase substrates (see above), thus, disruption of the ER localization of the DMPK protein may result in misregulated calcium signaling. The mislocalization of *DMPK* (CUG)n mRNA to sarcoplasmic masses (SM) explains the appearance of this key cytological feature of DM tissue. Indeed, the fully functional DM kinase in the new locale can explain the appearance of novel phosphoproteins common to DM patient tissue. Additionally, since phosphorylation is involved in cytoskeleton

assembly/disassembly, this may provide a clue to the generation of short segments of cytoskeleton that are a major component of SMs.

Third, for the first time our data delineate a physical and functional interaction between the gene products encoded by the two genes that can independently cause DM, when mutated. While these two genes reside on separate chromosomes, possess distinct mutations in divergent gene locations and encode diverse molecules (a kinase and an RNA binding protein), their physical and functional interaction provides a simple means whereby mutation of either disrupts this interaction and results in the same disease.

Fourth, disruption of *DMPK* mRNA localization due to RNAi knockdown of pDM2 unexpectedly resulted in the migration of MBNL to cellular nuclei. While we do not know the detailed mechanism for this movement, it is highly specific and mirrors the altered localization of a subset of Muscleblind proteins observed in DM1 tissue (Ornego 2008 and us). Similarly, it is well established that CUG-BP1 is underphosphorylated and migrates from the cytoplasm to the nucleus in DM1 patients (Philips 1998). As CUG-BP1 is a substrate for DM kinase *in vivo* and in vitro (Roberts 1997, reviewed in Kaliman 2008), the mislocalization of *DMPK* (CUG)n mRNA that results in the mislocalization of the DMPK protein and alters signal transduction cascades, may account for this property of DM tissue. This suggests that both of these molecular alterations in DM tissue may be downstream consequences of *DMPK* mislocalization.

Finally, and significantly, our experimental analyses described here provide the basis for the next steps toward rationale drug design/therapy. Factors responsible for mutant *DMPK* mRNA mislocalization may represent good drug targets. Additionally, 3'UTR signals responsible for mistargeting diseased messages may ultimately facilitate the target of specific therapeutic agents (kinase inhibitors) to the same cellular location to function most effectively.

DM2 PROTEIN HOMOLOGS IN DEVELOPMENT

The requirement of the wild type DM2 protein for proper *DMPK* RNA localization provides a function for this protein in mammalian mRNA biogenesis. DM2 homologs exist throughout evolution and are required for critical developmental steps in several model systems. However, the exact function of these proteins have never been elucidated. Our data offer a new perspective for evaluating their biological functions in developmental processes and the normal regulation of cellular RNAs.

In mice, chickens and zebrafish, the loss of the DM2 homolog during embryogenesis prevents the complete formation of the forebrain and results in craniofacial disfigurement (Chen 2003, Abe 2006, Wiener 2007). Specifically, the DM2 protein homologue is involved in the regulation of the cascade of neural crest development and proliferation (Wiener 2007). In one of these cases of neural crest development, loss of the RGG box domain of the DM2 homolog resulted in a dominant negative protein that could not function in its developmental role (Armas 2008). This finding suggests that RNA binding is crucial to its developmental function. The documented importance of pDM2 homologs in these processes, the fact that RNA localization plays a key part in embryogenesis and neurogenesis in developmental systems and the documentation that several known localization factors regulate groups of transcripts with which they interact, raises the distinct possibility that the DM2 homologs may likewise function in the localization of other developmentally significant messages. Thus, our findings may ultimately facilitate increased understanding of several developmental pathways.

A FAMILY OF RNA LOCALIZATION DISEASES?

Myotonic Dystrophy belongs to a group of diseases classified as triplet repeat diseases. This class represents a large group of afflictions with diverse etiologies and clinical presentations. Some members of this group, such as Huntington's disease, have been very closely studied for many years, while others, such as SCA8, are new horizons whose study has just been begun. The data presented here have demonstrated the primary role of mRNA mislocalization in the etiology of Myotonic Dystrophy, a repeat expansion disease and may lend insight into the etiology of other triplet repeat diseases. This is the first time that RNA localization has been shown to underlie a human disease, although it has been known to cause birth defects and infertility in model systems. The importance of this process in the normal function of nerves and muscles in humans indicates a high probability of finding aberrations in this system as a cause of other neuromuscular diseases. We believe that DM is a founding member of a class of localization diseases dictated by non-coding triplet repeat expansions. These could include several other neuromuscular disorders including Fragile X Syndrome, Huntington's-Like 2 and SCA8. While little is known about SCA8 other than its causative mutation, a 3'UTR triplet repeat expansion, a considerable amount of study has gone into Fragile X syndrome and , to a much lesser extent, Huntington's Like 2.

Fragile X Syndrome

Fragile X Syndrome (FXS), one of the most common causes of mental retardation and autism in humans, affects 1 in 4000 men (Crawford 2001). The clinical presentation is almost entirely neurological, resulting in mild to severe learning disabilities, reduced IQ, obsessive compulsive disorders and hyperactivity (reviewed in Garber 2008). The

causative CGG expansion in the 5'untranslated region of the *FMR1* gene results in the loss of FMRP (Fragile Mental Retardation Protein) by gene silencing due to CpG island hypermethylation (Hagerman 2002). The symptoms of FXS and DM are divergent; however, close examination of Fragile X Syndrome reveals several interesting parallels between the players in these two diseases and may likewise indicate an RNA localization process contributing to FXS etiology.

FMRP is an RNA binding protein containing three classic RNA binding domains (two KH domains and an RGG box (Darnell 2005)) and binds to a G-rich tertiary structure called a G Quartet within target mRNA via its RGG box (Darnell 2001). FMRP, which shuttles between the nucleus and cytoplasm (Tamanini 1999), is believed to shuttle in association with discrete transcripts from the nucleus until their translation in the cytoplasm. It functions in the translational repression of its associated mRNA as the latter is transported until its final destination and is translationally deployed. Identified FMRP regulated messages encode neuronal specific proteins which are spatially restricted to synapses (Bardoni 2006, Davidovic 2007). FMRP also functions to recruit the abundant, small, non-coding RNA BC1, to the mRNP. The BC1 RNA specifically binds to the message being localized, FMRP and eIF4a giving specificity to FMRP's repression function facilitating the message translational repression (Zalfa 2003, 2005, Iacoangeli 2008).

Similarly, the DM2 protein is a small RNA binding protein containing 7 predicted CCHC type Zinc fingers and a predicted RGG box. Similar to FMRP, my data has mapped DM2 protein target to an extremely G rich sequence within the *DMPK* message which, although not a G Quartet, abuts a region whose sequence predicts a G Quartet. While FMRP may function in the last step of mRNA localization, translational control,

my data suggest that DM2 acts at an earlier stage in the process and does not directly effect translational deployment (Croft, preliminary results).

The myriad of data on FXS etiology has not previously been examined from an RNA localization perspective. When we approached it with this point of view and combined it with the similarities to the DM2 protein, it is apparent that FMRP may be a localization factor for several synaptically localized neuronal messages. It likely functions in the translational inhibition of the localizing message as it travels from the nucleus through the cytoplasm. Potential mislocalization of the FMRP regulated messages must be examined; however, the parallels with the DM2 protein strongly suggest that the loss of FMRP will result in their mislocalization.

Huntington's Disease Like-2

Huntington's Disease Like-2 (HDL-2), a neuromuscular disease caused by a CTG repeat expansion in the 3'UTR of the *junctophilin-3* mRNA (Holmes 2001). The expression of *JPH-3* mRNA is highly restricted to specific cells in the brain, somatodendrites of hippocampal pyramidal neurons. Although the subcellular localization of the *JPH-3* mRNA has not been examined, it has been suggested that the message is transported into dendritic processes in the hippocampus (Nishi 2003). The corresponding JPH-3 protein is specifically localized to the ER outer membrane and composes part of the junctional membrane complex (Nishi 2003). This complex functions to transduce signals from external cell stimuli to the ER in excitable cells, such as neurons and muscle cells. This signaling is crucial for calcium control by enabling cross-talk between the plasma membrane and intracellular calcium channels (Takeshima 2000).

Neither the *JPH-3* message nor protein have been fully characterized, but the little data that is known suggests that the *JPH-3* message could be an ER localized message. This raises the possibility that the 3'UTR mutation expansion could cause the mislocalization of the message and result in HDL-2 pathology.

Further experimentation is required to determine if the process of mRNA localization is the basis for other diseases, but Fragile X Syndrome and Huntington's Disease Like-2 suggest that the RNA mislocalization underlying Myotonic Dystrophy may be a paradigm for other non-coding triplet repeat diseases.

Appendix 1- Mouse models of Myotonic Dystrophy

DM1 MODELS

DMPK knockout

Early attempts at a DM1 mouse model began with *Dmpk* knockout mouse models (Jansen 1996, Reddy 1996). These models showed only subtle defects, including some cardiac conduction abnormalities and muscle fiber degeneration, suggesting that DMPK may have a minor role in some DM pathology. Dmpk +/- mice showed almost no pathology except a first step A-V heart block (Berul 1999). Moreover, recall that no DM patient was discovered with a deletion or mutation of the *DMPK* coding region. Therefore the relevance of this system to recapitulate the disease situation is questionable.

DMPK with the CTG Triplet Repeat Expansion

Attention was then focused on the disease causing (CTG)n expansion However, it was realized that mice do not naturally have non-coding triplet repeat diseases; they possess efficient molecular mechanisms to prevent their occurrence. Therefore, to express the DMPK expansion in a mouse it must be within the context of human sequence, it cannot simply be inserted into the mouse *DMPK* gene. Several mouse models were then designed to express the human genes with the CTG repeat mutation. In two of these systems, DM300 (Seznec 2001) and EpA960/HSA-Cre-ERT2 (Ornego 2008), the human *DMPK* gene with a CTG expansion in its 3'UTR was inserted into the mouse genome. A third system, HSA^{LR} (Mankodi 2000), inserted the human A*ctin* gene with CTG repeats in the last exon into the mouse genome. All three of these systems showed a variety of

histological symptoms such as central nuclei and muscle fiber pathology that were similar to the histology of DM patients. Their muscle physiology and gene expression changes also corresponded to DM patients; however, all of them failed to produce the vast majority of DM pathology and clinical presentation (reviewed in Wansink 2003). Therefore, the (CTG)n expansion does have some pathological function, but DM is not due solely to the presence of this repeat.

RELATED MODELS

Six5 Knockout

To address the possibility that the full constellation of DM pathology is due to the aberrant actions of other factors besides DMPK, knockout models of several other proteins were created. The first of these models addressed the potential role of the *Six5* gene, which resides just downstream of *DMPK* on chromosome 19 and whose *Drosophila* homologues are known to effect eye (Serikaku 1994) and muscle development (Kirby 2001). Two groups independently made Six5 knockout mice (Sarkar 2000, Klesert 2000) and reported the presence of cataracts in these mice as proof of the relevance of a role for Six5 in DM etiology. However, closer examination revealed that the type of cataracts in these mice was not the distinct posterior subcapsular iridescent cataracts found in DM patients, therefore, despite initial conclusions, neither Six5 knockout mouse model showed any DM phenotype.

Muscleblind Protein Knockouts

The next potential disease-related factors examined were the Muscleblind proteins. Muscleblind was originally identified in *Drosophila* as a gene required for eye

development and muscle formation (Begemann 1997). The DM disease relevance of this group of proteins (3 in humans) comes from their identification as factors that specifically bind the CUG repeat expansion in the *DMPK* message within the context of the *DMPK* 3'UTR (Miller 2000). Molecular examination of these proteins revealed that they are alternative splicing factors, shown to regulate exon inclusion, depending on the other factors bound, in a variety of transcripts (reviewed in Pascual 2006). The first Muscleblind mouse model (Kanadia 2003) created a deletion in exon 3 of *Mbnl1*, which eliminates the production of any MBNL1 isoforms that could bind the *Dmpk* (CUG)n repeat expansion; therefore reproducing a hypothesized diseased situation where MBNL1 binds to the repeat tract rather than associating with its normal splicing targets. Interestingly, this mouse model recapitulated several distinct features of DM pathology, including centralized nuclei and muscle fiber abnormalities, myotonia, misregulated splicing and, most notably, unique posterior subcapsular iridescent cataracts, a symptom not seen in the previous models. This mouse demonstrated that the MBNL1 protein has a definite role in the pathology of DM; however, it did not address the other MBNL proteins which have also been shown to associate with the *DMPK* mRNA CUG repeat expansion (Fardaei 2002). Therefore, a MBNL2 knockout mouse was created to address its potential role in DM disease development (Hao 2008). This mouse had many of the same histological abnormalities shared by most of the DM mouse models, but, intriguingly, it did not share the distinct features of the MBNL1 mouse. In fact, this mouse showed only very minor *Calcium chloride channel 1* (*Clc1)* mRNA splicing abnormality, a hallmark of the MBNL1 mouse, and instead had an overall decrease in *Clc1* mRNA expression. Thus, these two proteins may share the ability to bind the DM repeat expansion, but they do not share roles in the pathology of the disease. Transgenic knockout MBNL3 mice have not yet been created.

DM2 Knockdown

Similar to DM1, the first mouse model for DM type 2 focuses on a possible role of the loss of DM2 protein in the etiology of DM (Chen 2007). In this mouse model, one copy of the *Znf9* gene has been disrupted by retroviral insertion, rendering the mouse with only one wild type *Znf9* allele which simulates this dominant disease. The phenotype of these mice dramatically combines many of the disease features seen in the DMPK mice, both knockout and expansion containing models, with the distinct features of the MBNL knockout mice, suggesting a vital role for the loss of DM2 in DM pathology. DM2 knockdown mice show several DM specific symptoms including: unique posterior subcapsular iridescent cataracts and both distal and proximal muscle wasting. There is, however, a noticeable lack of the MBNL1 and CUG expressing mice splicing abnormalities, suggesting 1) the DM2 protein does not function in splicing regulation and 2) the interaction of MBNL1 with the CTG expansion is a gain of function mechanism that specifically extends the pathology of DM to include splicing misregulation. Interestingly, the total *Clc1* mRNA levels are decreased in DM2 knockdown mice, just as they were in MBNL2 mice, indicating that the loss of either protein results in the disruption of a common process.

Despite the many attempts to create a comprehensive mouse model for DM, the field is left with a variety of different models that re-create various subsets of the disease. Even the models recapitulating specific DM symptoms, those of the protein factors MBNL1 and DM2, only represent certain aspects of DM pathology. The lack of specific symptoms from the CTG expansion models may represent the fact that non-coding triplet repeat expansions are only naturally seen in humans, so a mouse model to completely recapitulate DM based solely on the expansion mutations may not be possible.

Mouse Line	DMK -/-	HSA LR	DM300	EpA960/HSA-Cre-ERT2		Mbnl1 E3/E3	Mbnl2 -/-	Znf9 +/-
Reference	Jansen 1996, Reddy 1996	Mankodi 2000	Seznec 2001	Orengo 2008		Kanadia 2003	Hao 2008	Chen 2007
Mouse Creation Strategy	Replaced first seven exons of *DMPK* gene with antibiotic casette	Random insertion of genomic DNA containing human skeletal actin gene with 250 CTG repeats in last exon	Random insertion of 45kB of human genomic DNA containing *DMPK* with repeats	960 interrupted repeats in human DMPK 3'UTR expressed in inducible lox-Cre system		Disruption of *Mbnl1* gene at exon 3, preventing the production of CTG binding MBNL1 isoforms	Inserted Neomycin cassette and B-galactosidase gene into *MBNL2* exon 2	Retrovirus insertion disruption of *ZNF9* gene
Tissue Histology and Muscle Effects								
Central Nuclei	yes	yes+ increased number	yes	yes		yes	yes	yes+ increased number
Variable Fiber Diameter	yes	yes	yes	no		splitting muscle fibers	yes	yes+ increased number
Fibrosis	yes	no	yes + infiltration of fatty tissue in fibers	yes		no	yes + increased collagen	heart fibrosis
Other Histological Changes	mitochondrial morphology changes	ring fibers + sarcoplasmic masses	mitochondrial morphology changes	none		none	none	none
Myopathy	yes	yes	no	yes + fiber necrosis		no	no	distal and proximal muscle wasting
Myotonia	weak	yes	yes	yes		Myotonia with "warm-up" phenomenon (decreases with continuous usage of muscle)	Myotonia with "warm-up" phenomenon (decreases with continuous usage of muscle)	yes
Expression Changes	increase in MyoD protein expression	none	muscle regeneration-expression of fetal MyHC protein	switch from slow mycsin to both fast and slow protein expression		none	Clc1 mRNA expression decrease	Clc1 mRNA expression decrease
				CUBBP1 protein level increase				
Splicing Abnormalities	n/a	*Clc-1*	*Tau*	*Clc1, Serca, Cypher, Cap2b, Ank2, Fxr1*		*Clc1, Tnnt2, Tnnt3*	minor *Clc1*	none
Other DM-like Physical Features	cardiac conduction abnormalities-A-V block in heart	none	none	none		posterior sub-capsular iridescent opacities progressing into mature cataracts	Spinal curvature *common in other muscular dystrophies	posterior sub-capsular iridescent opacities progressing into mature cataracts
								hypertrophic cardiomyopathy-50% enlarged heart walls 1.5X thicker
								A-V block in heart
								cardiac arrhythmia

Table 1.4 Mouse models of DM.

Several mouse models have been created to try to recapitulate Myotonic Dystrophy; however, no one model exhibits more than just a subset of pathology. This table describes the different models created, the strategies used to make them and the major DM-like symptoms seen in the mice.

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Vita

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