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Myotonic Dystrophy: A Study of the Expression of the Myotonic Dystrophy Gene in Affected Tissues and Cells.

A Thesis Submitted to the School of Graduate Studies

University of Ottawa

In Partial Fulfilment of the Requirements for the Degree of

Doctor of Philosophy

Department of Microbiology and Immunology

Faculty of Medicine

By

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Abstract

Recently, the molecular basis of myotonic dystrophy (DM) has been characterized as an unstable trinucleotide CTG repeat amplification in the 3' untranslated region of a gene encoding a protein with serine/threonine kinase activity. As a first step towards understanding the molecular mechanisms underlying DM, we have analyzed the amplification of the CTG repeat and the DM kinase (DMK) mRNA steady state levels in tissues and cell lines obtained from normal and congenital DM individuals. Southern blot analysis of DNA samples from a severely affected neonate showed somatic heterogeneity of the CTG repeat in all tissues analyzed. Interestingly, RNA slot blot experiments on these tissues combined with a PCR-based expression analysis designed to look at transcripts derived from the mutant allele, showed a marked increase in DMK steady state mRNA levels relative to control. This demonstrated that the mutant DMK allele was expressed regardless of the number of CTG repeat and that the increase in DMK mRNA levels was due to elevated mutant mRNA levels. These results suggested that the dominant inheritance pattern of DM could be due to elevated DMK levels.

As these studies were ongoing, other groups reported under-expression of the mutant allele, as well as up- and down-regulation of the normal DMK mRNAs as an effect of CTG amplification in DM patients. Furthermore, using a variety of DMK anti-peptide antibodies, reduced levels of a 54 kDa protein in affected tissues had been reported. The size of this reactive species is significantly smaller from that predicted by the full length cDNA sequence (about 70 kDa). Furthermore, data from our laboratory and others have demonstrated that the 54 kDa protein previously shown to react to DMK anti-peptide antibodies is not a product of

the DMK gene. We have, therefore, raised polyclonal antibodies against human DMK fusion protein and undertook DMK protein expression analysis in freshly sampled muscle tissues from normal and DM individuals. Our antibody detected DMK protein isoforms of 72 and 84 kDa, for which the levels and distribution were not significantly altered in tissues from adult and congenital DM patients. In addition, we have demonstrated that the previously reported decrease in DMK mRNA expression in affected tissues may be the result of a significant loss of type I myofibers, which preferentially express DMK. In contrast to previous reports, our results also showed that the mutant DMK allele was clearly transcribed as a high molecular weight mRNA in muscle tissue of a severely affected patient. These observations raised the interesting possibility that the basis for the dominant inheritance pattern of DM may not involve the DMK product, but rather, may be mediated directly by a dominant effect, through the expanded CTG repeat in the 3' UTR.

Taken together, our data then showed that the mutant mRNA was over-expressed in muscle tissues from affected individuals but that the total levels of DMK protein in these tissues remained relatively unchanged. In agreement with this, Taneja et al., (1995) reported that mutant DMK mRNAs accumulated in the nuclei of fibroblasts and muscle cells derived from DM patients while the normal transcript did not, suggesting that the mutation alters the normal processing and/or metabolism of the mutant DMK mRNA. Therefore, CTG amplification may increase the steady state mRNA levels of the mutant DMK transcript and alter its export from the nucleus, whereby the total levels of DMK protein, translated mostly from the wild type mRNA, remain relatively constant. Therefore, our working hypothesis was

that this accumulation of DMK mRNA (and possibly DMK protein) could alter the normal physiology of muscle cells *in vivo*.

The observation that individuals with the most severe congenital form display a marked delay in muscle development *in vivo* was used to test our hypothesis in an *in vitro* skeletal muscle differentiation system. Initially, in order to gain insight into the role of DMK during myogenesis, we have examined DMK expression during muscle differentiation *in vitro* and subsequently investigated the effect of DMK over-expression on the terminal differentiation of the murine myoblast cell line C2C12. We demonstrated that DMK is up-regulated 2 to 3-fold during skeletal myogenesis and that constitutive over-expression of DMK mRNA in myoblasts caused a marked inhibition of myoblast terminal differentiation. Surprisingly, this activity mapped to the 3'UTR of the DMK transcript. When the DMK 3'UTR was placed downstream of a hygromycin resistance gene, the same inhibition of myogenesis was observed.

Differentiation and proliferation are considered to be mutually exclusive (reviewed in Olson et al., 1991). However, over-expression of the DMK 3' UTR in NIH 3T3 fibroblasts did not have any effect on their proliferation, suggesting that the 3' UTR does not prevent cell cycle withdrawal and differentiation by promoting growth. Further characterization of the 3' UTR sequences mediating the observed inhibition of terminal differentiation mapped these elements to a 239 bp conserved segment of the 3' UTR located upstream of the CTG repeat. Furthermore, the DMK 3' UTR did not have any significant effect on the activity of the myogenic regulator MyoD when co-transfected into 10T1/2 cells along with a reporter

construct bearing a muscle specific enhancer element. This suggested that the 3' UTR did not interfere with the ability of MyoD to transactivate muscle-specific genes. However, when the mRNA levels for two other early myogenic regulators were analysed, myoblast clones over-expressing the 3'UTR expressed normal levels of MEF-2C , but showed reduced myogenin mRNA levels compared to controls, following the induction of differentiation. In addition, in contrast to controls, myogenin protein levels were found to be unchanged during myogenesis in these clones. These data suggested that over-expression of the DMK 3' UTR may alter the expression of specific mRNAs leading to a delay in terminal differentiation.

Therefore, CTG amplification in the DMK 3' UTR may alter the nuclear metabolism of the mutant DMK transcript causing its accumulation in muscle cell nuclei, which may then affect a wide range of physiological processes such as differentiation and the excitation-contraction coupling mechanism in myofibers.

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DEDICATION

To Cathy, Vicky and my family, Daniel, Alfred and Nicole.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER V:
OVER-EXPRESSION OF THE MYOTONIC DYSTROPHY KINASE (DMK) 3' UNTRANSLATED REGION INHIBITS MYOBLAST TERMINAL DIFFERENTIATION *IN VITRO*.

LIST OF ABBREVIATIONS

bp	base pair
°C	degree Celsius
cDNA	complementary DNA
Ci	Curies
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DM	dystrophia myotonica/myotonic dystrophy
DNA	deoxyribonucleic acid
dpm	desintegration per minute
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
h	hour
kb	kilobase
l	liter
LB	Luria-Bertani medium
min	minute
ml	millilitre
mM	millimolar

mmol	millimole
mRNA	messenger ribonucleic acid
ng	nanogram
pg	picogram
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulfonyl fluoride
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SSC	standard saline citrate
TBE	tris-borate EDTA
uv	ultraviolet
µCi	microcuries
µg	microgram
µM	micromolar

Chapter I: Introduction

The molecular basis of Myotonic Dystrophy

1. Clinical presentation of Myotonic Dystrophy

Myotonic dystrophy (Dystrophia Myotonica; DM) or Curschmann Steinert disease is the most common inherited form of adult neuromuscular disease and affects 1 in 8000 individuals globally (Harper, 1989). Canadians have the highest reported incidence of DM with frequencies of 1 in 529 in some regions of Quebec (Mathieu et al., 1990). DM is a multisystemic, autosomal dominant disorder for which affected individuals present with a wide range of symptoms (reviewed extensively by Harper, 1989).

There are two clinically distinct forms of DM, an adult onset and a congenital form. Affected adults present mainly with myotonia, a delay in muscle relaxation, and muscle weakness and wasting. These typically involve the muscles of the face, jaw, neck, arms and legs. Furthermore, adult patients frequently display cardiac conduction defects, premature balding, cataracts, retinal degeneration, gonadal atrophy and mental retardation.

The clinical presentation of congenital DM, the most severe form of the disease, differs significantly from the adult form (Vanier, 1960). Affected neonates suffer from

marked hypotonia, feeding difficulties and respiratory distress. Mental impairment ensues in the children that survive the neonatal period. In the surviving patients, an improvement is noted during childhood, followed by a gradual deterioration during adolescence with the development of symptoms characteristic of the adult onset. Congenital DM has been observed to be exclusively transmitted by affected mothers (Mulley et al., 1993). Whether specific maternal factors contribute to the development of the congenital DM phenotype remains to be elucidated.

Histological studies and protein profile analyses on muscle tissues from congenital DM children have demonstrated an abundance of satellite myoblasts and altered expression of mature muscle-specific protein isoforms, suggesting impaired muscle terminal differentiation (Sarnat and Silbert, 1976; Farkas-Bargeton et al., 1988; Soussi-Yanicostas et al., 1991). Observations indicative of developmental delay have also been reported for other organs such as brain and kidneys (Young et al., 1981).

DM individuals have been observed to present with highly variable phenotypes ranging from an assymptomatic condition to the most severe and frequently fatal congenital form. Furthermore, within families, genetic anticipation (an increase in disease severity in successive generations) has been associated with DM (Howeler et al., 1989). Before the cloning of the genetic defect responsible for DM, these phenomenon were not understood.

2. Cloning of the molecular defect underlying DM

Prior to the identification of the DM gene, the function and properties of the corresponding protein were unknown. Therefore, the identification of the molecular defect underlying DM was achieved through positional cloning (reviewed in Shutler, G.G., Ph.D thesis, submitted to the department of Microbiology and Immunology, University of Ottawa). Initial genetic studies had mapped the DM gene to the long arm of chromosome 19 (Hulsebos et al., 1985; Myklebost and Rogne, 1988; Smit et al., 1988). Later, using specific RFLP analysis, the DM locus was more precisely mapped to subregion q13.3 of chromosome 19 (Schonk et al., 1989). Following extensive genetic and physical mapping the essential genomic region containing the DM gene was bracketed by two genetic markers, ERCC1 (Smeets et al., 1989) and X75b (Jansen et al., 1992a), and subsequently cloned in a 700 kbp contig derived from overlapping cosmids and yeast artificial chromosomes (YACs) (Aslanidis et al., 1992; Jansen et al., 1992a; Shutler et al., 1992). Using sequence analysis of patient DNA, candidate genes comprised within this region were screened for mutations in order to identify the DM gene. The observation that single copy probes derived from the candidate region revealed an unstable genomic fragment in DNA from DM individuals greatly facilitated the search for the DM locus (Aslanidis et al., 1992; Buxton et al., 1992; Harley et al., 1992a; Mahadevan et al., 1992; Fu et al., 1992; Brook et al., 1992). Indeed, in DM individuals, this genomic segment was found to increase in length, beyond the predicted

size for the normal allele. Further characterization of the genomic region and corresponding cDNA clones revealed that this increase in size observed in DM patients was due to the amplification of a CTG trinucleotide repeat in the 3' UTR of the gene. The amplification of this trinucleotide repeat in affected individuals forms the molecular basis for DM.

3. The DM mutation

Analysis of genomic DNA from normal individuals has shown that the CTG repeat is polymorphic in the normal population, ranging from 5 to approximately 40 repeats (see review by Wieringa, 1994). However, beyond 40 and up to 80 repeats, no or minimal symptoms, such as cataracts, can be observed. Furthermore, these alleles have been observed to remain fairly stable during transmission to offspring (Barcelo et al., 1993). Although some cases of mutation reversions have been reported (O'Hoy et al., 1993; Brunner et al., 1993; Shelbourne et al., 1992), expansions exceeding 80 repeats have been found to be extremely unstable and observed to increase in size during transmission in successive generations. The degree of expansion has been inversely correlated with the age of onset and positively correlated with the severity of the disease (Tsilfidis et al., 1992; Harley et al., 1992b), suggesting that the observed genetic anticipation is a true phenomenon in the case of DM.

Individuals that present with classical DM carry CTG expansions ranging from

80 to approximately 1000 repeats. In the case of congenital DM individuals, CTG amplifications have been observed to vary between 500 and over 2000 repeats. Significant overlap has been demonstrated between DM patients with respect to CTG repeat length, making it unreliable as a prognostic criterion (Harley et al., 1992b; Thibault et al., 1993). Remarkably, the size of the repeat shows significant heterogeneity upon genomic DNA analysis of affected individuals, suggesting that the repeat is mitotically unstable (Aslanidis et al., 1992; Buxton et al., 1992; Harley et al., 1992a). Significantly, the length of the repeat in skeletal muscle and other tissues has been found to be consistently larger than that of peripheral blood leukocytes (Thornton et al., 1994; Anvret et al., 1993) supporting the hypothesis that mitotic instability contributes to the observed heterogeneity within and between tissues. Interestingly, when sperm DNA from affected fathers was compared to blood DNA isolated from their offspring, no overlap in repeat size was observed, suggesting that the repeat can also be meiotically unstable during gametogenesis (Jansen et al., 1994).

During intergenerational transmission, genetic studies have demonstrated that maternal transmissions give rise to much greater expansions in the next generation when compared to offspring of fathers bearing similar initial repeat length (Harley et al., 1992b; Lavedan et al., 1993). One explanation for this difference is that sperm cells bearing large expansions are not viable or give rise to non-viable embryos (Mulley et al., 1993).

4. Structure of the DM gene and its encoded product

Characterization of cDNAs and cosmid clones containing the DM locus revealed significant homology between the DM gene coding sequence and members of the serine/threonine kinase family (Brook et al., 1992; Fu et al., 1992; Mahadevan et al., 1993b; Jansen et al., 1992b). For this reason, the DM protein will now be referred to as the DM kinase throughout this thesis (DMK; Sabourin et al., 1993; Mahadevan et al., 1993b). The DMK gene is comprised of 15 exons and is highly homologous (more than 90%) to the murine gene (Mahadevan et al., 1993b; Jansen et al., 1992b). Both proteins share 86% identity and 90% similarity at the amino acid level. The N-terminal first exon encodes a leucine rich region, presumably involved in protein-protein interaction. The kinase catalytic domain is encoded by exons 2 to 8 and displays all the signature motifs of serine/threonine kinase family members (Hunter, 1987; Hunter and Sefton, 1991; Kemp and Pearson, 1990). However, DMK shows more significant homology to protein kinase A and C (Brook et al., 1992; Fu et al., 1992;Jansen et al., 1992b). In agreement with this, serine/threonine-specific kinase activity has been demonstrated *in vitro* on histone H1 substrate (Dunne et al., 1994). Following the kinase region, a domain with a predicted α-helical coil structure homologous to those found in myofibrillar proteins is encoded by exons 9 through 12. Except for a predicted hydrophobic domain in exon 15, the remaining 3 exons do not show any similarity to

any known polypeptides. The polymorphic CTG trinucleotide repeat is also found in exon 15. Interestingly, the mouse gene displays an imperfect repeat (5'-CTGCTGCAGCAGCTG-3') which has not been found polymorphic in mice (Jansen et al., 1992b).

Various human DMK cDNAs representing different alternatively spliced variants have been isolated (Fu et al., 1992; Jansen et al., 1992b; Mahadevan et al., 1993b). In addition to the full length cDNA encoding a protein with a predicted size of approximately 70 kDa, cDNAs corresponding to the splicing of exons 13 and 14 have been cloned. The predicted size of this isoform is approximately 10 kDa smaller (about 60 kDa) than the one encoded by the full length mRNA. Additional isoforms have been detected in murine libraries or by RT-PCR of human muscle RNA (Jansen et al., 1992b; Fu et al., 1992). However, the mRNAs encoding these putative isoforms can only be detected by RT-PCR, suggesting that they might represent intermediates from incomplete heteronuclear RNA processing.

5. Trinucleotide repeats and genetic diseases

Several other important dominant genetic diseases have been shown to be associated with trinucleotide repeat amplification (see table 1). The fragile-X mental retardation syndrome (FRAX-A) has been demonstrated to involve the expansion of a CGG trinucleotide repeat in the 5' UTR of the X-linked FMR-1 gene (Verkerk et al.,

Table 1-1.

Dynamic mutations with triplet repeat expansion

Condition	Repeat sequence	Repeat localization	Repeat number
FRAXA	CGG	5' UTR	large (200-1000)
FRAXE	CGG	?	large (200-1000)
FRAXF	CGG	?	large (300-500)
FRA16A	CGG	?	very large (1000-2000)
Kennedy	CAG	ORF	small (<100)
Huntington	CAG	ORF	small (<150)
SCA1	CAG	ORF	small (<100)
DRPLA	CAG	ORF	small (<100)
Machado-Joseph	CAG	ORF	small (<100)
Myotonic Dystrophy	CTG	3' UTR	very large (200-4000)

Table 1-1. Dynamic mutations with triplet repeat expansion showing the repeat sequence and localisation within the respective gene. Reproduced from Willems (1994).

1991). Similarly, other X-linked syndromes, FRAX-E (Knight et al., 1993), FRAX-F (Parrish et al., 1994) and FRA16A (Nancarrow et al., 1994) are associated with the amplification of a CGG repeat. However, these diseases have not yet been demonstrated to involve any specific transcribed sequences (Willems, 1994). In FRAX-A, CGG amplification induces hypermethylation of these CpG residues and causes transcriptional repression of the FMR-1 gene (Pieretti et al., 1991; Bell et al., 1991; Sutcliffe et al., 1992).

Other genetic defects such as Kennedy's disease, or X-linked spinal and bulbar muscular atrophy (SBMA; LaSpada et al., 1988), and Huntington's disease (HD; The HD Collaborative Research Group, 1993) involve the amplification of a CAG repeat. Similarly, spinocerebellar ataxia type 1 (SCA1; Orr et al., 1993), dentatorubral pallidoluysian atrophy (DRPLA; Koide et al., 1994; Nagafuchi et al., 1994) and Machado-Joseph disease (Kawaguchi et al., 1994) are also caused by the expansion of a CAG repeat. In contrast to DM and fragile-X, these genetic diseases are associated with the amplification of a trinucleotide repeat in the coding region of their respective genes, resulting in the lengthening of a polyglutamine tract. Furthermore, the degree of these expansion are markedly less than what has been observed for DM or fragile-X syndromes (see review by Wieringa, 1994)

The effect of CAG expansion on protein function or activity is unknown. However, as longer polyglutamine tracts appear to increase the transactivational activity of transcription factors (Gerber et al., 1994), it has been postulated that CAG expansion

causes a gain of function of the respective protein (Housman, 1995). Although unstable in humans, mutant alleles derived from SBMA patients were found to be stable in transgenic mice bearing mutant human cDNAs (Bingham et al., 1995), suggesting that the chromosomal location of the mutation or specific trans-acting factors unique to human cells may affect its stability.

Functions of 3' untranslated regions (UTR).

The presence of the DM mutation in the 3' UTR may alter one or several of the functions mediated by 3' non-coding sequences. These include the control of mRNA metabolism, translation or transport.

1. Control of mRNA stability

The 3' UTR of mRNAs have been demonstrated to play important roles in the regulation of translation (see Jackson, 1995; Curtis et al., 1995 for review), mRNA metabolism (reviewed by Belasco and Brawerman, 1993) and in the control of growth and differentiation (Rastinejad and Blau, 1993; Rastinejad et al., 1993; Sabourin et al., submitted). More importantly, numerous studies involving mRNAs with short half lives, have helped to delineate the RNA sequences and the corresponding binding factors that control mRNA degradation. Although coding elements have been mapped, most stability

determinants have been shown to reside in the 3' UTR of labile mRNAs and to function as stability determinants or turnover elements.

The steady state levels of a given mRNA are determined by the equilibrium between the rate of synthesis and the rate of degradation for that mRNA. Extensive studies on specific mRNAs such as estrogen-regulated mRNAs and early growth response genes, including the proto-oncogenes *c-fos* and *c-myc* have provided insights into the mechanisms that govern mRNA turnover. The analysis of cell cycle-regulated genes such as histone H3 and the transferrin receptor have also uncovered important RNA stability determinants and novel RNA binding proteins. These stability elements, involved in the control of mRNA degradation, have been mapped to the 3' UTR of these mRNAs.

Vitellogenin mRNA levels have been shown to be destabilized upon estrogen treatment of chick liver cell cultures. However, estrogen has been demonstrated to stabilize other mRNAs in the same system (Brock and Shapiro, 1983). The hormone-induced destabilization has been found to be mediated through the 3' UTR of the vitellogenin mRNA by inducing a degradative activity. Similarly, other hormones such as glucocorticoid are known to affect the stability of several mRNAs through 3' UTR sequences. Elements in the 3' UTR of hormone-regulated mRNAs have been demonstrated to specifically bind protein factors of 60 kDa and 34 kDa involved in the control of mRNA stability (Belasco and Brawerman, 1993). However, these factors have not yet been characterized. The activity of the protein factors involved in the

control of mRNA stability have been shown to be regulated, in part, by agents that act through protein kinase C and A, which are important components of signalling pathways.

The mRNAs encoding the proto-oncogene *c-fos* and *c-myc* have been more extensively studied and the sequence elements mediating the control of their degradation have been mapped. When arrested fibroblasts are stimulated with growth factors such as platelet derived growth factor (PDGF), phorbol esters (PMA) or high serum, early growth response (EGR) genes are rapidly induced, owing to an increase in their transcription rate and stability (Greenberg and Ziff, 1984; Cochran et al., 1983; Lau and Nathans, 1987). Shortly after stimulation, the steady state levels of these transcripts decline sharply. This decrease in mRNA levels is known to be mediated by turnover determinants, termed AU-rich elements (ARE) in the 3' UTR of these mRNAs (Belasco and Brawerman, 1993). These sequences have also been identified in the short-lived mRNAs of several cytokines. Sequence swapping experiments have shown that AREs do not affect the transcription rate of the genes in which they are found, but instead, they cause a dramatic decrease in the half-life of the corresponding mRNA, suggesting that they act as destabilizing sequences (Bonnieu et al., 1990; Kabnick and Housman, 1988; Treisman, 1985). Whereas some AREs, upon PMA treatment, enhance the degradation of certain mRNAs like *c-fos*, other AREs will induce the stability of other transcripts such as granulocyte-macrophage colony stimulating factor (GM-CSF), suggesting the existence of two classes of AREs. Refined deletion analysis of AREs has

revealed that regions flanking the AU-rich segment are also important in the control of mRNA stability, suggesting that these sequences may be important in the preservation of RNA secondary structure. Removal or mutation of the ARE in *c-fos* has little effect on its decay rate following growth factor stimulation, suggesting that other determinants may act in concert with the AREs. Supporting this, stability determinants have been identified in the coding region of *c-fos* and *c-myc* (Treisman, 1985; Wisdom and Lee, 1991). Importantly, mRNA degradation through the ARE is dependent on protein synthesis, suggesting that the binding factors mediating decay are labile or induced upon stimulation of arrested cells.

Several factors binding 3' UTR AREs have been characterized. Interestingly, although they all bind AREs, these factors have been shown to have differential affinity for the various AREs. The ARE-binding protein AU-B/C shows high affinity for lymphokine ARE but not to *c-myc*, whereas another factor, Auf, binds both ARE and stimulates the decay of *c-myc*. Interestingly, AU-A binds both types of ARE in the nucleus of stimulated cells.

3' UTR stability determinants are also known to be important in the control of the cell cycle-regulated transferrin receptor (TfR) and histone H3 (H3) mRNAs. The steady state levels of TfR mRNA are known to be regulated by intracellular iron. Iron depletion induces a 10 to 20-fold increase in TfR mRNA stability (Mullner and Kuhn, 1988). This increase is mediated by conserved 3' UTR sequences termed iron responsive elements (IRE; Mullner et al., 1989). Upon iron starvation, the IRE has been

demonstrated to bind a 69 kDa iron-sulphur IRE-binding protein (IREBP) which dramatically increases the stability of the tfR mRNA. Interestingly, IREs have been found to function in the same manner when placed downstream of heterologous mRNAs, suggesting that they are sufficient to mediate mRNA stability under conditions of iron depletion.

At the end of each S phase of the cell cycle, histone mRNAs are rapidly degraded and cells proceed to M phase. The cell cycle-regulated degradation of H3 also involves a stem loop structure at the 3' end of the mRNA. However, the degradation of replication-regulated histones is dependent on the position of this loop relative to the termination codon. A protein factor, stem loop binding protein (SLBP) has been identified that binds the loop during S phase. Following the completion of DNA synthesis, SLBP is modified and that triggers the degradation of histone mRNAs, suggesting that SLBP promotes histone mRNA stability by masking a nuclease attack site in the 3' UTR during S phase (Belasco and Brawerman, 1993).

It is now well documented that the 3' UTRs play important functions in the control of mRNA stability. This is accomplished *in trans* by the binding of distinct protein factors to specific sequence elements found in the 3' UTR of mRNAs.

2. 3' UTRs and translational control

The control of mRNA translation occurs, for the most part, in the 5' UTR of

most mRNAs. However, recently, the 3' UTR of mRNAs have been implicated in important aspects of translational control. For instance, the existence of a poly-A tail appears to dramatically increase translational efficiency. This is thought to be mediated, in part, by the binding of the poly-A binding protein (PABP) to the poly-A tail (Keller, 1995; Jackson, 1995; Curtis et al., 1995). The binding of PABP seems to facilitate the assembly of the 60S ribosomal subunit and its subsequent interaction with the 40S subunit. In addition, the binding of the PABP complex to the poly-A tail appears to protect the mRNA 3' end from degradation (Sachs, 1993; Beelman and Parker, 1995).

The formation of the poly-A tail during transcription requires the activity of several protein factors. Poly-A polymerization is mediated by the cooperative interaction of the cleavage and polyadenylation specificity factor (CPSF) and the cleavage stimulatory factor (CstF). These proteins form a stable complex on the canonical AAUAAA polyadenylation signal of pre-mRNAs. Subsequent to the interaction of CPSF and CstF, the poly-A polymerase (PAP) is required for polyadenylation. This elongation step is facilitated by auxiliary proteins such as the poly-A binding protein II (PABP II; Manley and Proudfoot, 1994)

The extent of polyadenylation has been shown to be a controlling step in gene expression. In both *X. laevis* and mouse, changes in the length of poly-A tails on certain maternally derived mRNAs are known to occur in the cytoplasm during oocyte maturation and early development (Curtis et al., 1995; Jackson, 1995). Prior to translational activation, maternal mRNAs have a short poly-A tail and are "masked" by

protein factors that prevent their translation. Significantly, these masking elements are found in the 3' UTR of developmentally regulated mRNAs. During both oogenesis and embryogenesis, the translational activity of specific mRNAs is regulated by the poly-A tail length, and specific sequences in the 3' UTR have been shown to control polyadenylation. Therefore, 3' UTR sequences can repress or activate ("unmasking") translation by modulating the length of the poly-A tail, thus regulating translational efficiency. At the onset of maturation, the poly-A tails of some mRNAs are elongated, allowing "unmasking" and translation. Cytoplasmic polyadenylation requires two sequence elements: a U-rich cytoplasmic polyadenylation element (CPE) and the nuclear polyadenylation signal AAUAAA. Furthermore, precise spacing between the CPE and the poly-A signal is required for efficient extension of the poly-A tail. In the absence of these 3' UTR motifs, no poly-A elongation occurs and the mRNAs remain translationally repressed. The presumption is that protein-RNA interactions at the CPE or other 3' UTR elements regulate the changes in poly-A tail length. Other specific sequences, distinct from the CPE have been identified in the 3' UTR of several other developmentally regulated genes such as the reticulocyte-specific 15-lipoxygenase mRNA (Ostareck-Lederer et al., 1994). These novel elements are composed of tandem repeats of a pyrimidine-rich motif, called R sequence that mediate translational repression. Therefore, the combination of "masking" elements and CPE (and others) in the 3' UTR of mRNAs likely contributes to the tight regulation of mRNA translation.

Efficient translational control has also been demonstrated for maternal mRNAs

that are not subject to poly-A tail elongation. For example, *nanos* (*nos*) mRNA localizes to the posterior pole during oogenesis in Drosophila (see section below on mRNA localization). During early development, *nos* protein is only translated from the localized mRNA (Gavis and Lehman, 1994; Curtis et al., 1995). Sequences that mediate the translational repression of *nos* have been mapped to the 3' UTR of the mRNA. It has been proposed that a uniformly distributed repressor inhibits the translation of unlocalized *nos* RNA. Similarly, *oskar* mRNA localization and translation are mediated through sequences in its 3' UTR and like *nos*, *oskar* translation and localization are coordinately regulated. During embryogenesis *nos* protein represses translation of the maternal *hunchback* (*hb*) mRNA. Sequence motifs, the *nos* response elements (NRE), in the *hb* 3' UTR are necessary and sufficient for *nos*-mediated translational repression. During the production of sperm cells or eggs in the C. elegans hermaphrodite, a similar mechanism may be operational during cell-type switches (Hodgkin, 1987).

Finally, translational repression through an mRNA antisense type mechanism has been observed in C. elegans. The association of a small non-coding complementary mRNA (*lin-4*) with repetitive elements in the 3' UTR of *lin-14* has been demonstrated to regulate its translation (Curtis et al., 1995).

3. mRNA degradation: role of the 3' UTR

The degradation of mRNAs is a crucial step in the control of gene expression.

As discussed above, distinct sequences can mediate the specific breakdown of certain mRNAs through the binding of sequence-specific factors. The degradation of mRNAs can occur through two independent mechanisms: deadenylation-dependent or -independent decays. During deadenylation-independent decay, endonucleolytic cleavage (or 5' decapping) of the mRNA occurs, usually within a region that harbors a stability determinant, and rapid degradation of the intermediates follows by an exonuclease activity (Sachs, 1993; Beelman and Parker, 1995). This type of decay seems to control the degradation of the TfR mRNA, where endonucleolytic cleavage appears to occur in the 3' UTR, in the region containing the IRE. Therefore, binding of the IREBP may act to protect the mRNA from nuclease attack. In contrast, deadenylation-dependent decay involves the shortening of the poly-A tail as the rate limiting step of the pathway (Beelman and Parker, 1995). Several studies have demonstrated that RNA sequence elements such as AREs may stimulate deadenylation of unstable mRNAs, making these shortened mRNAs good targets for subsequent degradation steps (Belasco and Brawerman, 1993). It would then appear that specific 3' UTR sequences can enhance deadenylnation activity, which is a prerequisite for the decay of certain class of mRNAs. mRNAs are not degraded until the poly-A tail are shortened to 10-12 residues. This presumably causes a loss of PABP binding which appear to protect the 3' end of polyadenylated mRNAs from degradation. The ribonuclease involved in the shortening of poly-A tails has been purified from yeast. This poly-A nuclease (PAN) requires a protein-RNA complex as a substrate (Manley and Proudfoot, 1994). PAN deadenylation

activity *in vitro* can be regulated by 3` UTR elements of the polyadenylated substrate mRNA, possibly through the AREs or other destabilizing sequences. Following deadenylation, processes such as decapping, 5`→ 3` as well as 3`→ 5` decay have been observed to occur for some mRNAs, suggesting that the stimulation of deadenylation of mRNAs through specific 3` UTR sequences is the first rate limiting step in the decay of certain mRNAs.

4. Role of the 3` UTR in mRNA localization.

Twelve years ago, it was demonstrated that actin mRNA is enriched in the myoplasm of Ascidian eggs (Jeffrey et al., 1983). Since then , many mRNAs have been demonstrated to localize to specific sites within cells, such as fibroblasts, myoblasts, neurons, or specific cell population within developing embryos. It is presumed that mRNA localization is an efficient way to target protein expression and to avoid protein translocation, which requires more energy than RNA transport.

Most of our knowledge on mRNA localization comes from numerous studies involving *Drosophila* and primary cell cultures (reviewed in St-Johnston, 1995). Several of these studies have demonstrated that the localization of mRNAs is mediated by determinants found in their 3' UTR. For example, when β-actin mRNA localization is disrupted using antisense oligonucleotides directed against the localization signals in the 3' UTR, β-actin protein is no longer concentrated at the leading edge of migrating

fibroblasts (Kislauskis et al., 1994). mRNA localization in the *Drosophila melanogaster* oocyte plays an important role in the generation of polarity. More than ten maternal mRNAs have been found to localize to specific regions of the oocyte. Messenger RNAs such as *gurken* localize to the dorsal anterior corner of the oocyte. Similarly, *oskar* mRNA localizes to the posterior pole of the egg. Another class of maternal mRNAs that are localized in the oocyte includes *bicoid* and *nanos* which are anchored at the anterior and posterior poles, respectively, and act as localized sources of diffusible proteins. Similar to its Drosophila counterpart, the *Xenopus laevis* oocyte sorts various maternal mRNAs to specific regions of the egg. A common feature of all localized mRNAs studied to data is the mapping of localization determinants to the 3' UTR of the transcript (St-Johnston, 1995; Jackson, 1995). These localization signals are relatively large and are likely to contain multiple protein binding sites. The *bicoid* mRNA has been extensively studied and is predicted to have a complicated secondary structure conserved during evolution, potentially involved in localization. At egg activation, *bicoid* RNA is released from the cortex and anchored in the anterior cytoplasm. This retention at the anterior pole requires the activity of *staufen* protein (Ferrandon et al., 1994). Extensive mapping of *bicoid* 3' UTR has mapped a 440 nucleotide segment, corresponding to three stem-loops, that associates with *staufen*. Similarly, posterior transport of *oskar* mRNA requires *staufen*. Again sequences that mediate *oskar* localization are comprised within its 3' UTR. As for the Drosophila *bicoid* and *oskar*, the localization of Xenopus *Vg1* mRNA is mediated by a 340 nucleotide region of the

3' UTR, shown to interact with a 69 kDa protein (St-Johnston, 1995). However this protein has not yet been identified. In contrast, *staufen* protein has been demonstrated to contain five copies of a double-stranded RNA (dsRNA)-binding motif and binds dsRNA *in vitro* (St-Johnston et al., 1992).

Although the 3' UTR localization signals are conserved, mRNAs appear to be targeted to their final location by various pathways. Specific localization of some mRNAs involve the spatial control of mRNA stability. For example, *hunchback* mRNA in early Drosophila embryos is specifically degraded in regions where the product is not required, establishing an anteroposterior mRNA gradient. Other localization pathways include the anchoring of mRNAs at localized binding sites, vectorial nuclear export and directed transport (St-Johnston, 1995).

5. mRNA export

The microinjection of mRNA into Xenopus oocytes and the study of yeast mutants have provided much insights into the process of mRNA export and the protein factors that mediate transport (reviewed in Izzauralde and Mattaj, 1995). Among the most abundant nuclear RNA-binding factors are the heterogeneous ribonuclear particle proteins (hnRNP). One member of this family, A1, has been shown to be associated with poly-A$^+$ mRNA and shuttles rapidly between the nucleus and cytoplasm. Several other hnRNPs, in yeast and mammals, have been observed to behave like A1 and could

mediate export of mRNAs. Interestingly, the export of several classes of RNA (tRNAs, 5S ribosomal RNA, U1 small nuclear RNA [snRNA] and mRNAs) is specifically saturable, that is that a given RNA can competitively inhibit the export of some RNAs, without affecting others (Jarmolowski et al., 1994). These results suggest that sequence-specific binding factors can be "titrated out" by other RNAs bearing similar binding sites. Therefore, mRNA mutations may alter the binding of such factors resulting in the loss of interaction with export proteins. Alternatively, novel interaction with some nuclear component may arise, leading to mRNA retention. This has been previously reported by Legrain and Rosbash (1989), who demonstrated that interaction with splicing components prevented pre-mRNA export. More recently, the U3 snRNA has been shown to be retained in the nucleus by interaction with a saturable factor (Terns and Dahlberg, 1994).

Genetic screening of yeast mutants has revealed the existence of several genes encoding export factors. The yeast *rat* and *mtr* genes have been found to be implicated in the transport of poly-A^+ RNA to the cytoplasm. In addition, nucleoporins, which are part of the nuclear pore complex (NPC), have been identified as mediators of mRNA export. The products of three nucleoporin genes, *NUP145, NUP116* and *NUP100* share a common motif, the nucleoporin RNA-binding motif (NRM) which has been shown to bind homopolymeric RNA *in vitro* (Izzauralde and Mattaj, 1995). The proteins that mediate RNA export through the NPC are thought to interact more specifically with other sequence-specific RNA binding proteins such as those described in the previous

section.

The occurence of the expanded CTG repeat, the only DM mutation identified to date, in the 3' non-coding region of the DMK gene may very well disrupt any of the normal functions associated with its 3' UTR. Including transcription, any of the processes described above, such as the control of mRNA stability, translation or localization of the mutant DMK mRNA may be altered by the presence of the amplified CTG repeat.

The molecular biology of muscle development

1. Muscle fiber diversification

Adult skeletal muscle is a highly specialized tissue composed of differentiated muscle fibers. Different fiber types within the same muscle have different contractile properties which are related to the various myosins and other structural protein isoforms that they express (reviewed in Miller, 1991; Buckingham, 1992). The various adult fiber types and their specific content of contractile protein isoforms are generated during the perinatal period (Buckingham, 1992; Feldman and Stockdale, 1992). During development, muscle fibers arise from the fusion of tens to hundreds of mononucleated myoblasts and these fibers can be classified as slow twitch (type I) or fast twitch (type II) fibers. Fast twitch fibers are further subdivided into types IIA, IIB and IIX. These

fibers are most often defined by the myosin heavy chain (MHC) isoform that they express (e.g. type I fibers express type I MHC) and their intrinsic ATPase activities (e.g. slow fibers have low ATPase activity) (Miller, 1991). Different muscles have a specific proportion of type I and type II fibers as determined by MHC isoform content. In addition to the adult isoforms of MHC, an embryonic and a neonatal isotype have been identified (Miller, 1991). These isoforms are slowly replaced by adult ones as the various muscles mature, indicating that the maturation process of different muscle groups involves the isotype switching of MHC isoforms at specific times in specific muscles (Hughes et al., 1993). In addition, there is increasing evidence to show that this switching is muscle type-specific (Brozanski et al., 1993; Soussi-Yanicostas et al., 1990; Vazquez et al., 1993).

During embryonic development in mammals, fiber type diversity is postulated to arise from the fusion of primary myoblasts that are restricted to the formation of fast, slow or mixed primary myotubes (Stockdale, 1992; Miller, 1992; Donoghue and Sanes, 1994). The generation of primary myotubes does not require innervation by motor neurons. On the other hand, secondary myotubes, seem to arise from the fusion of secondary myoblasts which appear later during development. Furthermore, these myotubes are initially homogeneous for the expression of MHC, but at later stages, they appear to preferentially express one MHC isoform. This final MHC isoform specification of secondary myotubes appears to be influenced markedly by the types of neurons that innervate them (Donoghue and Sanes, 1994)

In the mouse, the formation of myotubes and muscle maturation is accompanied by the expression of various contractile and structural protein isoforms that are specific to each fiber type. First, around embryonic day 8.5, α-cardiac and α-skeletal actins are synthesized along with myosin light chain isoforms. Subsequently, between embryonic day 9.5 and 10.5, embyonic and neonatal MHC isoforms and muscle creatine kinase (MCK) are induced. Later on, after birth, the muscles mature and express fiber type-specific adult MHC as well as numerous other muscle-specific regulatory and structural genes (Buckingham, 1992).

2. Control of myoblast differentiation

Terminal differentiation of myoblasts is specified by environmental cues such as growth factors and thyroid hormones as well as cell-cell interactions (Buckingham, 1992). Mesodermal stem cells become determined to the skeletal muscle lineage by giving rise to multiple types of myoblasts. After limited cell proliferation, committed myoblasts exit the cell cycle to a growth arrest state and fuse to form terminally differentiated multinucleated myotubes with diverse phenotypes (Miller, 1991, Miller, 1992). Remarkably, this last step can be performed *in vitro* using murine or human derived skeletal myoblasts, providing an excellent tool to investigate the mechanisms controlling muscle terminal differentiation.

Cell growth and differentiation are usually mutually exclusive or natural

antagonists (Olson, 1990; Olson et al., 1991). Accordingly, cell cycle arrest appears to be an early pre-requisite for terminal differentiation. This is further evidenced by the observed suppression of tumorigenicity when normal cells are fused to actively proliferating tumor cells. Differentiation has been proposed to be the mechanism by which the malignant cells stop dividing (Olson et al., 1991). Furthermore, transformation of normal muscle progenitors by oncogenic viruses prevents growth arrest, thereby inhibiting differentiation (Taylor et al., 1993; Braun et al., 1992a; Olson et al., 1987). Independently of the experimental conditions, myogenesis is accompanied by the induction of several muscle-specific genes that appear to be temporally regulated (Buckingham, 1992) and their sequential induction requires the activation of specific myogenic regulators (reviewed in Olson, 1990; Weintraub et al., 1991a, Olson and Klein, 1994, Emerson, 1993).

Analysis of the mechanisms underlying commitment and differentiation in 10T1/2 mesodermal precursor cells has shown that a single locus can activate both the determination and differentiation programs. The cloning of corresponding cDNAs led to the identification of MyoD, a central regulator of myogenesis (Davis et al., 1987). Subsequently, using *in vitro* differentiation systems, three other myogenic regulators were identified: myogenin (Edmonson and Olson, 1989; Wright et al., 1989), myf5 (Braun et al., 1989a) and MRF4 (Rhodes and Konieczny, 1989; Miner and Wold, 1990; Braun et al., 1989b), all of which share some homology with each other. When expressed ectopically, each of these genes can activate myogenesis in a variety of

nonmuscle cell types (Weintraub et al., 1989).

MyoD family members have been identified in several species, and are expressed exclusively in skeletal myoblasts and myotubes. Interestingly, they can autoregulate as well as regulate one another's expression (Olson and Klein, 1994; Thayer et al., 1989; Hollenberg et al., 1993; Braun et al., 1989b). All share a conserved *c-myc* homology region containing a 68 amino acid basic helix-loop-helix motif (bHLH) involved in DNA binding and activation of myogenesis (Olson and Klein, 1994; Lassar et al., 1989). The functional activity of these regulators requires the bHLH domain and heterodimerization, through the HLH, to other ubiquitously expressed bHLH factors such as E12 and E47 (Lassar et al., 1991; Thayer and Weintraub, 1993).

Functional bHLH regulators bind to a consensus DNA sequence called the E box (CANNTG), first identified in the immunoglobulin *k* enhancers and found in most muscle-specific genes (Weintraub et al., 1991b; Olson and Klein, 1994). Studies in tissue culture have led to a model in which the bHLH protein/E12 heterodimers activate muscle-specific transcription by binding to E boxes in muscle gene control region or by inducing the expression of downstream intermediate regulators that can trigger the expression of other genes through E-box-independent pathways. Non-E box binding regulators (MEF-2, MAPF-2) have been isolated (Ernst et al., 1991; Cserjesi and Olson, 1991; Gossett et al., 1989; Martin et al., 1993; Leifer et al., 1993) and may be responsible for the activation of muscle-specific genes lacking E boxes such as skeletal α-actin and MLC. Recently, the N-terminal 50 amino acids of MyoD and MRF-4 have

been shown to mediate cooperative binding between adjacent E boxes and to confer subtle specificities on individual factors (Weintraub et al., 1991b; Mak et al., 1992).

3. Expression pattern of the myogenic regulators

MyoD or myf5 is preferentially expressed in proliferating myoblasts. The presence of one regulator rather than the other in established cell lines suggest that they might inhibit one another's expression (Weintraub, 1993). Upon the induction of differentiation of cultured myoblasts, the expression of MyoD or myf5 remains unchanged. However, shortly after induction, myogenin is expressed at high levels and is maintained in myotubes (Edmonston and Olson, 1989). In contrast, MRF4 is induced later, 48-72 h after induction, in myotubes (Rhodes and Konieczny, 1989). Surprisingly, a different pattern of expression has been observed *in vivo* in the developing mouse embryo. The first regulator to be expressed in the somites (a compartment destined to the formation of skeletal muscle, dermis and cartilage) is myf5 at day 8 post-coitum (p.c.) followed by myogenin, at day 9.5 p.c., which remains throughout fetal development. MRF4 mRNA is transiently expressed on days 10 and 11 and is re-expressed at day 16 throughout the life of the animal. Finally MyoD appears at day 10.5 p.c. and is expressed thereafter throughout development (reviewed in Olson and Klein, 1994).

4. Specific functions of the individual myogenic regulators

Since all the bHLH regulators bind the same consensus E-box sequence, a central issue has been to determine the specific function of each of them. The generation of muscle-specific bHLH-null mutant and double mutant mice has provided insights into the functional role of each of these factors (see figure 1-1). Remarkably, MyoD-null mice are viable and show no obvious muscle abnormalities, suggesting that redundancy exists among the various factors (Rudnicki et al., 1992; Weintraub, 1993). Supporting this is the observation that MyoD mutant mice display a significant increase in myf5 expression, suggesting that myf5 is negatively regulated by MyoD and that it can presumably substitute for MyoD during development. Interestingly, myf5-null mice display normal muscles but die at birth as a result of an apparent rib defect which impairs breathing (Braun et al., 1992b). Remarkably, the levels of myogenin and MRF4 mRNAs in both the MyoD and myf5 mutant mice are normal, suggesting that the presence of at least MyoD or myf5 is sufficient for their expression. In marked contrast, double mutant MyoD/myf5 mice show no muscle or muscle products (Rudnicki et al., 1993). In addition, myoblast markers such as desmin fail to be expressed in these mice, suggesting the complete absence of myoblasts. These data suggest that MyoD and myf5 are more likely to be involved in the determination of myogenic precursors and maintenance of the committed state.

Myogenin null mice also die at birth and display severe skeletal muscle

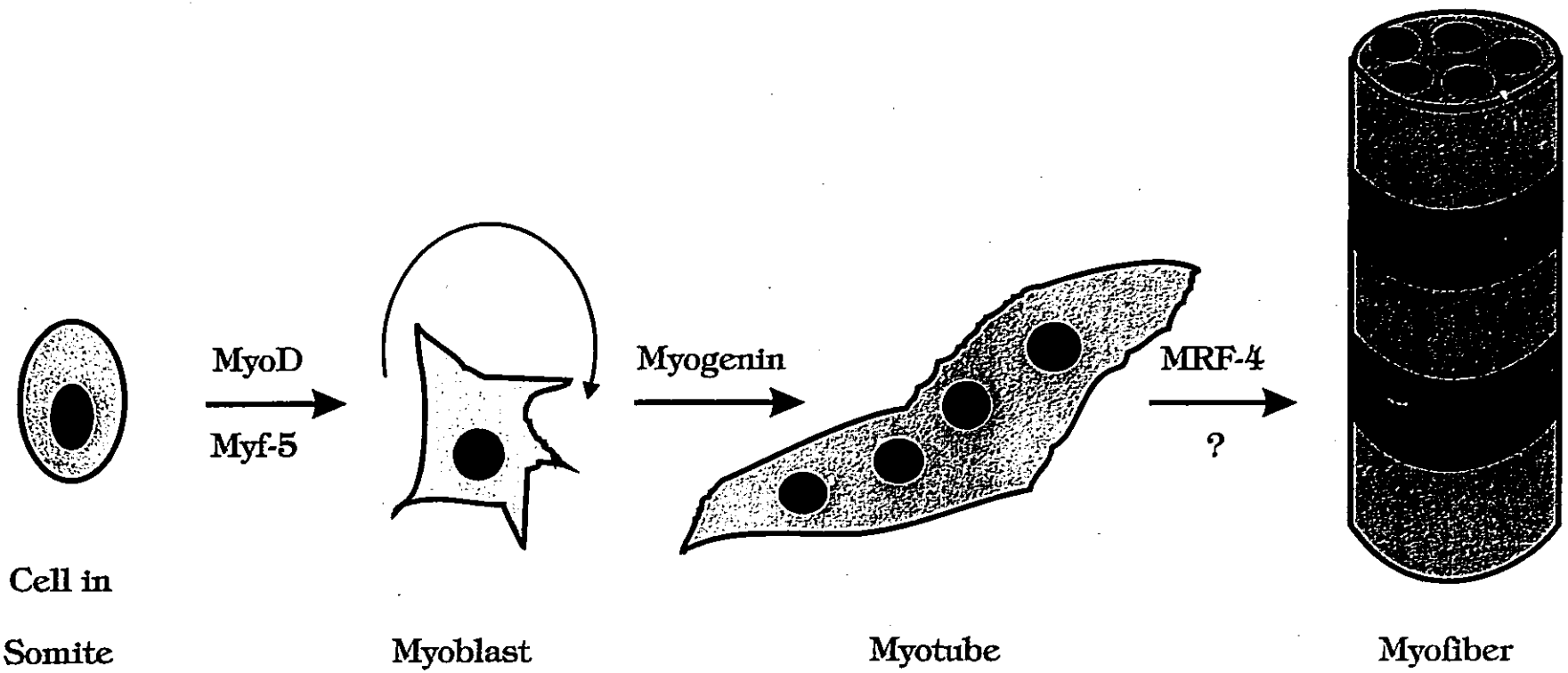

MyoD
Myf-5
Myogenin
MRF-4
?
Cell in
Somite
Myoblast
Myotube
Myofiber

Figure 1-1. Diagrammatic representation of the action of the individual myogenic regulators on myoblast determination and terminal differentiation. Myogenic precursors (cell in somite) become determined upon the activation of Myf-5 or MyoD. Upon induction of myogenin, replicating myoblasts differentiate into multinucleated myotubes and mature into myofibers under the action of MRF-4.

deficiencies (Nabeshima et al., 1993; Hasty et al., 1993). Mainly, the neonates show mononucleated cells and very few myofibers. Interestingly, these animals do not express appreciable levels of mRNAs for several muscle-specific genes, but express others, suggesting that myogenin is required for the expression of only a subset of these genes. Myogenin mutant mice express normal levels of MyoD mRNA, indicating that myogenin is not strictly required for MyoD expression. The fact that myogenin-null mice have myoblasts suggests that myogenin acts downstream of MyoD and myf5 in a regulatory cascade.

Recently, the generation of MRF4 mutant mice has demonstrated that its inactivation results in apparently normal muscles and a significant up-regulation of myogenin, suggesting that it is negatively regulated by MRF4 (Zhang et al., 1995). As is the case of the myf5-null mice, MRF4 mutant animals display some rib anomalies.

Therefore, MyoD or myf5 appear to activate the determination program and to initiate differentiation. Subsequently, myogenin would appear to be required for the terminal differentiation and myoblast fusion. MRF4 appears to act downstream of myogenin, based upon tissue culture studies, and therefore would seem to be more important for the maturation process of the myofibers. The fact that MyoD is expressed later than myf5 in development may simply reflect the temporal appearance of distinct myoblast populations.

5. Activation of terminal differentiation: the cell cycle connection

As for several other systems, cell cycle exit is required for myogenesis to occur (Olson et al., 1991). Furthermore, terminal differentiation of myoblasts appears to be determined by a balance of positive (MyoD activation) and negative (exogenous growth factors) signals. Consistent with this hypothesis, factors that promote growth appear to downregulate MyoD activity. Moreover, MyoD itself can inhibit cell growth, supporting the notion that both differentiation and proliferation are mutually exclusive. Two recent studies have now shown that MyoD activation inhibits cell cycle progression directly (Sorrentino et al., 1990; Crescenzi et al., 1990). A clear demonstration of this was the inhibition of serum-induced G_0 to S phase transition and DNA synthesis as a result of MyoD overexpression in transfected NIH 3T3 fibroblasts (Sorrentino et al., 1990). In addition, MyoD was observed to block DNA synthesis in the non-differentiating CV1 cell line, suggesting that the growth and differentiation activities of MyoD are controlled separately. Moreover, high serum concentrations were found to inactivate MyoD and block muscle-specific gene expression in cells that express bHLH regulators (Olson and Klein, 1994). As for MyoD, myocyte enhancer factor 2 (MEF2) activity is also suppressed by the presence of serum or growth factors (Cserjesi and Olson, 1991).

The growth inhibitory effects of MyoD have been mapped to the HLH region of the bHLH motif as mutant MyoD protein that cannot bind the E box element also blocks growth (Crescenzi et al., 1990). It is possible, then, that MyoD interacts with

specific but different bHLH regulators controlling differentiation and growth arrest. These experiments support the model that MyoD induces cell cycle exit prior to differentiation rather than causing growth arrest as a consequence of myogenesis (Crescenzi et al., 1990). Consistent with these data, a recent study has shown that MyoD and myogenin are co-expressed, *in vivo*, with p21, a cell cycle inhibitor (Parker et al., 1995). Furthermore, transfection of 10T1/2 cells with MyoD induces p21 and arrests the cells (Halevy et al., 1995) and co-expression of the proliferation-associated G_1-specific cyclin D1 and MyoD in 10T1/2 prevents myogenesis (Skapek et al., 1995).

It is now evident that myoblast growth arrest, prior to differentiation, is mediated by a specific activity of MyoD. Consistently, growth related processes have been shown to inhibit MyoD activities and consequently, myogenesis. Upon serum stimulation of arrested fibroblasts, a large number of proteins undergo phosphorylation (Cooper, 1990). This type of post-translational modification has been demonstrated to inhibit myogenesis by directly inactivating members of the bHLH family (Olson and Klein, 1994). Furthermore, inhibition of protein phosphatases by okadaic acid was observed to block myogenesis by first abolishing MyoD DNA binding activity (Kim et al., 1992). Cultures of myoblast cell lines in high mitogenic medium have also been shown to maintain high levels of the Id protein, a negative regulator of the bHLH factors (Benezra et al., 1990). Id is strongly related to the myogenic regulators with the exception that it lacks the basic DNA binding domain and is expressed in most cell types. The Id protein can form inactive heterodimers with MyoD and E12, but is normally found to be associated with

the E12 factor (Weintraub et al., 1991a). Upon serum depletion, Id levels are rapidly downregulated, releasing E12, allowing the formation of MyoD-E12 heterodimers which can then activate transcription of muscle-specific genes (Benezra et al., 1990).

The expression of several proto-oncogenes has been demonstrated to be tightly linked to cell proliferation (Cooper, 1990). Interestingly, overexpression of several proto-oncogenes or activated oncogenes inhibits MyoD activity and myogenesis. The *c-myc* proto-oncogene is structurally similar to the bHLH regulators and plays an important role in the control of cell proliferation and can suppress the MyoD-initiated differentiation (Miner and Wold, 1991). This also prevents upregulation of myogenin normally expressed at the onset of differentiation. The *c-myc*-mediated inhibition of myogenesis appears to be independent of Id, suggesting that *c-myc* is part of a different negative regulatory pathway. Furthermore, overexpression of *c-fos* and *c-jun*, both involved in cell cycle entry from an arrested state, suppresses transactivation of the MCK gene by myogenin and MyoD (Li et al., 1992a). This was also observed with *jun*B but not *jun*D which is expressed in cycling myoblasts. The repression was observed to be targeted at the bHLH region of MyoD (Bengal et al., 1992; Li et al., 1992a). However, conflicting results have been reported with respect to the region of *c-jun* interacting with MyoD. Both the amino-terminus end and the leucine zipper domain have been independently shown to mediate MyoD repression. Regardless, these data suggest the existence of cross-talk between transcription factors involved in myogenesis and cell proliferation. Furthermore, MyoD can repress the *c-jun*-mediated

transactivation from an AP-1 site, suggesting a functional involvement of MyoD in the maintenance of cell cycle arrest (Trouche et al., 1993).

The *ras* proto-oncogene, encoding a member of the GTP-binding proteins is induced at the mid-G_1 phase of the cell cycle. As is the case for the immediate early genes, overproduction of an oncogenic form of *ras* in C2 myoblasts inhibits myogenesis as evidenced by the absence of expression of MCK and acetylcholine receptors (Olson et al., 1987). The expression of normal *ras* genes had no effect on differentiation, suggesting that the inhibition of myogenesis occurs through a mechanism that is independent of proliferation. Consistent with these observations, transforming growth factor ß (TGF-ß), acting through a *ras*-mediated pathway, inhibits myogenin-induced myogenesis (Brennan et al., 1991). In contrast to results obtained with total serum, TGF-ß does not affect the DNA binding ability of myogenin, demonstrating that TGF-ß acts through a mechanism distal to DNA binding, independent of the negative regulator Id.

Cell cycle entry is accompanied by the synthesis of several second messenger molecules such as cyclic nucleotides and inositol derivatives (Cooper, 1990). One of these second messenger systems generates cyclic AMP (cAMP) through the *ras*-mediated activation of adenylate cyclase. High concentration of cAMP in turn activates the cAMP-dependent protein kinase A (PKA). Supporting the findings that growth associated processes block differentiation, overexpression of the catalytic subunit of PKA prevents transactivation of a reporter gene from the MCK enhancer through an

indirect pathway involving PKA (Li et al., 1992b). Recently, overexpression of protein kinase C (PKC) in myoblasts and fibroblast growth factor (FGF) treatment of myogenic cultures have been found to inhibit the activity of the bHLH regulators (Li et al., 1992c). FGF-mediated PKC activation causes phosphorylation, at a consensus PKC site, in the basic region of myogenin, abolishing its DNA binding activity. Mutants lacking this PKC site were found to bind to E boxes and transactivate a reporter gene under the control of the MCK enhancer. Therefore, there is a direct link between growth related events and inhibition of myogenesis, through the myogenic regulators. This has been further demonstrated by the interaction of MyoD with the negative cell cycle regulator retinoblastoma (Rb; Gu et al., 1993). In cycling cells it was observed that the presence of an inactive Rb, through hyperphosphorylation or SV40 large T antigen binding, resulted in the inhibition of skeletal myogenesis. However, in its active state, early in G_1, the Rb protein can directly associate with MyoD and cause growth arrest, allowing myogenesis to ensue.

A functional MyoD protein, therefore, is required for growth arrest and terminal differentiation of myoblasts. Inactivation of myogenic regulators by growth related processes delays or completely inhibits terminal differentiation. Hence, it is possible that the observed delay in muscle maturation in congenital DM muscle tissues and that the muscle atrophy noted in adult DM cases may be directly associated with altered expression of the DM gene, a protein kinase. Alternatively, the mutation may indirectly affect the activity of the muscle-specific bHLH proteins required for the onset of

differentiation (such as MyoD, myf5 or myogenin) or the maintenance of the differentiated state (such as MRF4).

Summary of objectives

The cloning of the DM gene and the identification of the genetic defect underlying DM have allowed us to analyse the effects of CTG amplification on the expression of the mutant DMK gene. As a first step into the characterization of DMK expression in affected tissues, a critical mRNA expression and quantitation was undertaken. These studies revealed that, in tissues isolated from congenital DM individuals, mutant DMK mRNAs are expressed and their steady state levels were significantly increased.

The second part of the plan was to examine the levels and distribution of DMK protein isoforms in adult and congenital DM tissues. These studies showed that the levels of DMK protein isoforms were not significantly altered in these patients although mutant DMK mRNA levels were found to be elevated in a congenital DM individual. This suggested that the DM mutation may alter the levels and the processing of the mutant DMK transcripts.

Therefore, in order to better understand the pathophysiology of DM, human DMK was over-expressed in cultured murine myoblasts. Cells over-expressing DMK mRNA were found to display a marked reduction in their differentiation potential, as

previously observed in skeletal muscles of congenital DM patients. Significantly, this effect was mapped to the 3' UTR of the DMK transcipt, suggesting that over-expression of the 3' non-coding region of DMK is sufficient to mediate the observed phenotype. These results are consistent with the hypothesis that the pathophysiology of DM may not involve the DMK protein but rather, may be due to a *trans* effect of the mutant 3' UTR.

Chapter II: General Materials and Methods

1. DNA preparation

Genomic DNA

Genomic DNA for determination of CTG expansion size was extracted from either patient lymphoblastoid cultures or tissues sampled from DM patients on an automated ABI DNA extractor according to the manufacturer's instructions. DNA samples were reconstituted in water and aliquots were digested in standard restriction enzyme reactions prior to Southern blot analysis.

Plasmid DNA preparation

Plasmid DNA was isolated by standard alkaline/SDS method as described by Birnboim and Doly (1979). Clones were grown in 100 ml of LB medium (1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl) containing 50 μg/ml ampicillin for 12 to 14 h at 37°C with vigorous shaking. In some preparations, following the final ethanol precipitation, the DNA pellet was resuspended in 2.2 ml of TE buffer (10 mM tris-HCl, 1 mM EDTA) and 2.75g of CsCl (Gibco/BRL) was added. Ethidium bromide was then added to the samples at 0.8 mg/ml. The samples were then transfered to ultracentrifuge tubes (Beckman) and banded at 75,000 r.p.m. for at least 12 h at 20°C. Plasmid DNA bands were isolated and extracted as described (Sambrook et al., 1989). Final DNA pellets were resuspended in water and used in all other subsequent manipulations.

2. DNA analysis

Southern blot analysis of genomic DNA

For analysis of patient DNA, aliquots corresponding to 5 µg of DNA were digested with specific restriction enzymes (Gibco/BRL), fractionated onto 0.8% agarose gels in standard 1 x TBE buffer (Sambrook et al., 1989) and Southern blotted to nylon membranes (Amersham Hybond-N). The transfered DNA was fixed to the membranes by uv cross-linking using a Bioslink 312 apparatus (BIOS).

Membranes were hybridized to ^{32}P-labelled DNA probes (Feinberg and Vogelstein, 1984) in 6X SSC, 0.5% SDS, 5X Denhardt's solution and 100 µg/ml salmon sperm DNA at 65°C for a minimum of 12 h. Following hybridization, the final wash conditions consisted of 0.2X SSC/0.1% SDS at 55°C for 30 min. The filters were then exposed to Kodak X-AR films with intensifying screens (Dupont, Cronex) at -80°C for 1 to 4 days.

3. RNA analysis

RNA preparation

Total RNA from cultured cells was prepared according to the method of Birnboim (1988). Briefly, the cells were collected and lysed in RES-1 buffer (1.0 M urea, 0.5 M LiCl, 20 mM sodium citrate, 5 mM EDTA and 1% SDS). The lysates were then sonicated for 15 sec and incubated with 100 µg/ml proteinase K for 30 min at 50°C. Following ethanol precipitation the pellets were resuspended in 0.4 ml of RES-1

and subjected to a second proteinase K digestion. After a phenol/chloroform and chloroform treatment, the RNA was recovered by ethanol precipitation and stored at -80°C in CES buffer (2 mM citrate, 1mM EDTA and 0.1% SDS) until use. Total RNA from tissue samples was prepared using a modification of Chomczynski's procedure, as described by Puissant and Houdebine (1990).

Northern blot analysis

For Northern blotting, total RNA in 10 µl of CES (usually 10 µg), was mixed with 5 µl of formamide and 5 µl of a mixture of formamide: 37% formaldehyde: 1 M sodium phosphate (47:50:3.3). The samples were subsequently denatured for 20 min at 55°C and cooled on ice for 2 min. The denatured RNA was then fractionated onto 1.2% agarose gels (Hoeffer SE400 vertical slab unit) containing 0.6% fromaldehyde. The buffer system consisted in 20 mM sodium phosphate and 1 mM EDTA. Following electrophoresis, the RNA was transferred to nylon membranes (Amersham Hybond-N) using a vacuum blotting apparatus (LKB). The transferred RNA was then fixed and hybridized to radiolabelled probes as for Southern blotting.

4. DNA probes

DNA probes used in radiolabelling reactions for hybridizations to Southern or Northern blots were restriction fragments obtained from various plasmids. Following enzyme digestion, the fragments were purified by fractionation through low melting

point agarose gels or by purification on glass powder according to the manufacturer's instructions (BIO 101, Geneclean kit). The specific DNA probes used for Southern and Northern blot hybridizations are described in subsequent chapters.

5. DNA sequencing

Following cloning protocols or in vitro mutagenesis, purified plasmid DNA was sequenced on an ABI 373A automated sequencer using Taq polymerase and fluorescent dye-labelled dideoxynucleotides, according to the manufacturer's specifications (Applied Biosystems). The sequence data was analyzed by ABI software on a Macintosh PC.

6. Oligonucleotide synthesis

Oligonucleotides for PCR amplification and sequencing were generated on an automated DNA synthesizer (PCR-Mate, model 391, Applied Biosystems). The oligos were synthesized according to the manufacturer's instructions using the phosphoramidite method of synthesis. At the end of the synthesis, the oligos were removed from the solid support and deblocked from the protective group using ammonium hydroxide at 55°C overnight. The resulting DNA solution was dried and reconstituted in water for spectrophotometric determination of DNA concentration.

7. Plasmid constructions

Plasmid vectors for DMK expression were generated by standard recombinant

DNA techniques (Sambrook et al., 1989). Gel purified (Geneclean kit; BIO 101) restriction fragments were ligated into the desired vectors using T4 ligase and buffer (Gibco/BRL) at 14°C for 4 to 16 h. Ligation reactions were used to transform E. coli DH5α (Hanahan, 1985) and positive clones were identified by restriction enzyme (Gibco/BRL) mapping of miniprep plasmid DNA. The constructions of the vectors used in the expression studies are detailed in chapter V.

Chapter III: Effect of the myotonic dystrophy mutation on the mRNA levels of the DM gene.

1. Introduction

Myotonic dystrophy (DM) is the most common form of inherited neuromuscular disease in adults, with a global incidence of approximately 1 in 8000 individuals. DM is an autosomal dominant, multisystemic disorder characterized mainly by myotonia and progressive muscle weakness and wasting. Clinically, affected individuals present with a highly variable phenotype, ranging from an asymptomatic condition to a severe congenital form that is frequently fatal shortly after birth (Vanier, 1960; Harper, 1989). The main clinical signs of congenital myotonic dystrophy are hypotonia, neonatal respiratory distress and, in most cases, severe mental retardation. Significantly, this form of DM is associated with a delay or an arrest of muscle maturation (Sarnat and Silbert, 1976; Farkas-Bargeton et al., 1988; Soussi-Yanicostas et al., 1991).

Recently, the molecular basis of DM was identified as an unstable CTG trinucleotide repeat in the 3' untranslated region of an mRNA encoding a putative serine/threonine protein kinase (Aslanidis et al., 1992; Mahadevan et al., 1992; Brook et al., 1992; Buxton et al., 1992; Fu et al., 1992; Jansen et al., 1992b). It has been demonstrated that over 98% of individuals affected with DM display CTG amplification, the degree of which correlates with disease severity (Harley et al., 1992b;

Tsilfidis et al., 1992; Barcelo et al., 1993; Caskey et al., 1992; Richards and Sutherland, 1992). Furthermore, within families, DM displays genetic anticipation, an increase in disease severity in successive generations (Howeler, 1989). It is postulated that CTG amplification forms the basis for anticipation.

Trinucleotide repeat expansion has been demonstrated to be associated with several other important genetic diseases. The fragile X-A (FRAX-A; Fu et al., 1991; Verkerk et al., 1991), fragile X-F (FRAX-F; Parrish et al., 1994), FRA16A (Nancarrow et al., 1994) and fragile X-E (FRAX-E; Knight et al., 1993) syndromes have been shown to be associated with a CGG repeat. Huntington's disease (HD; Huntington's disease collaborative research group, 1993), spinocerebellar ataxia type I (SCA1; Orr et al., 1993; Koide et al., 1994), dentatorubral-pallidoluysian atrophy (DRPLA; Nagafuchi et al., 1994), X-linked spinal and bulbar muscular atrophy (SBMA; La Spada et al., 1991) and Machado-Joseph disease (MJD; Kawaguchi et al., 1994) have all been demonstrated to be associated with an expanded CAG repeat in their coding region, presumably resulting in lengthened polyglutamine tract in the respective gene. Unlike HD, SCA1, DRPLA, SBMA and MJD, the trinucleotide repeat in DM, as well as in FRAXA and FRAXE, can undergo very large expansions (Wieringa, 1994). In contrast to fragile X and SBMA, the DM mutation in the 3' untranslated region of the myotonic dystrophy kinase (DMK) gene may represent a novel mechanism for this type of dynamic mutation.

In order to gain insight into the molecular mechanisms by which such a mutation mediates the dominant expression of the DM phenotype, we compared DMK mRNA levels in tissues isolated from normal individuals and a case of congenital DM. We show that DMK mRNA steady state levels are markedly increased in congenital DM tissues and present evidence that the increases are due to elevated levels of transcripts derived from the mutant DMK allele.

2. Methodology

RNA preparation and analysis

Tissue fragments were isolated post-mortem (24 h) from a 1.5 month-old congenital DM patient and frozen until use. Normal fetal brain tissue was obtain from the Canadian brain tissue bank. Total RNA from frozen tissue was isolated by a modification of Chomczynski's procedure (Puissant and Houdebine, 1990). Total RNA from cultured cells grown in α-MEM supplemented with 15% fetal calf serum, was extracted according to the method of Birnboim (Birnboim, 1988). For Northern analysis, RNA was denatured (Sambrook et al.,1989) size fractionated on 1.2% formaldehyde agarose gels and transferred to Hybond-N membranes (Amersham). Hybridizations with ^{32}P-labeled fragments were performed for at least 15 hr at 65°C as described (Sabourin and Hawley, 1990). All probes used were restriction fragments purified from agarose gels by electrophoresis onto Whatman DE81 paper (Dretzen et al., 1981). Fragments

were labeled by random priming to 3 x 10^8 to 10^9 dpm/ug using α-^{32}P-dCTP (Feinberg and Vogelstein, 1983). After hybridization, filters were washed under high stringency conditions and exposed at -80°C for 1 to 5 days to Kodak XAR-5 films with intensifying screens. Normal adult RNA was purchased from Clontech and analyzed as above. For slot blot analysis, 10 µg of denatured total RNA was applied onto nylon membranes (Hybond-N; Amersham) using a BRL Hybri-Slot manifold under 50 cm H_2O pressure vaccuum. Filters were hybridized and washed as described for Northern blots. Autoradiograms were scanned by laser densitometry on a LKB ultroscan densitometer and the relative value for the steady state level of mRNA was determined by normalization to the level of GAPDH (Piechaczyk et al., 1984). Poly-A$^+$ RNA was selected through one round of oligo dT cellulose chromatography (Sambrook et al., 1989) and analyzed by Northern blot.

Genomic DNA preparation and analysis.

For isolation of congenital DNA, tissue fragments were finely minced in phosphate buffered saline (PBS) and high molecular weight genomic DNA was extracted on an ABI Genepure 341 nucleic acid purification system according to the manufacturer's instructions. Control DNA was prepared from cultured lymphoblasts. For CTG expansion analysis, 5 µg of genomic DNA was restricted with EcoRI and subjected to Southern blot analysis (Sambrook et al., 1989) using a 2.2 kb BamHI-EcoRI genomic fragment derived from the human DM kinase gene (Mahadevan et al., 1992; Mahadevan

et al., 1993b) as probe. Post-hybridization washes were in 0.2 x standard saline citrate (SSC) containing 0.1% sodium dodecyl sulfate (SDS) at 65°C. Membranes were exposed to x-ray film for 1 to 4 days.

Reverse Transcriptase PCR.

For reverse transcription, 100 ng or 2 μg of DNaseI-treated total RNA was used in a 20 μl reaction containing 50 ng of downstream primer, 50 mM Tris-HCl (pH 8.0), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1mM each dNTP, 20 units of each RNA Guard and M-MLV reverse transcriptase (Pharmacia). Reactions were incubated for 60 min at 37°C and a fraction corresponding to 100 ng of RNA-equivalent was then added to standard PCR reactions containing 50 ng of both downstream and upstream primers. Amplification was carried out for 30 cycles using the following conditions: 94°C/1 min, 55°C/1 min and 72°C/1 min. For DMK tissue distribution analysis, exon 15-specific primers were used: 5'-CTCCGAGAGCAGCGCAAGTGA-3' (downstream) and 5'-GTTCGCAAAGTGCAAAGCTT-3' (upstream). CTG repeat-flanking primers (5'-CAGAGCAGGGCGTCATGCACA-3'; downstream and 5'-GAAGGGTCCTTGTAGCCGGGAAT-3'; upstream) were used to monitor the level of expression of the wild type allele in congenital tissues. As negative control, in each case, non-reverse transcribed RNA was added to PCR reactions.

Competitive PCR assays

Competitive PCR assays were performed according to Gilliland's method (Gilliland et al., 1990). Briefly, total cDNA generated from 100 ng of RNA was mixed with 0.1, 0.5 and 5 ng (figure 3-5) or 250, 25, 2.5, 0.25 and 0.025 pM (figure 3-6) of competitor plasmid DNA containing the primer binding sites flanking intron 1 of the DM gene. An exon 2-specific 22-mer (5'-CGTCCGATCACCTTCAGAATCT-3') was used as a dowstream primer. The upstream primer (5'-GTGGCCGACTTCTTGCAGTGG-3') was specific to exon 1. All PCR reactions were allowed to go through 30 cycles of amplification as above and products were resolved by agarose gel electrophoresis, transferred to nylon filters and hybridized to end-labelled upstream PCR primers. Autoradiographs were scanned by laser densitometry and the amount of input cDNA prior to PCR was estimated as described (Gilliland et al., 1990). Competitor plasmid DNA consisted of either a DMK genomic region containing exons 1 and 2 interrupted by intron 1 (figure 3-4) or a vector harboring intron 3 flanked by exon 1 and 2-specific primer binding sites (figure 3-5). Control PCR reactions contained an equivalent amount of non-reverse transcribed RNA for determination of genomic DNA contamination.

Exon polymorphism assay

For expression analysis of mutant alleles, total RNA (250 ng) from congenital lymphoblasts was reverse transcribed as described using 50 ng of downstream primer (5'-CATCCTGTGGGGACACCGAGG-3') and subsequently added to standard PCR

reactions and subjected to 35 cycles of amplification using 50 ng of each downstream and upstream (5'-CTGTCGGACATTCGGGAAGGT-3') primers. For the amplification of genomic DNA, an intron-specific oligo was used as upstream primer (5'-CTGCAGAAGGTTTAGAAAGAGC-3'). PCR conditions consisted of 94°C/1 min, 55°C/1 min and 72°C/1 min for 10 cycles followed by 94°C/1 min, 60°C/1 min and 72°C/1 min for 25 cycles. Genotypes were determined by digestion of the PCR products with 1 unit of Bpm I (New England Biolabs) followed by gel electrophoresis of total digests through 3% agarose.

3. Results

Expression of Normal and Mutant DMK Transcripts.

Prior to DMK expression analysis in congenital tissues, we sought to establish the extent of CTG amplification in various congenital tissues by Southern blot analysis. As shown in figure 3-1, all tissues except heart showed a similar degree of expansion which is classified here as E4 (expansion greater than 4.5 kb), according to nomenclature reported elsewhere (Mahadevan et al., 1992; Tsilfidis et al., 1992). The CTG amplification ranged in size from 10 to 12 kb (brain, muscle, diaphragm, liver) indicative of somatic heterogeneity within single tissues, as previously demonstrated in DM leukocytes (Mahadevan et al., 1992; Brook et al., 1992; Fu et al., 1992; Harley et

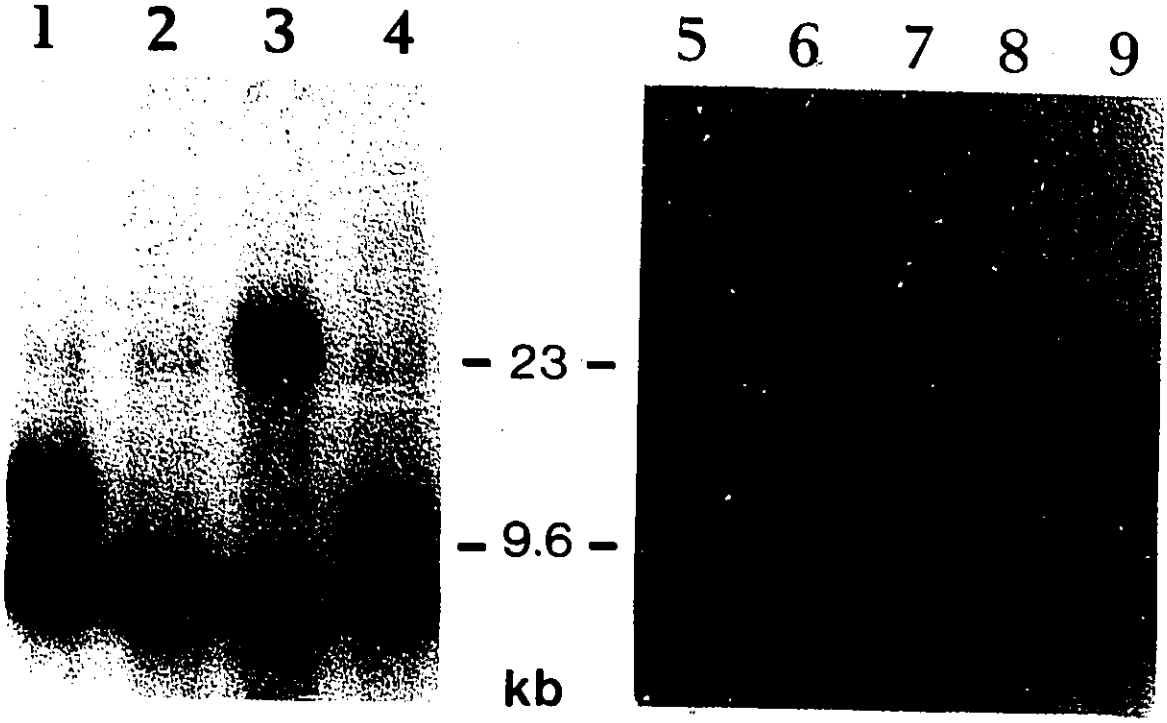

1 2 3 4
5 6 7 8 9
23
9.6
kb

Figure 3-1. CTG trinucleotide expansion status in genomic DNA. EcoRI restricted genomic DNA (5 µg) was analyzed for expansion by Southern blot with a 2.2 kb BamHI-EcoRI genomic fragment derived from the DM gene (Mahadevan et al., 1992). Lane 1: affected E1 individual, Lane 2: normal homozygote for the 9 kb allele, Lane 3: affected E4 individual, Lane 4: normal heterozygote, Lanes 5-9: congenital DM tissues from a single individual; heart, brain, diaphragm, skeletal muscle and liver, respectively. Positions of the 23 and 9.6 kb lambda HindIII markers are indicated. The analysis shows somatic heterogeneity ranging between 10 and 16 kb.

al., 1992a). Heart genomic DNA displays a slightly larger and relatively more heterogeneous expansion (12 to 16 kb) but the basis and significance for this difference is unknown. Somatic heterogeneity was confirmed by Southern blotting of patient DNA restricted with enzymes flanking the repeat region. This results in greater resolution of smaller fragments bearing expanded repeats (data not shown).

As determined by Southern blot, heterogeneous genomic (CTG) repeat amplification was observed in various congenital tissues and sized between 10 and 16 kb. Mature mutant DMK transcripts from these tissues should be in the range of approximately 13 to 19 kb in size. Total RNA samples prepared from normal and affected tissues were analyzed by Northern blotting experiments using a human derived cDNA probe. Analysis of normal human tissue RNA samples (not shown) detected a single 3.3 kilobase DMK transcript with the highest level of expression in heart and muscle samples. A relatively less abundant DMK mRNA can be observed in brain, liver and lung tissues, similar to the findings in murine and baboon tissues (Brook et al., 1992; Jansen et al., 1992b). This pattern of expression correlates well with the primary involvement of muscle tissues in DM and is consistent with the multisystemic nature of the disease.

Surprisingly, Northern analysis of RNA from affected tissues failed to show the existence of high molecular weight mutant DMK mRNA (13-19 kb) as a smear above wild type DMK or in the form of a diffuse band as anticipated from our Southern blot results (figure 3-2). However, hybridizing species of approximately 7 kb were detected

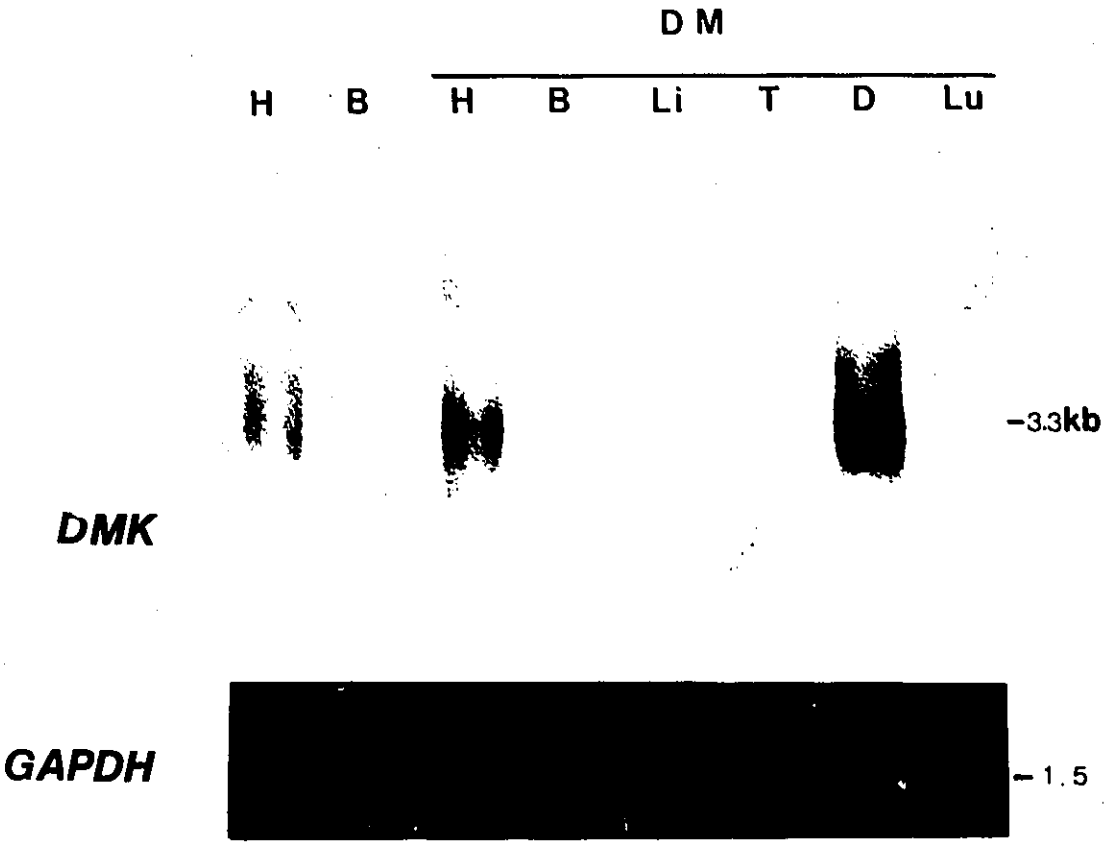

D M
H
B
H
B
Li
T
D
Lu
-3.3kb
DMK
GAPDH
- 1.5

Figure 3-2. Expression of DMK mRNA in congenital tissues. Total RNA (20 μg) isolated from various congenital DM tissues from a single individual was analyzed for the expression of DMK. Control hybridization was performed using a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (Piechaczyk et al., 1984). The analyzed tissues were: H, heart; B, brain; Li, liver; T, testis; D, diaphragm; Lu, lung. The 'DM' bracket indicates congenital DM tissues. Normal adult heart and brain samples were used for comparison. Exposure times were 48 hr and 17 hr for DMK and GAPDH, respectively. No differences in the levels of wild type DMK mRNA were observed. The mRNA corresponding the the mutant DMK transcript could not be detected.

in both DM and normal heart tissue samples that are likely to correspond to heteronuclear transcripts. The apparent absence of mutant transcripts may suggest that the DM allele is not expressed, as is the case for the fragile X FMR-1 gene. However, it is possible that agarose gel fractionation of the heterogeneous population of mutant DMK transcripts has a dilution effect, rendering them undetectable by Northern blot analysis. Furthermore, the isolation of intact high molecular weight RNA requires fresh tissues and under the circumstances, the congenital tissues sampled may have undergone limited RNA degradation.

In order to overcome the potential degradation problem of the high molecular weight tissue mRNA, poly-A$^+$ RNA was prepared from fibroblasts derived from the same congenital patient and then subjected to Northern blot analysis (figure 3-3). For comparison, RNA samples from a human rhabdomyosarcoma (TE.32; McAllister et al., 1969), HeLa and human lymphoblasts were also analyzed. Significantly, in addition to the 3.3 kb message, congenital DM fibroblast RNA showed a hybridization signal corresponding to a 10 kb transcript, likely to represent a major species of mutant DMK mRNAs. These data demonstrate that, in congenital fibroblasts bearing large CTG expansions, the mutant DMK allele is transcribed at levels comparable to the 3.3 kb mRNA.

Normal and Affected Tissue RNA Levels

Because of the inherent difficulties in quantitating the levels of DMK transcripts

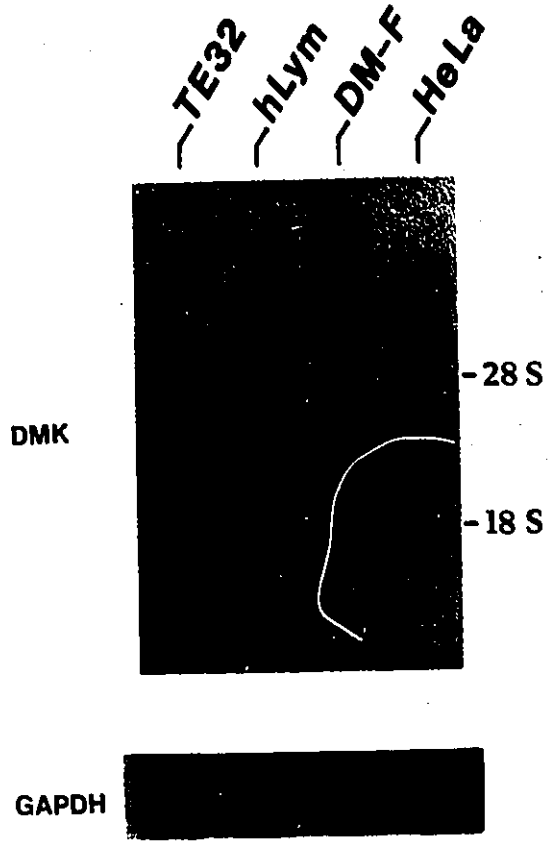

TE32
hLym
DM-F
HeLa
DMK
28 S
18 S
GAPDH

Figure 3-3. Expression of DMK transcripts in human cell lines. Poly-A^+-selected RNA (5 μg) from several human and rodent cell lines was analyzed for DMK expression by Northern blot. A rat GAPDH probe was used for control hybridization. Human cell lines are: rhabdomyosarcoma TE32, normal lymphoblast (hLym), DM fibroblast (DM-F; derived from the congenital patient described in the text) and HeLa cells. Exposure time was 20 hr. A mutant 10 kb DMK mRNA was readily observed in the DM-F RNA sample. Lower levels of slightly smaller hybridizing species are present in HeLa cell RNA, presumably corresponding to partially processed immature transcripts.

by Northern blot analysis, an additional test for the quantitation of DMK mRNA steady state levels was performed using a slot blot procedure on total RNA samples obtained from various normal and affected tissues. As shown in figure 3-4a, after normalization to GAPDH, the steady state levels of DMK mRNA were found to be increased by approximately 14-fold in DM brain tissue relative to the normal adult control. However, only a 2 to 4-fold increase was consistently observed in DM heart and muscle samples, and a 4 to 6-fold increase in the liver and lung, when compared to normal adult tissues. In contrast to Northern analysis, slot blot experiments are likely to have a concentration effect on all forms of DMK transcript allowing for the simultaneous detection of wild type and all mutant mRNAs. Surprisingly, no significant differences were observed in lymphoblast cell lines derived from normal or congenital DM patients, suggesting that the increase in DMK steady state levels is tissue specific.

In order to verify that the increase in DMK mRNA levels was due to differential developmental regulation of the DM gene, the levels of DMK transcripts were analyzed by the competitive PCR (Gilliland et al., 1990) using normal adult and fetal brain RNA samples. The PCR products (figure 3-4b) were Southern blotted and hybridized to one radiolabelled PCR primer followed by densitometric scanning of the autoradiographs. Quantitative measurements on heart, brain and fetal brain samples demonstrate that the ratios of DM heart to normal heart and DM brain to fetal brain were approximately 3 and 12 fold, respectively, in agreement with the findings obtained from slot blot

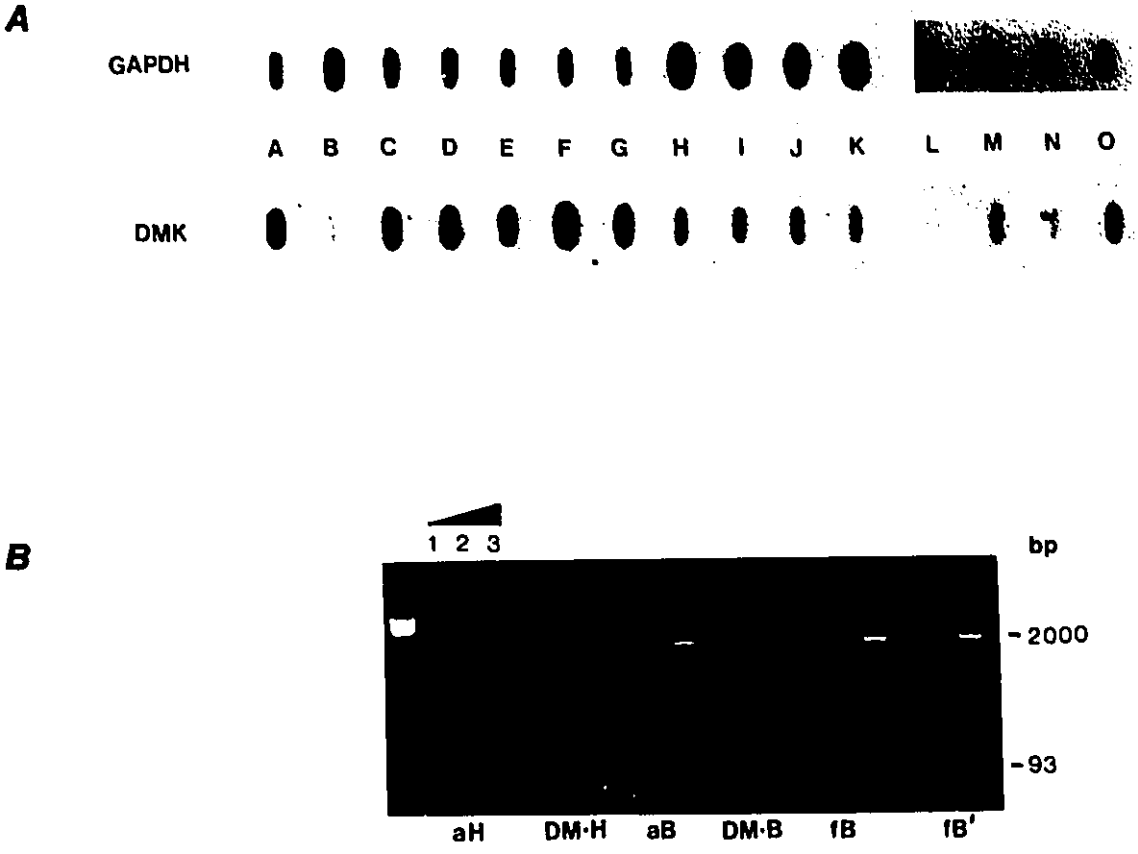

A
GAPDH
A B C D E F G H I J K L M N O
DMK
B
1 2 3
bp
2000
93
aH
DM·H
aB
DM·B
fB
fB'

Figure 3-4. Increased DMK mRNA steady state levels in congenital tissues. a) Total RNA (10 μg) was immobilized on nylon filters using a slot blot manifold (Bethesda Research Laboratories) and hybridized sequentially to a human DMK and rat GAPDH probe. Slot A: normal adult heart; B: normal adult brain; C: normal adult skeletal muscle; D: congenital heart; E: congenital brain; F: congenital muscle; G: congenital diaphragm; H: normal lymphoblastoid cells; I-K: congenital lymphoblastoid cells; L: normal adult liver; M: congenital liver; N: normal adult lung; O: congenital lung. Similar results were obtained in 4 independent experiments. Results from one representative analysis is shown. Exposure time was 23 hr for each probe. b) Competitive DMK-specific RT-PCR. Total RNA (100 ng) was reverse transcribed and added to PCR reactions containing various amounts of competitor plasmid DNA. Products were visualized by ethidium bromide agarose gel electrophoresis. Each set of three reactions contained 0.1 ng (lane 1), 0.5 ng (lane 2) or 5 ng (lane 3) of competitor DNA. Densitometric readings were obtained after Southern blotting and autoradiography of the corresponding gel. Analyzed samples were: aH, adult heart; DM-H, congenital heart; aB, adult brain; DM-B, congenital brain; fB, normal fetal whole brain; fB', normal fetal temporal and occipital cortex. cDNA (93 bp) and competitor (2000 bp) products are indicated.

analysis. Furthermore, the levels of DMK transcripts in normal adult brain did not differ significantly from normal fetal brain. These data demonstrate that the observed increase in DMK mRNA steady state levels in affected tissues is not likely caused by a difference in DMK steady state levels between two distinct developmental stages.

In order to confirm these results, we performed competitive PCR on brain RNA samples using an altered DMK-derived genomic fragment as a competitor over a broader concentration range (figure 3-5a). Quantitation of both products was done as described above. By plotting the ratio of genomic competitor to DMK cDNA versus the input concentration of competitor DNA (Gilliland et al., 1990) (figure 3-5b), we obtained, at the equivalence point (ratio=1), a 10-fold increase in DMK mRNA in the case of the DM brain sample, demonstrating that both PCR assays support the slot blot analysis result.

Expression of wild type and mutant alleles

If the DMK gene is transcribed equally from both genomic copies, each allele should account for 50% of all DMK mRNAs. Surprisingly, comparison of heart and brain RNA samples from normal and affected tissues did not show any detectable differences in dosage of wild type DMK trancripts (figure 3-2) after normalization to GAPDH mRNA levels. Furthermore, neither the slot blot nor competitive PCR analyses,

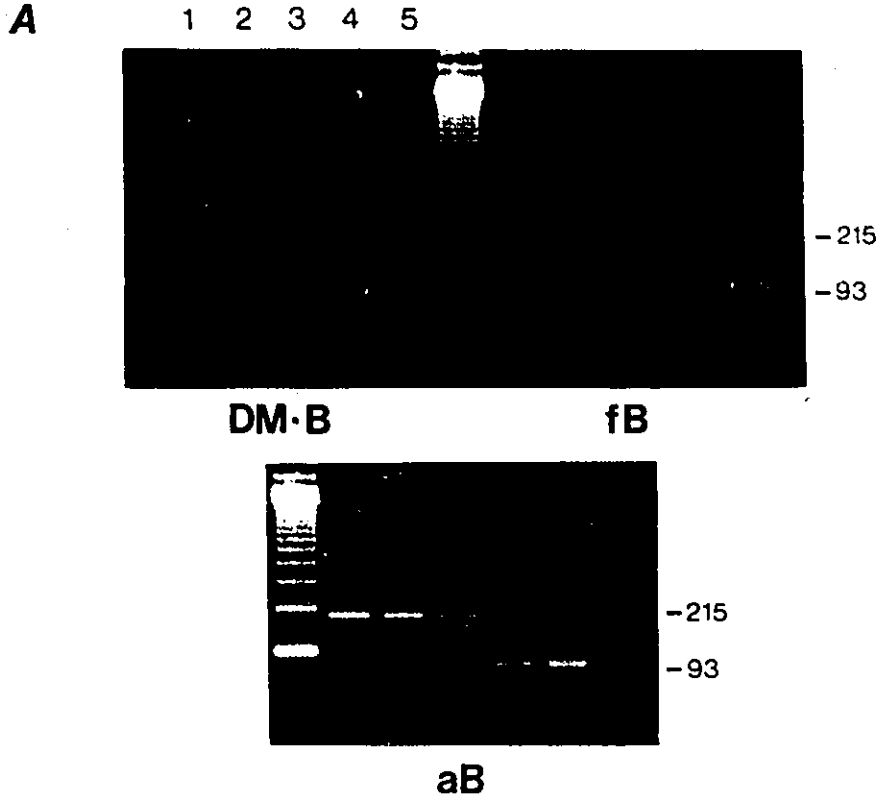
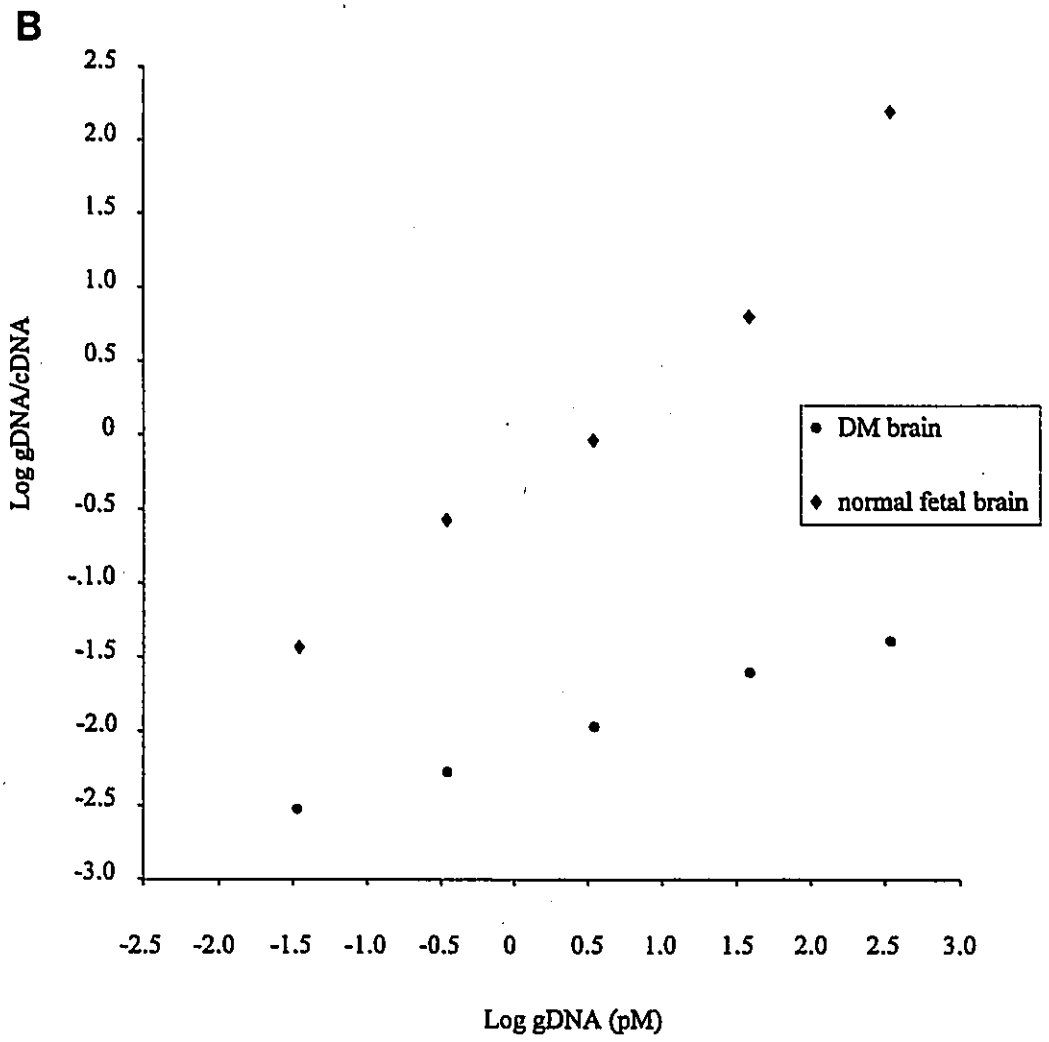
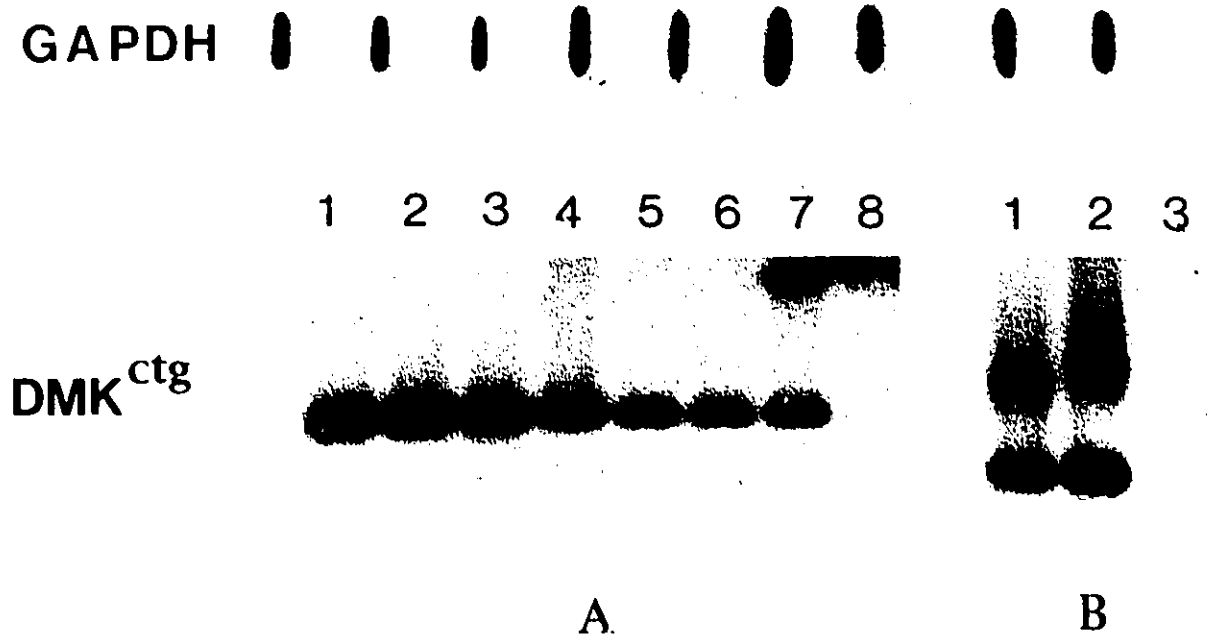

A
1 2 3 4 5
215
93
DM·B
fB
215
93
aB

Figure 3-5. Competitive DMK-specific RT-PCR. a) Total RNA (100 ng) was reverse transcribed and added to PCR reactions containing ten-fold serial dilutions of competitor DMK plasmid. Each set of five reactions contained 250, 25, 2.5, 0.25, and 0.025 pM of competitor genomic DNA (gDNA,lanes 1-5). Thirty cycles of PCR and electrophoresis of samples were performed. The tissues analyzed were congenital brain (DM-B), normal fetal brain (fB) and normal adult brain (aB). b) Following hybridization to a radiolabelled oligo, autoradiographs, were scanned by laser densitometry and the ratio of O.D. units for the competitor (gDNA) products (215 bp) to cDNA (93 bp) was calculated and plotted as log gDNA/cDNA vs. log input gDNA. To correct for differences in molecular weight, the O.D. units obtained from competitor DNA were multiplied by 215/93. At the equivalence point, a concentration of approximately 1.2 pM and 12 pM was observed for normal brain and DM brain samples respectively. Similar results were obtained in three independent experiments.

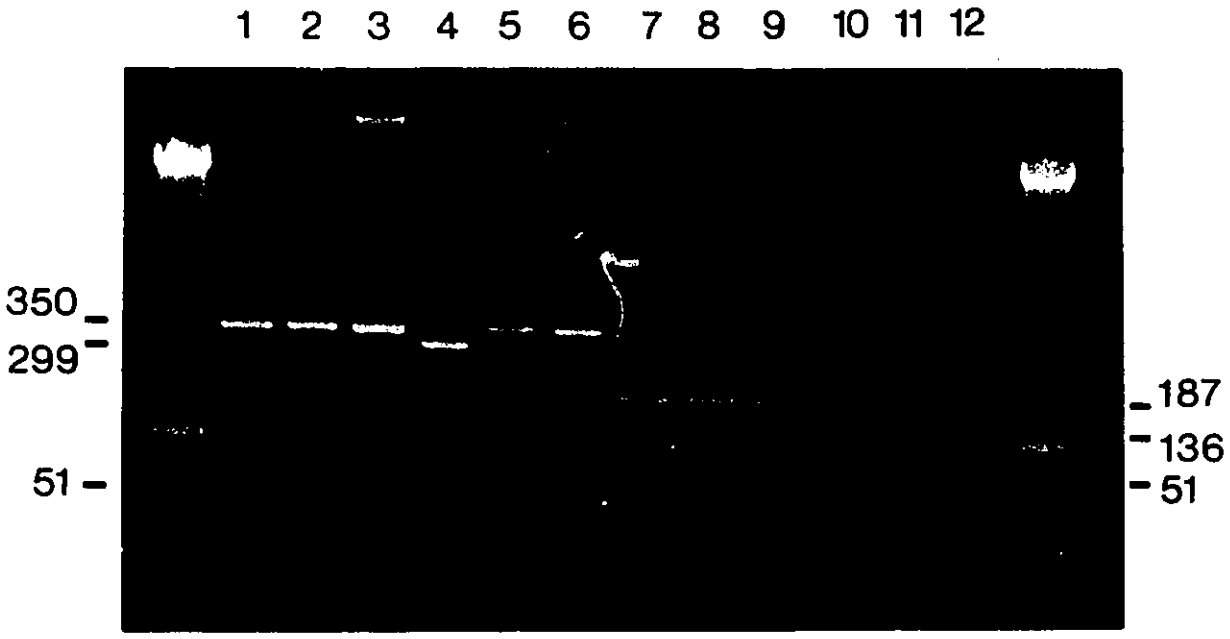

B
2.5
2.0
1.5
1.0
0.5
0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
Log gDNA/cDNA
-2.5 -2.0 -1.5 -1.0 -0.5 0 0.5 1.0 1.5 2.0 2.5 3.0
Log gDNA (pM)
DM brain
normal fetal brain

as described above, were able to discriminate between the mutant and the wild type allele. Therefore, we analyzed the levels of wild type DMK transcripts in various congenital tissues using a direct RT-PCR analysis of the CTG repeat region. During the course of this study, we found that reverse transcription could not proceed through the very large CTG repeat sequence of a mutant DM allele. However, RT-PCR of this region from normal, and even from protomutation size alleles (50-80 CTG repeats; Barcelo et al., 1993), was not problematic. As a result, we sought to monitor the relative levels of the wild type transcripts from normal and affected tissues. To control for input RNA, an identical aliquot of total RNA was slot blotted and probed for GAPDH. After normalization to GAPDH levels, densitometric scanning showed that the levels of the wild type transcript (having 8 CTG repeats) in all affected tissues surveyed represented 50% of the total DMK mRNAs found in normal control tissues (5 and 13 CTG repeats; figure 3-6a). In parallel, RNA from two individuals bearing CTG repeat sizes of 5/83 and 5/90, respectively, were subjected to the same RT-PCR assay. As shown in figure 3-6b, alleles containing up to 90 repeats are expressed, suggesting that this level of CTG amplification does not abolish expression of the mutant DMK allele.

To test for the expression of greatly expanded loci, a diallelic exon polymorphism (frequency of the rare allele equals 0.15) was used to discriminate between wild type and mutant alleles. Unfortunately, the congenital patient analyzed throughout this study was found to be homozygous for the common allele. Therefore,


GAPDH
1 2 3 4 5 6 7 8
1 2 3
DMK ctg

A B


1 2 3 4 5 6 7 8 9 10 11 12
350
299
51
187
136
51

C

Figure 3-6. Expression of wild type and protomutation size alleles. Total RNA from lymphoblasts or various normal and DM tissues (2 μg) was reverse transcribed and subjected to 30 cycles of PCR using primers flanking the polymorphic CTG repeat. PCR products were transfered to nylon filters and subsequently hybridized to an end labelled $(CTG)_{10}$ oligo followed by densitometric scanning of the autoradiographs. An equivalent amount of RNA was used for a GAPDH control hybridization. a) RT-PCR on normal and affected tissues. Lanes 1-3: normal adult heart, brain and muscle, respectively. Products are 224 bp (5 repeats) and 238 bp (13 repeats). Lanes 4-7: congenital heart, brain, muscle and diaphragm, respectively. Wild type products are 224 bp (5 repeats). Lane 8: negative control. b) RT-PCR on lymphoblast RNA obtained from individuals bearing protomutation size CTG amplification. Lane 1: 83 (348 bp) and 5 repeat (224 bp) alleles. Lane 2: 90 (469 bp) and 5 (224 bp) repeat alleles. Lane 3: negative control. c) Expression of mutant DM allele in congenital lymphoblasts. Reverse transcribed RNA (250 ng) and genomic DNA (1 μg)from lymphoblastoid cell lines derived from severely affected patients was subjected to 30 cycles of PCR using DMK exon and intron-specific primers. PCR products were digested with Bpm I and analyzed by ethidium bromide agarose gel electrophoresis. Lane 1: undigested genomic product from a normal homozygote. Lanes 2 and 3: undigested genomic product from congenital heterozygotes. The respective digested genomic products are shown in lanes 4-6. The corresponding cDNA products are shown in lanes 7-9 (undigested) and lanes 10-12 (digested). Wild type (350 and 187 bp) and mutant (299 and 136 bp) alleles are indicated. Regardless of the size of the CTG amplification, expression of the mutant DMK gene was observed.

RNA extracted from lymphoblastoid cell lines derived from two other congenital DM patients (possessing E3 genotypes; Tsilfidis et al., 1992) was added to RT-PCR reactions and the resulting products digested using the restriction enzyme BpmI, which detects the relevant sequence polymorphism. As demonstrated in figure 3-6c, the DM-associated allele is present, indicating that even very large CTG amplifications do not repress the expression of the mutant DMK gene. Furthermore, an equal expression of both wild type and mutant DMK alleles was found in these cell lines, a result consistent with that observed using slot blot analysis of lymphoblastoid cell RNA, as described above.

These data demonstrate that mutant DMK alleles are not repressed by CTG trinucleotide amplification and that elevated DMK mRNA levels are likely due to an increase in the steady state levels of mutant DMK transcripts.

4. Discussion

As an initial step into the analysis of DMK expression in affected tissues, we performed Southern blot analysis in order to determine the expansion status in somatic tissues from a congenital patient. Our experiments show CTG expansion ranging between 10 and 16 kb in the various tissues analyzed. The observed heterogeneity is in accordance with previously reported results for DM lymphoblastoid cell lines

(Mahadevan et al., 1992; Brook et al., 1992; Buxton et al., 1992; Fu et al., 1992; Harley et al., 1992a; Tsilfidis et al., 1992; Barcelo et al., 1993) and demonstrates the high degree of mitotic and meiotic instability since the 12 kb expansion was inherited from the mother who displayed less than a 2.0 kb amplification of the CTG repeat as determined by Southern blot analysis (data not shown).

As a first step towards the characterization of the molecular mechanisms underlying DM, we have used Northern and slot blot analysis to evaluate the steady state levels of DMK transcripts in normal and congenital DM tissues as well as in lymphoblastoid cell lines. In fragile-X, hypermethylation of the CGG repeat in the 5' untranslated region has been demonstrated to repress expression of the FMR-1 gene in affected individuals (Sutcliffe et al., 1992). In marked contrast to fragile-X, slot blot and competitive PCR analysis of tissue RNA samples obtained from normal and affected individuals showed a significant increase in the steady state DMK levels in DM brain and, to a lesser extent , in heart, muscle, liver and lung samples. Surprisingly, RNA samples from lymphoblastoid cells derived from normal and congenital individuals did not show any detectable difference in DMK levels, suggesting that the increase in DMK steady state levels is tissue specific. PCR analysis of congenital and normal tissue RNA shows that the wild type allele in congenital samples is expressed at 50% the levels of total DMK mRNAs found in normal control tissues. Furthermore, in PCR-based assays, we find that mutant alleles are expressed independently of the number of CTG repeats.

Consistent with these results, Northern blot analysis of poly-A$^+$ RNA from fibroblasts of the congenital DM patient showed expression of the mutant allele as a 10 kb message that is likely to represent a predominant species of mutant mRNA. Therefore the increased DMK steady state levels observed by slot blot analysis and competitive PCR is due to elevated levels of the mutant transcript. These results are in direct contrast to those reported by Fu et al. (1993), who demonstrated decreased levels of DMK mRNA in affected tissues. An explanation for these conflicting results may the fact that the present study examines DMK expression in cells and tissues from severely affected neonates, while Fu and co-workers studied tissues from DM adults. Nevertheless, it is difficult to reconcile how different mechanisms of DMK expression could explain the pathogenesis of the adult versus congenital form of the disease. An alternative explanation for these contrasting results may lie in the different methods used to quantitate the levels of normal and mutant DMK transcripts. In the study by Fu et al. (1993), RT-PCR using primers flanking the CTG repeat was used to measure message levels. As shown by the present study, reverse transcription through amplified repeat regions is difficult and would be problematic in the quantitation of DMK mRNA. Therefore, the detection of decreased message levels in the adult cases may, in fact, be due to a technical artifact.

It is not clear at this point whether the elevated DMK levels observed in this study are due to increased stability or higher transcription rate of the mutant DMK allele. The establishment of DM and normal myoblast cell lines, in conjunction with

nuclear run-on and mRNA stability experiments, will allow these possibilities to be assessed.

Computer analysis of mRNA secondary structure using the RNAfold algorithm (Zucker and Stiegler, 1981), predicts a thermodynamically stable stem loop structure in the repeat region by C:G base pairing. Such structures have been identified in the 3' untranslated region of the human and mouse transferrin receptor, conferring protein mediated mRNA stability in the absence of iron (Mullner and Kuhn, 1988; Mullner et al., 1989). In addition, stability determinants have been identified in several proto-oncogene and cytokine mRNAs (reviewed in Belasco and Brawerman, 1993; Peltz et al., 1991; Bernstein et al., 1992). Therefore, a possibility is that the amplification of the CTG repeat causes the aberrant binding of a tissue specific RNA binding factor increasing DMK mRNA half-life by a polysome-dependent mechanism. This would then directly lead to increased levels of the DM kinase protein product, similar to what has been postulated to account for the results found with the transferrin receptor (Mullner et al., 1989). Alternatively, it is conceivable that the CTG repeat is bound by a RNA-binding protein, that may directly interfere with translation efficiency, thereby leading to polysome-dependent mRNA stability (Kaspar et al., 1992; Sengupta et al., 1990).

In various systems, translational efficiency is coupled to post-transcriptional

regulation (reviewed in Peltz et al., 1991; Sengupta et al., 1991; McCormack et al., 1992; Wisdom and Lee, 1990). Whether the increase in DMK transcripts produces elevated kinase protein levels remains to be clarified. Nevertheless, one can envisage a mechanism by which aberrant binding of a protein factor to the expanded CTG repeat that increases DMK mRNA stability leading to an elevated kinase concentration. DNA sequence alignment reveals that DMK is highly homologuous to cyclic nucleotide dependent kinases. In DM, high levels of the putative DMK catalytic subunit would result in an alteration of the catalytic to regulatory subunit ratio (Taylor et al., 1990). The ultimate net effect would be the non-regulated hyperphosphorylation of its substrate by the DM kinase protein. This would explain how an unstable mutation in the 3' UTR of a gene encoding an enzyme would result in the dominant inheritance pattern and variable phenotype observed in Myotonic Dystrophy.

A delay of terminal muscle differentiation has been observed in the congenital form of DM (Sarnat and Silbert, 1976; Farkas-Bargeton, 1988; Soussi-Yanicostas et al., 1991), suggesting a functional involvement of DMK. In this regard, a recent study has demonstrated that overexpression of the catalytic subunit of protein kinase A (Li et al., 1992b) and protein kinase C (Li et al., 1992c) inhibits muscle cell differentiation by indirectly silencing the activity of the MyoD family of regulatory factors. Therefore, in congenital DM patients, terminal muscle differentiation may be delayed as a result of hyperphosphorylation by the DMK gene product along the differentiation pathway.

Analysis of the activity of MyoD family members in transfected myoblasts from congenital DM patients and *in vitro* differentiation systems will allow these different hypotheses to be assessed.

Chapter IV: Expression of the Myotonic Dystrophy Kinase (DMK) in Muscle Tissues and Primary Cell Cultures from DM Patients

1. Introduction

The mutation underlying DM has been identified as the amplification of a polymorphic $[CTG]_n$ trinucleotide repeat in the 3' non-coding region of the gene (Aslanidis et al., 1992; Mahadevan et al., 1992; Brook et al., 1992; Buxton et al., 1992; Fu et al., 1992; Harley et al., 1992a). In the normal population, repeat sizes range from 5 to approximately 40 (reviewed in Wieringa, 1994). This polymorphic region was shown to be significantly amplified in DM individuals, ranging from approximately 50, in mild DM cases, to several thousand repeats in severely affected patients. A strong positive correlation has been demonstrated between the size of the repeat and the severity of the disease (Harley et al., 1992b; Tsilfidis et al., 1992; Barcelo et al., 1993).

The DM gene product (DMK) shows extensive homology to the catalytic domain of serine/threonine protein kinases and more limited homology, in a predicted alpha helical coil structure, to myofibrillar proteins (Mahadevan et al., 1993b; Jansen et al., 1992b; Brook et al., 1992; Fu et al., 1992). Although serine/threonine specific kinase activity has been demonstrated (Dunne et al., 1994), the exact function of DMK is unknown. Immunolocalization experiments have shown that it is a component of neuromuscular junctions (van der Ven et al., 1993, Whiting et al., 1995) and

intercalated disks (Whiting et al., 1995), suggesting a functional role for DMK in the excitation-contraction coupling mechanism.

Conflicting observations have been reported with respect to the effect of the expanded repeat on DMK expression. Over-expression (Sabourin et al., 1993) and reduced expression (Fu et al., 1993; Carango et al., 1993; Hofmann-Radvayani et al., 1993) of the mutant allele has been reported using different assays. In addition, trans up-regulation (Radvayani et al., 1994) and down-regulation (Hofmann-Radvayani et al., 1993) of the wild type allele has been documented as an effect of $[CTG]_n$ amplification. More recently, RT-PCR analyses have shown that the levels of both the wild type and mutant DMK transcripts in DM individuals were not affected in total RNA preparations but were significantly reduced in the RNA poly-A^+ fractions (Wang et al., 1995). Similarly, it has been observed, by western blot analysis using anti-DMK peptide antibodies, that the levels of a 54 kDa protein were reduced in affected tissues (Fu et al., 1993; Koga et al., 1994). The size of this polypeptide is significantly smaller than the predicted size of 70 kDa (Mahadevan et al., 1993b).

In order to resolve these discrepancies, we have analysed RNA from freshly isolated DM muscle tissue and generated rabbit polyclonal anti-DMK antibodies that react specifically to human and murine DMK. Mutant DMK mRNA is readily detectable by Northern blot analysis of intact RNA prepared from freshly sampled muscle tissues from affected patients. However, western blotting experiments demonstrate that the levels of DMK are not significantly altered in congenital or adult

DM skeletal muscle samples and myoblast cell lines derived from these patients. In addition, immunofluorescent studies failed to show any differences in the distribution of DMK protein in muscle sections from affected individuals. These data suggest that the dominant inheritance pattern of DM is not due to a significant alteration in DMK protein levels but may be due to a *cis* or *trans* dominant effect of the amplified repeat.

2. Methodology

RNA and genomic DNA preparation and analysis.

Tissue fragments were isolated and total RNA was extracted immediately and analysed by Northern blotting as described (Sabourin et al., 1993). Genomic DNA was prepared from frozen tissue fragments or cell pellets and subjected to Southern blot analysis as described (Sabourin et al., 1993).

Tissue culture and cell lines.

All cell lines described in this study were purchased from the American Type Culture Collection (ATCC) and maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cells were grown in α-MEM supplemented with 15% fetal calf serum (FCS; Gibco/BRL). For transfection experiments, a full length human DMK cDNA, cloned into the pcDNA3 expression vector (Invitrogen), was introduced into COS-1 cells by the calcium phosphate method as described (Sambrook et al., 1989). 48 hours following

transfection, cells were harvested and lysed as described below. To establish primary myoblast cultures, a fragment or needle biopsy of skeletal muscle was obtained from affected and normal individuals and minced into small pieces in cold phosphate buffered saline (PBS). The minced fragments were then incubated in trypsinization solution (0.25% trypsin and 53 mM EDTA (Gibco/BRL) supplemented with 10 mM glucose) at 37°C, with shaking, for 1 hour. Debris were allowed to settle by gravity and the cells contained in the supernatant were spun down at 400xg and subjected to a second trypsinization. Again, the resulting supernatant, containing satellite myoblasts, was plated and the cells were maintained in F-12 medium (Gibco/BRL) containing 20% FCS and 5μg/ml epidermal growth factor (Sigma). Cell pellets were subjected to Southern and western blot analysis.

Protein preparation and analysis.

Tissue fragments and cell pellets were homogenized in a buffer consisting of 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 2 mM benzamidine HCl and 10 μg/ml aprotinin. The lysates were rotated at 4°C for 1 hour and centrifuged at 10,000xg for 5 min. The resulting supernatant was quantitated for total protein concentration using a BCA protein assay system (Pierce) according to the manufacturer's instruction. For western blot analysis, 20μg of cytosolic protein was subjected to 10% SDS-PAGE and transferred (Sambrook et al., 1989) to polyvinylidene difluoride membranes (PVDF; Immobilon-P). Electrophoresis and transfer was

performed using a Bio-Rad mini-protean II system. Efficiency of transfer was evaluated by staining the filters with 0.2% Ponceau S in 0.1% acetic acid. Membranes were blocked with 5% skim milk powder in TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) and incubated for 1 hour at room temperature with 140 ng/ml of DMK antibody, designated anti-DMK-1 (or anti-DMK-2). For normalization, an anti-α-sarcomeric actin monoclonal antibody (5C5; Sigma) was used at a 1:10,000 dilution as suggested by the manufacturer. Anti-MHC type I monoclonal antibodies (clone 4A9; a kind gift form P. Merrifield) were used at a 1:10 dilution of hybrydoma supernatant. Detection was performed using a horse radish peroxidase-labelled goat anti-rabbit (or anti-mouse) secondary antibody at a 1:3000 dilution as recommended by the manufacturer (Amersham). Post-incubation washes (3 times/10 min) were in TBST at room temperature. Following the last wash, membranes were subjected to chemiluminescence detection using ECL reagents according to the manufacturer's specifications (Amersham). Signals were visualized following a 2 to 5 min exposure to Kodak XAR-5 films. Autoradiograms were scanned on a Bio-Rad GS-670 imaging densitometer.

Immunofluorescence analysis.

Tissues were obtained at autopsy (6 hours post mortem), flash frozen in liquid nitrogen and stored at -80°C until sectioned. Cryostat sections (16 μm) were cut and thaw mounted on slides for immunostaining as described (Whiting et al., 1995). Stained

sections were viewed and photographed using a Zeiss fluorescence microscope. For N-CAM and desmin staining of myoblast cultures, the cells were plated on 2 cm x 2 cm coverslips and grown for 24 hours. Coverslips were fixed for 3 min in pre-chilled methanol (-20°C), rinsed with phosphate buffered saline and incubated with anti-N-CAM monoclonal antibody (5H.111) or an anti-desmin monoclonal antibody (ZSD1; Pierce). Detection was performed using a fluorescein-labelled goat anti-mouse antibody.

3. Results

Generation of DMK-specific antibodies

Rabbit polyclonal antibodies to DMK were obtained following immunization with bacterially expressed DMK as a GST fusion protein (described in Whiting et al., 1995). Affinity purified antibodies (anti-DMK-1 and anti-DMK-2) were used in all subsequent analyses. Western blotting experiments of normal human tissue extracts, using anti-DMK-2, results in the detection of two major reactive species of 74 kDa (DMKα) and 82 kDa (DMKβ, see Fig 4-1). Similar DMK protein species of 70-72 kDa have been reported using different antibodies (Timchenko et al., 1995; Sasagawa et al., 1994). Interestingly, the 82 kDa isoform is the predominant species in human skeletal muscle, whereas the 74 kDa DMK protein is found to be more abundant in cardiac tissues. Relatively low levels of these two isoforms are seen in total human and murine

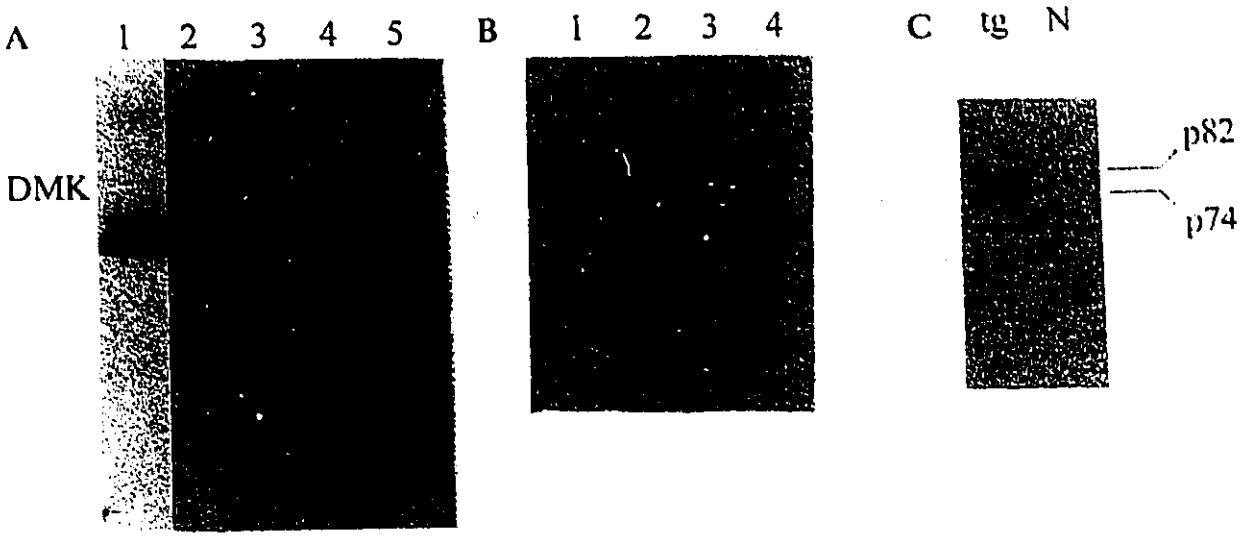

A
1
2
3
4
5
DMK
B
1
2
3
4
C
tg
N
p82
p74

Fig. 4-1. Expression of DMK protein in tissues and cell lines. a, Total cytosolic protein extracts (20μg) isolated from various normal human tissues were analysed for DMK expression by western blot analysis. The tissue extracts were: lane 1, heart; lane 2, adult pectoralis; lane 3, neonatal pectoralis; lane 4, brain; and lane 5, liver. b, Expression of DMK in cell lines: lane 1, COS-1 cells; lane 2, 2μg of protein extract from COS-1 cells transfected with a full length DMK cDNA; lane 3, C2C12 murine myoblasts; and lane 4, C3H10T1/2 fibroblasts extract. c, Expression of DMK in a brain sample from a DMK transgenic mouse; tg, transgenic brain extract; N, normal mouse brain extract. All filters were probed with anti-DMK-2. and detection was performed using an enhanced chemiluminescent system with a horse radish peroxidase-coupled goat anti-rabbit antibody. The sizes of the observed DMK product are indicated in kiloDaltons (kDa).

brain samples. However, significantly higher levels were observed in the choroid plexus and ependyma regions (Whiting et al., 1995), suggesting a cell type-specific expression of DMK in the brain. Liver tissue does not show appreciable levels of either DMK isoforms. These relative protein levels are consistent with the observed abundance of DMK mRNAs in the corresponding tissues (Brook et al., 1992; Jansen et al., 1992b). We have previously reported the existence of two major DMK isoforms in cDNA libraries prepared from human tissues which correspond to the alternative splicing of exons 13 and 14 (Jansen et al., 1992b). These mRNAs are predicted to encode products that differ by approximately 10 kDa and it is possible that DMKα and DMKβ represent these isoforms.

Further characterization of anti-DMK-2 was carried out on various cell lines and brain tissue of DMK transgenic animals. As shown in figure 4-1b, COS-1 control cells do not show any detectable levels of DMK protein. However, COS-1 cells transfected with a full length DMK cDNA display high levels of p82-DMK. The murine myoblast cell line C2C12 was found to express DMK at appreciable levels, in contrast to the mesodermal precursor fibroblast cell line C3H10T1/2. This pattern of DMK expression also correlates with the abundance of DMK mRNA detected in these cell lines (data not shown). Finally, other than a minor cross-reacting species at 66 kDa, anti-DMK-2 did not detect any DMK-specific protein in a total brain extracts from normal control mice. However, specific DMKα and β isoforms were readily observed in brain samples from transgenic mice bearing the entire human gene (Fig 4-1c and M.Narang, unpublished

data). These data, in conjunction with recent results from our laboratory (Whiting et al., 1995), strongly suggest that anti-DMK-2 specifically reacts with authentic murine and human DMK isoforms of 74 (DMKα) and 82 kDa (DMKβ). Whether the previously described 54 kDa reactive protein represents a product from an alternatively spliced variant of DMK mRNA or a processed isoform is unclear. However, DMK anti-peptide-derived antibodies (van der Ven et al., 1993), show the presence of the 54 kDa DMK protein in $DMK^{-/-}$ mutant mice, whereas the DMKα and β isoforms detected by anti-DMK-2 are absent (Be Wieringa, Nijmegan, Netherlands, personal communication). This suggests that the 54 kDa protein is not a DMK product.

Expression of mutant DMK mRNA

Previous studies have reported the absence of mutant DMK mRNA in tissues from affected individuals (Fu et al., 1993; Carango et al., 1993; Hofmann-Radvayani et al., 1993). One of the difficulties in the analysis of such mRNA is that reverse transcription PCR (RT-PCR) through the expanded $[CTG]_n$ repeat may be inefficient (Sabourin et al., 1993). In addition, because of the size (up to 20 kb) and heterogeneity of the amplified $[CTG]_n$ repeat, mutant DMK mRNAs may be more susceptible to degradation and therefore difficult to quantitate. In order to minimize the degradation problem we used freshly isolated muscle tissue from a congenital DM patient as a source of total RNA for northern blot analysis. The congenital patient suffered neonatal

respiratory distress, severe hypotonia and died of respiratory failure at 1 month of age. Prior to RNA analysis, the expansion status of the muscle tissue was examined. Southern blotting experiments of muscle DNA showed a $[CTG]_n$ repeat size of approximately 8 to 12 kb, predicting a mutant DMK mRNA ranging in size from 11 to 15 kb (Fig 4-2a). In contrast to previous reports in which mutant DMK transcripts were not detected, enlarged transcripts of approximately 10 kb were readily detectable in the congenital muscle sample (Fig 4-2b). Significantly, probes specific to intron 1 and intron 9 of the DMK gene did not show any hybridization signal (data not shown), suggesting that the enlarged transcript does not represent a heteronuclear RNA. Furthermore, the dosage of the 3.3 kb wild type message (50% of control) was not affected, a result also contrary to what has been previously reported (Hofmann-Radvayani et al., 1993; Radvayani et al., 1994; Wang et al., 1995). The 10 kb transcript is likely to represent a major mutant species among a heterogeneous population of DMK mRNAs. Consistent with these results, we have reported the detection of mutant DMK transcripts in a congenital DM fibroblast cell line (Sabourin et al., 1993). Furthermore, others have shown enlarged transcripts in tissue samples from adult and congenital patients (Roses et al., 1992). These combined data show that DMK mRNAs are transcribed from mutant DMK genes bearing large repeats and that the failure to detect them in affected tissue samples may be due to RNA degradation.

A

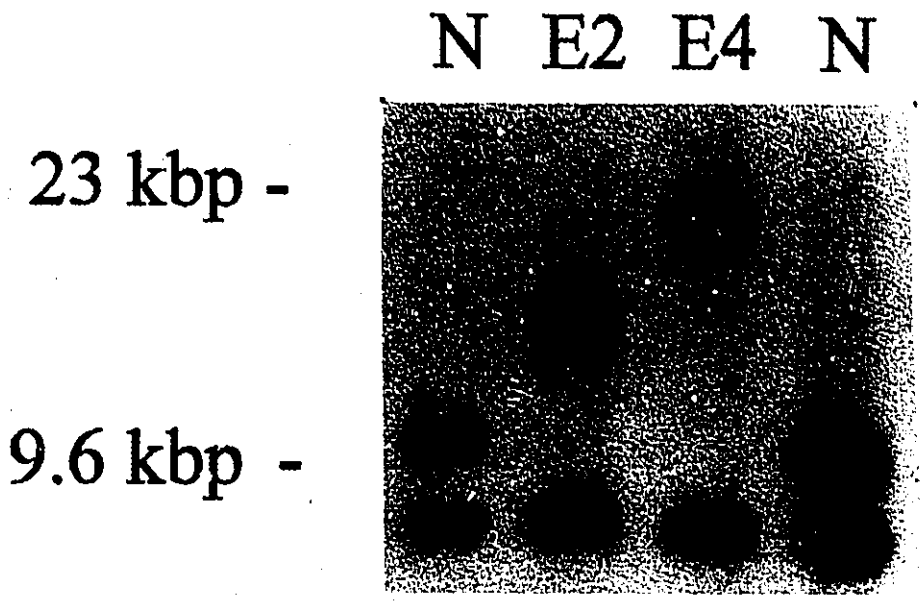
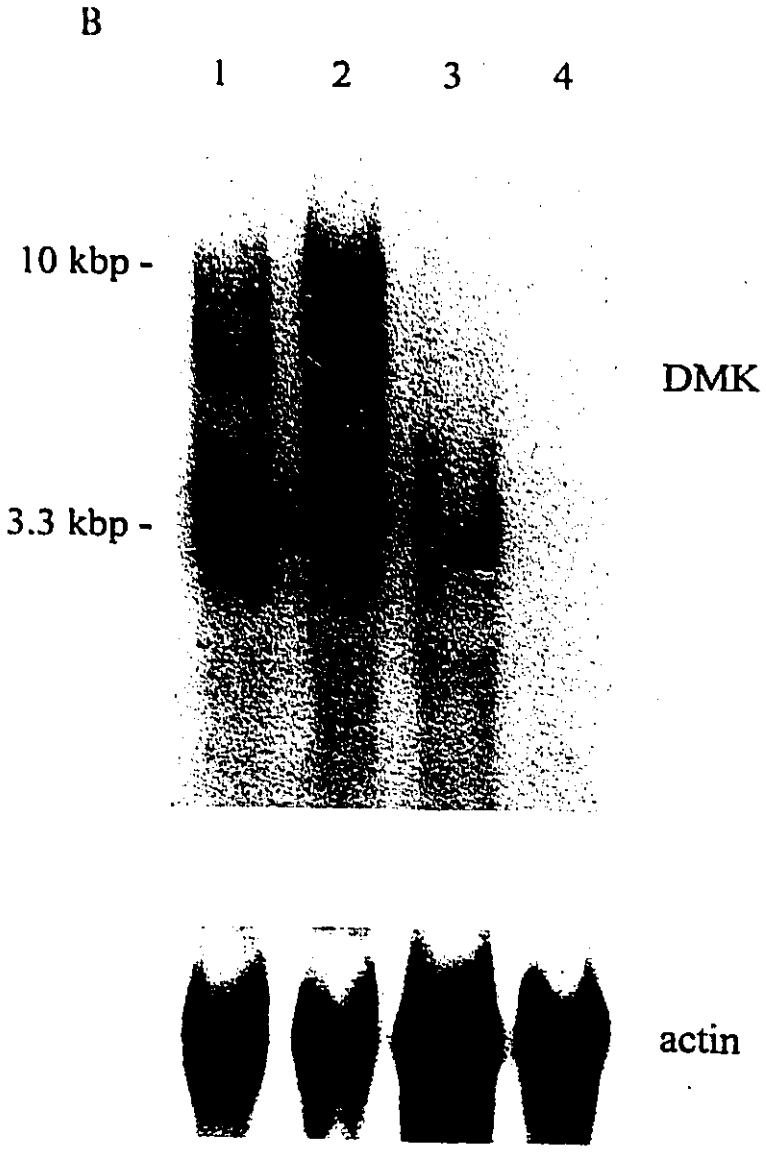


Fig. 4-2. Expression of mutant DMK mRNA in congenital muscle tissue. a, Southern blot analysis of EcoRI-restricted muscle genomic DNA (5μg) for determination of expansion size as described previously (Tsilfidis et al., 1992). N, normal controls; E4, congenital DM muscle sample. A sample (E2) known to have an expansion of about 1000 CTG repeats was included for comparison. Molecular weight markers are indicated in kilobasepairs (kbp). b, Northern blot analysis of total RNA (10μg) extracted from freshly sampled muscle tissue from the congenital DM patient described in figure 2a (lane E4); Lane 1, normal muscle tissue; lane 2, congenital DM muscle. For comparison an RNA sample from a human rhabdomyosarcoma cell line (TE32) and murine fibroblast (NIH 3T3) was included: lanes 3 and 4, respectively. The filter was probed with a 531 basepair Pst I fragment derived from a DMK cDNA. Control hybridization was performed using a β-actin cDNA probe.


B
1
2
3
4
10 kbp -
3.3 kbp -
DMK
actin

Expression of DMK protein in affected muscle tissue and myoblast cell lines

Having established that the mutant gene is transcribed, we sought to analyse the effect of the repeat on DMK protein expression and distribution in affected tissues. Several DM patients were surveyed. One patient was a 28 week old fetus obtained from a therapeutic abortion. The adult DM patient was a 56 year old female with myotonia and muscle weakness who died of heart failure. The congenital DM patient is as described above. The adult normal control was a 70 year old male who died of gastrointestinal perforation. As a control for the congenital DM samples, tissues from a 3 week old female, who died of pulmonary collapse, were used. Following Southern blot analysis of tissue DNA for determination of expansion size (Fig 4-3a), skeletal and cardiac muscle total proteins were prepared and analysed for DMK levels by western blotting. Using an anti-α-skeletal actin antibody for normalization, the total levels (α and β isoforms) of DMK protein were determined by densitometric scanning. As shown in figure 4-3b, DMK-specific bands were detected at 74 and 82 kDa. One cross-reactive species is consistently observed at about 46 kDa with anti-DMK-1. This reactive band is only detected when using anti-DMK-1 and can also be observed in non-expressing tissues or cell lines (data not shown). Finally, a band of approximately 22 kDa was observed in some samples and is likely to represent a proteolytic degradation product of DMK. Supporting this is the fact that degradation was also observed for skeletal actin in the same samples (Fig 4-3b). Following quantitation, no significant differences

A

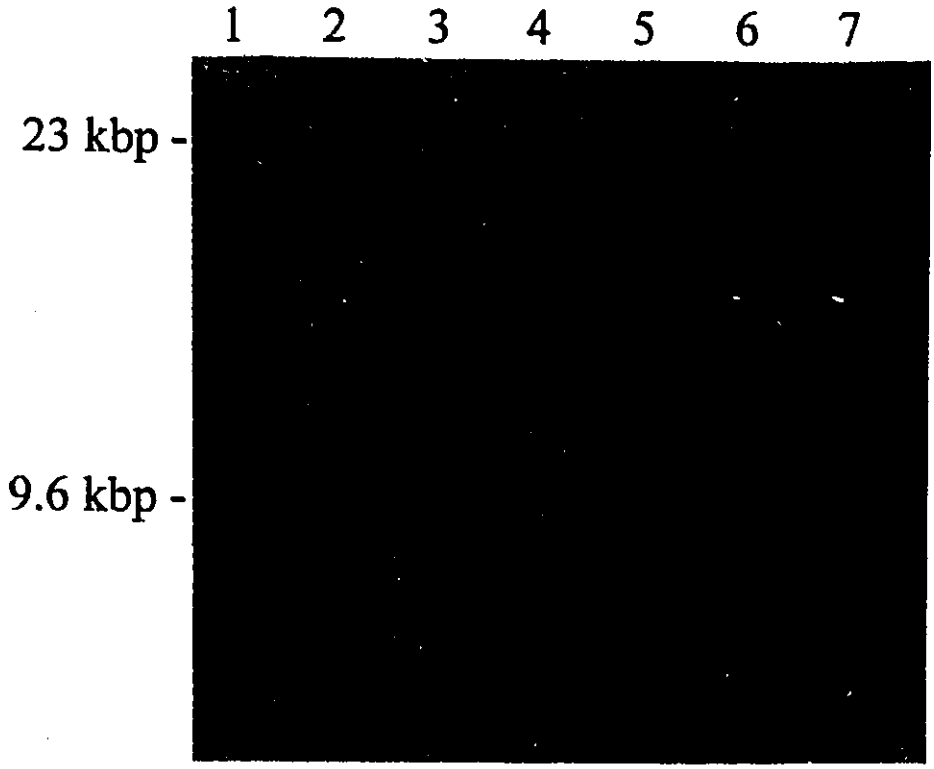
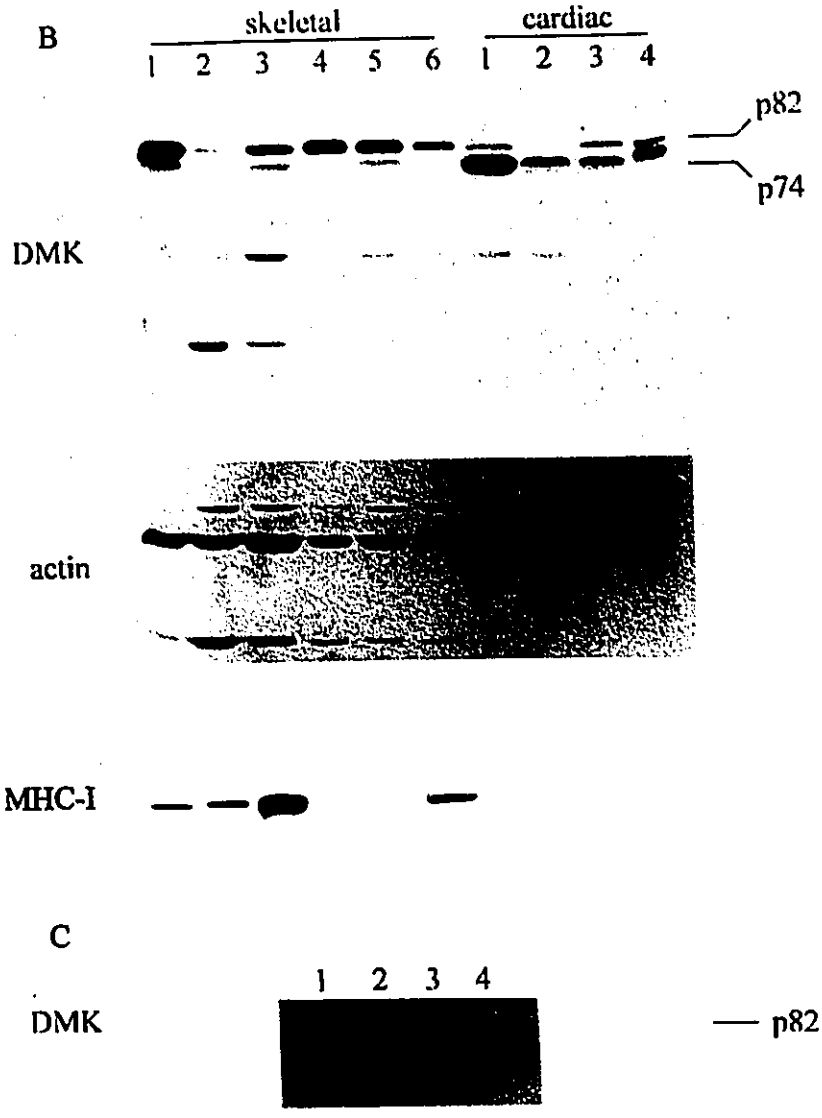


Fig. 4-3. Expression of DMK protein isoforms in affected adult and congenital DM tissues. a) Southern blot analysis of DNA (5μg) for determination of amplification size. DNA was digested with EcoRI prior to fractionation and blotting. Lane 2, normal myoblasts; lanes 4 and 6, lymphocyte DNA from two normal controls; lane 1, congenital DM-1 primary myoblast culture; lane 3, congenital DM-2 primary myoblast culture; lane 5, skeletal muscle from an E2 fetus; and lane 7, skeletal muscle from an adult DM. b) Western blot analysis of muscle samples from various DM individuals. The skeletal muscle samples analysed were: lane 1, E2 fetal muscle; lane 2, adult DM; lane 3, normal adult; lane 4, normal neonate (pectoralis); lane 5, congenital DM (pectoralis); and lane 6, congenital DM (diaphragm). The cardiac samples were: lane 1, normal adult; lane 2, adult DM; lane 3, normal neonate; and lane 4, congenital DM. The congenital DM samples were obtained from the individuals described in figure 3a. The membrane was probed with anti-DMK-1 and detection was performed as described. The same filter was stripped and incubated with an anti-α-skeletal actin antibody (Sigma), that reacts to both skeletal and cardiac isoforms, for normalization. For type I fiber normalization, equivalent amounts (as for figure 3b) of skeletal muscle total protein extracts were analysed for MyHC-I expression using a monoclonal antibody (4A9) to the MyHC type I isoform. c) Western blot analysis of normal and DM primary myoblast culture extracts. Primary myoblasts cultures from various DM patients were established and equivalent amounts of protein extracts (20μg) were subjected to western blotting. Equivalent protein loading was confirmed by coomassie blue staining of a replicate gel (not shown). Lane 1, normal myoblasts; lane 2, E2 fetal myoblasts; lane 3, congenital DM-1 myoblasts; and lane 4, congenital DM-2 myoblasts. Corresponding samples that were used for both DNA and protein analysis are as follows: E2 fetus, adult DM, normal adult, congenital DM-1 and congenital DM-2. Probing and detection was as in (b).


B
skeletal
cardiac
1 2 3 4 5 6 1 2 3 4
p82
p74
DMK
actin
MHC-I
C
1 2 3 4
DMK
p82

were observed, between a neonate control, an E2 fetus and congenitally affected skeletal tissues in the levels of both DMK isoforms (Fig 4-3b and Table 4-1). Interestingly, there appears to be a marked reduction of DMK levels in the normal neonate diaphragm (about 25% of normal pectoralis muscle control, when normalized to skeletal actin), suggesting significant tissue specific differences in DMK expression. Following α-skeletal actin normalization, total DMK protein levels in the adult DM muscle sample were found to be approximately 40% that of the normal adult control (Table 4-1). Quantitation is more difficult in this case because of possible proteolytic degradation of DMK as evidenced by the presence of the 22 kDa reactive species (Fig 4-3a). However, when this reactive species is included in the analysis, no significant differences were observed between the normal and DM adult patient (Table 4-1).

DM has been demonstrated to be associated with a paucity and a marked atrophy of type I myofibers (slow twitch fibers; Harper, 1989), suggesting that DMK may be expressed predominantly in slow fibers. Supporting this, immunofluorescence studies have shown that, in addition to neuromuscular junctions, DMK preferentially stains type I myofibers (H. Epstein, Houston, Texas, personal communication).

Taken together, these observations suggest that the observed reduced expression of DMK in adult skeletal muscle may be due to a reduced number of slow fibers. In order to evaluate slow fiber content, all skeletal muscle samples were analysed for the myosin heavy chain type I isoform (MHC-I). As shown in figure 4-3c, the levels of MHC-I isoform in the adult DM skeletal muscle sample were dramatically reduced

Table 4-1.

aDM-M/N-M	aDM-C/N-C	E2/neo-M	cDM-M/neo-M	cDM-C/neo-C
1.2/0.4*	0.6	1.0	1.4	1.0

Table 4-1. Relative DMK content in DM patients after α-skeletal actin normalization. Densitometric readings for DMK were normalized to those for α-actin and the DMK ratio of DM patients to the appropriate normal control was calculated using normalized DMK values. The results do not show any significant differences in the levels of DMK in a congenital DM patient or an E2 DM fetus. A reduction in DMK levels was observed in cardiac tissues from an adult DM case. Similar results were obtained in two independent experiments. The data obtained from one such experiment are tabulated above. DM-M and aDM-C designate adult DM skeletal muscle and cardiac muscle, respectively; N-M and N-C represent normal adult skeletal muscle and cardiac muscle, respectively. E2, fetal muscle; cDM-M and cDM-C correspond to congenital DM skeletal muscle and cardiac muscle, respectively; neo-M and neo-C are normal neonate skeletal muscle and cardiac muscle, respectively. The asterisk (*) corresponds to the obtained ratio when the readings for the degradation product were not included in the calculation.

(about 6-fold) compared to the normal adult control, suggesting a marked reduction in the number of type I fibers in the DM sample. More importantly, this suggests that the observed decrease in DMK protein levels is not due to under-expression of DMK but rather to a reduced slow fiber content. Furthermore, when the observed DMK levels in both adult samples were normalized to MHC-I content, the ratio of DMK levels between the adult DM sample and the normal control was found to be greater than 1.0, suggesting elevated DMK levels in the affected individual (Table 4-2).

Consistent with previous reports, no MHC-I was detected in both the neonatal and congenital DM skeletal muscles, which are still expressing the neonatal isoform of slow MHC (Hughes et al., 1993). In contrast, normal neonatal diaphragm tissue expressed adult MHC-I, consistent with muscle type-specific differential expression of slow MHC isoforms (Brozanski et al., 1993; Soussi-Yanicostas et al., 1990). However, the E2 fetal limb muscle sample expressed a low levels of MHC-I, again presumably reflecting the complex spatial and temporal expression pattern of MHC-I (Sutherland et al., 1991).

For cardiac muscles, neonatal tissues showed no difference between affected and normals (Fig 4-3a and Table 4-1). However a 40% reduction in total DMK levels as observed in an adult DM heart sample. Importantly, this individual died of heart failure and therefore this difference in DMK levels may be due to a secondary consequence of extensive tissue damage, although this does not exclude the possibility that DMK protein levels may be reduced in cardiac tissue of adult onset cases of DM. In

Table 4-2.

aDM-DMK[1]/aDM-MHC	N-DMK[1]/N-MHC	aDM-M[2]/N-M
4.7/1.3*	0.7/0.5*	5.8/2.0*

Table 4-2. Relative DMK levels in a DM adult patient after normalization to MHC type I. [1]DMK readings were normalized to α-skeletal actin and the ratio of DMK to MHC-I was calculated for the DM and normal samples. These values show an increase DMK content in the DM sample relative to the adult control. [2]Similar results were obtained when the DMK levels were normalized to MHC-I content prior to calculating the DM to normal ratio for DMK. aDM-DMK and aDM-MHC represent adult DM DMK and MHC-I content, respectively; N-DMK and N-MHC designate normal adult DMK and MHC-I content, respectively; aDM-M, adult DM skeletal muscle; N-M, normal adult skeletal muscle. The asterisk (*) corresponds to the obtained ratio when the readings for the degradation product were not included in the calculation.

conclusion, therefore, the current study demonstrates that DMK levels corresponding to the 74kDa and 82kDa isoforms in affected individuals are not significantly altered compared to controls, in contrast to previous reports on the quantitation of a 54 kDa protein (Fu et al., 1993; Koga et al., 1994).

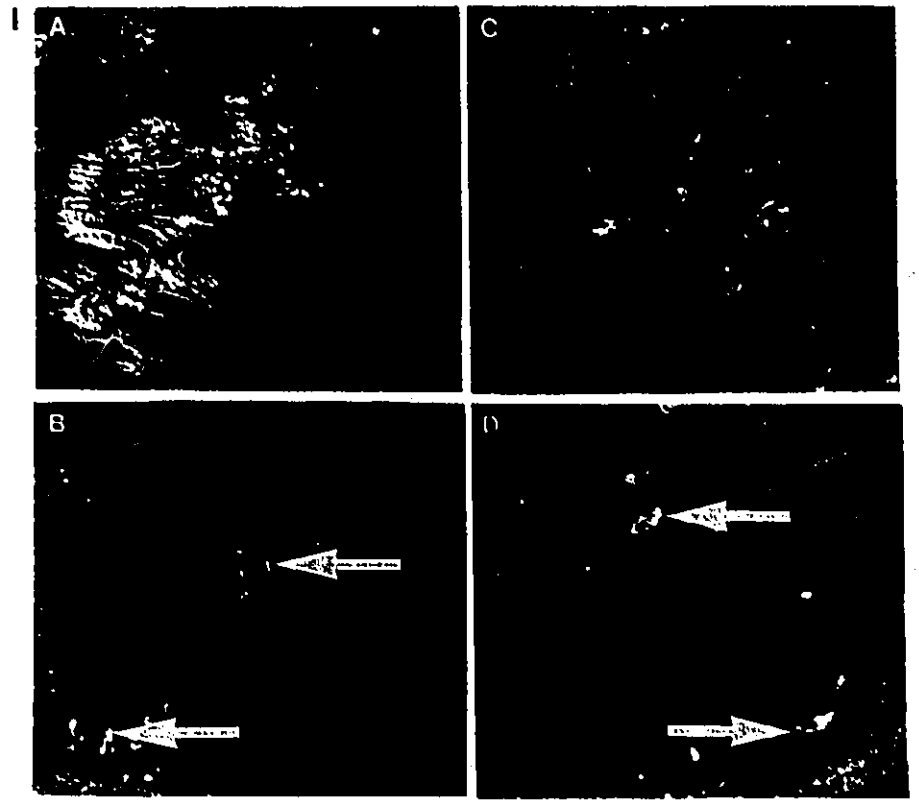
In order to further evaluate DMK protein levels in affected individuals, primary myoblast cell lines were established from limb muscle of an affected fetus (described above) carrying an E2 expansion (corresponding to approximately 1000 CTG repeats) and two 15 week old congenital DM fetuses (with a repeat size of approximately 3000-5000 CTG repeats, Fig 4-3a). Control myoblasts were purified from a needle biopsy performed on a 13 month old unaffected individual. The purity of the myoblast cultures was determined by immunofluorescence staining for N-CAM and desmin and found to be greater than 98%. For DMK protein analysis, myoblast extracts were quantitated for total proteins and 20μg was subjected to western blot analysis. Although significantly lower than normal skeletal muscle tissue, the levels of DMK in DM myoblasts were not altered in comparison to control (Fig. 4-3c). Interestingly, higher levels (approximately 4-fold) were observed in one of the two congenital myoblast samples bearing similar expansions. One explanation for this difference may be that each myoblast line was established at various stages of muscle differentiation reflecting differential DMK expression during muscle development. Alternatively, one sample may be enriched in a particular myoblast subset (Miller, 1991) in which DMK is expressed at higher levels. Nevertheless, these data imply that, regardless of the expansion status, DM muscle-

derived satellite cells express relatively normal levels of DMK protein.

Distribution of DMK in affected tissues

To determine whether or not the normal distribution of DMK would be altered in tissues derived from DM individuals, we performed immunofluorescence staining of frozen tissue sections. Normal adult cardiac muscle sections were from a 70 year old male (described above). In addition, heart sections were prepared from a 26 year old DM affected male who had pronounced myotonia and muscle weakness. For skeletal muscle analysis, tissue sections were obtained from a normal 3 week old female and a 28 week old DM affected fetus (both described above).

Normal and DM muscle sections showed brightly stained neuromuscular junctions with no obvious differences (Fig 4-4 panel I). Specific staining of neuromuscular junctions by anti-DMK antibodies has been previously reported in human and rodent skeletal muscle (van der Ven et al., 1993; Whiting et al., 1995). Also as previously shown (Whiting et al., 1995), staining of cardiac muscle sections revealed DMK-specific reactivity to the intercalated disks. Again, no major differences were found for the DM sample in comparison to the normal (Fig.4-4 panel II). To correct for autofluorescence due to lipofusion, adjacent sections were processed with secondary antibody without prior incubation with anti-DMK-1 (Fig.4-4 panel II-B and D). Our immunofluorescence analysis shows that relative DMK levels and distribution in


I
A
C
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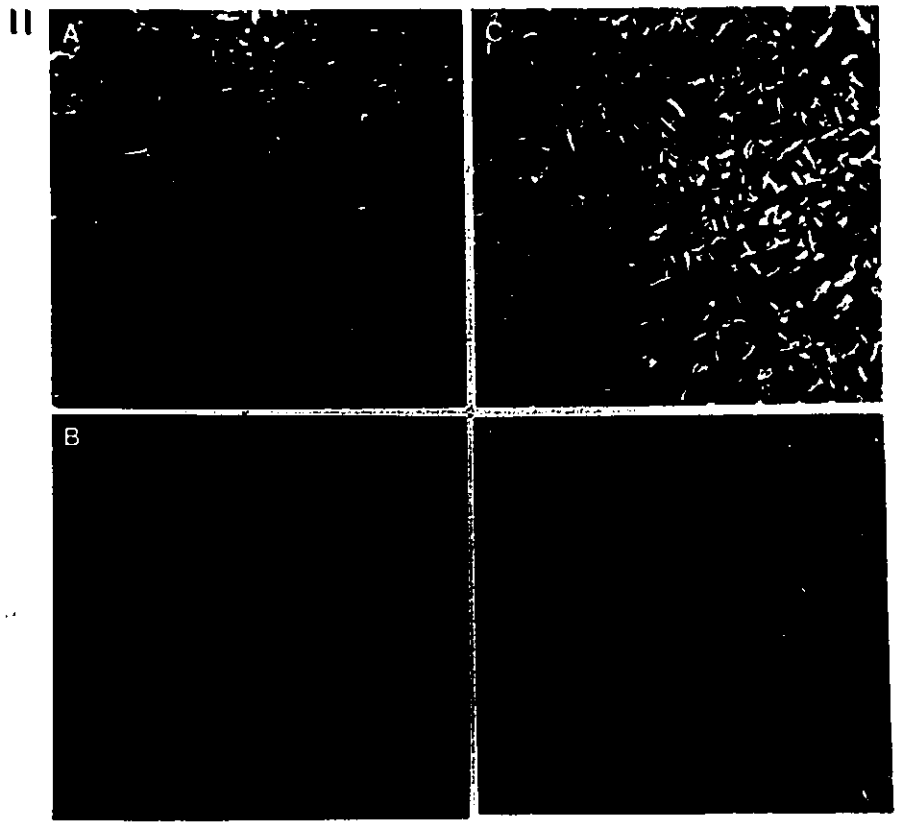
D


II
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C
B

Fig. 4-4. Immunolocalization of DMK in affected skeletal and cardiac tissues. Frozen sections from sampled tissues were stained with anti-DMK-1. Panel I, A and B, normal neonatal muscle sections; C and D, E2-DM fetal muscle sections. Magnifications were 20x (A and C) and 100x (B and D). Specific neuromuscular junction staining is seen at higher magnification (arrows). Panel II, A and C represent an anti-DMK-1-stained DM and normal cardiac muscle section, respectively. The autofluorescence control of an adjacent section is shown in B and D. More extensive autofluorescence is seen in the sample from the older individual (D) due to an age-related increase in lipofusion compared to that observed in the DM sample (B). DMK was shown to localize to intercalated disks and no differences was observed between affected and normal.

skeletal and cardiac muscle samples are not significantly altered in affected individuals.

4. Discussion

We have generated anti-DMK antibodies that react to human and murine DMK. The observations that the 74 and 82 kDa proteins are only found to be expressed in DMK transgenic organs such as brain (where it is normally undetectable) and that DMK "knock-out" mice do not express these isoforms, (Be Wieringa, personal communication), suggest that anti-DMK-1 and 2 react with authentic DMK. Our results clearly demonstrate that anti-DMK-1 and anti-DMK-2 react with major DMK species of 74kDa (DMKα) and 82 kDa (DMKβ) in both skeletal and cadiac tissues. Using different antibodies, previous reports have demonstrated reactivity to the 74 kDa but not to the 82 kDa isoform, suggesting that their antibody reacts more specifically to DMKα (Timchenko et al., 1995; Sasagawa et al., 1994).

The relative tissue levels of DMK protein observed in this study were found to correlate with previously reported DMK mRNA levels (Jansen et al., 1992b). Furthermore, DMK levels observed in the murine myoblast cell line C2C12 were found to be markedly higher than in the mesodermal precursor fibroblast line C3H10T1/2, suggesting that DMK is upregulated during myoblast conversion of mesodermal stem cells. Supporting this, P19 stem cells converted to myoblast-like cells by over-expression of MyoD1, express DMK (Skerjanc et al., 1994). Further studies on

myoblast differentiation and determination will help to delineate the role of DMK during myogenesis, particularly in the light of the observations that muscle cross-sections from congenital patients show an abundance of satellite cells, suggesting a delay of muscle maturation in these individuals (Farkas-Bargeton et al., 1988; Soussi-Yanicostas et al., 1991; Sarnat and Silbert, 1976).

Previous studies have failed to conclusively demonstrate the existence of mutant DMK transcripts. In addition, it has been demonstrated that the $[CTG]_n$ repeat acts as a strong nucleosome positioning element, suggesting that it may downregulate transcription (Wang et al., 1994; Wang et al., 1995; Otten and Tapscott, 1995). However, our Northern analysis of freshly isolated RNA from congenital muscle tissues bearing large $[CTG]_n$ expansions revealed the presence of a major species of mutant DMK mRNA. These results suggest that the DM mutation does not repress DMK gene expression as was demonstrated for the FMR-1 gene mutation (Pieretti et al., 1991; Sutcliffe et al., 1992). Furthermore, in contrast to previous observations (Hofmann-Radvayani et al., 1993; Radvayani et al., 1994; Koga et al., 1994; Wang et al., 1995) the levels of the wild type DMK mRNA were not altered in this congenital DM individual.

A common feature of adult myotonic dystrophy is the observation that muscle cross-sections show a paucity and atrophy of type I myofibers (Harper, 1989). Interestingly, DMK has been shown to be expressed preferentially in the cytoplasm of type I fibers (H.Epstein, Houston, Texas, personal communication).

Taken together, these observations suggest that the failure to detect mutant transcripts in RNA prepared from affected individuals may be due mainly to a significantly reduced number of type I fibers and/or the preferential degradation of high molecular weight RNA. Similarly, the recent findings that the levels of both the wild type and mutant DMK mRNAs are decreased in poly-A$^+$ preparations from DM muscles (Wang et al., 1995) may not be due to a disease-specific effect but rather to a specific reduction in type I fiber content. Supporting this is the fact that we observed a marked reduction in slow MHC-I isoform expression in adult DM patients (Fig 4-3b). Therefore, as cautioned by Roses (Roses, 1994), care should be taken in the interpretation of data obtained from the analysis of tissues because appropriately matched controls with respect to fiber type distribution, fatty infiltration and tissue disruption are difficult, if not impossible, to obtain. Alternatively, quantitative PCR of DMK mRNA through a polymorphic region (Sabourin et al., 1993), other than the repeat, could be performed on single fibers.

A slight variation in absolute DMK protein levels in affected tissues, which may not be readily detectable by western blot analysis, could be responsible for the DM phenotype. However, our data show that a significant alteration of DMK protein levels and distribution are not observed in muscles of a fetal, congenital and adult DM patient and three primary myoblast cell lines derived from various affected individuals. These results are in contrast to previous studies which have shown decreased levels of a 54 kDa cross-reactive protein in DM skeletal and cardiac muscle (Fu et al., 1993; Koga et

al., 1994) but as discussed above, this protein is not likely a product of the DM gene.

Therefore, a change in DMK protein level distribution or expression pattern may not constitute the molecular basis of DM. However, an alternate hypothesis is that genes surrounding the DM locus are affected by the amplified $[CTG]_n$ repeat (Wieringa, 1994). One such gene, DMR-N9, has been reported to lie within a few hundred base pairs upstream of the transcriptional start site of the DMK gene (Mahadevan et al., 1993b; Jansen et al., 1992b). However, expression studies looking at the effect of the DM mutation on the expression of the DMR-N9 gene have yet to be reported.

Although DMK protein levels may not be changed , the CTG expansion could still alter DMK mRNA levels. Consistent with this interpretation, we have previously shown that the amount of the mutant DMK mRNA was significantly increased in various tissues of a congenital DM patient (Sabourin et al., 1993). However, as shown by the present study, the total levels of DMK protein in affected individuals do not differ from the normal control, suggesting that the expanded mRNA is somehow inefficiently processed or translated. Recently, *in situ* hybridization studies have shown that aberrant post-transcriptional processing appears to be associated with mutant DMK transcripts (Taneja et al., 1995). Specifically, foci of mutant DMK transcripts were observed in the nuclei of DM fibroblasts and myofibers. These results suggest that mutant DMK messages may be inefficiently processed, that is, primary transcripts are not properly spliced and/or the mature messages are not transported to the cytoplasm. Hence, although DMK mRNA levels may be increased (as we previously reported;

Sabourin et al., 1993), total DMK protein levels remain relatively unchanged in the disease state, a prediction that is consistent with the findings of the present study.

The expanded repeat, nevertheless, may titrate away nuclear factors required for specific mRNA metabolism functions such as the control of mRNA stability or transport. Therefore, the dominant inheritance pattern of DM may be mediated, *in trans*, by the mutant mRNA itself and may not involve the DMK product. Studies concerned with RNA processing and transport of DMK mRNAs carrying expanded repeats will help to delineate if such a mechanism underlies or contribute to the pathogenesis of myotonic dystrophy.

Chapter V: Over-expression of the myotonic dystrophy kinase (DMK) 3' untranslated region inhibits myoblast terminal differentiation *in vitro*

1.Introduction

Myotonic dystrophy (DM), the most common form of inherited neuromuscular disease in adults, affects 1 in 8000 individuals globally. DM is an autosomal dominant and multisystemic disorder characterized mainly by myotonia and progressive muscle weakness and wasting (Harper, 1989). Affected individuals present with a highly variable phenotype, ranging from an assymptomatic condition to a severe and frequently fatal congenital form. Congenital DM patients display marked hypotonia, neonatal respiratory distress and severe mental retardation. Significantly, histochemical studies of muscle cross sections from these patients has demonstrated the presence of immature myofibers and an abundance of satellite cells. Furthermore, protein profile analyses have shown an altered content of contractile protein in these muscles, suggesting that the congenital form of DM is associated with a delay or an arrest of muscle maturation (Sarnat and Silbert, 1976, Farkas-Bargeton et al., 1988 Soussi-Yanicostas et al., 1991,).

In research concerning the expression of the DMK gene, controversial observations have been reported on the effect of CTG amplification. Using different assays, over-expression (Sabourin et al., 1993) and reduced expression of DMK (Fu et al., 1993; Carango et al., 1993; Hofmann-Radvayani et al., 1993) have been observed

in muscle tissues from affected individuals. More recently, expression studies using RT-PCR have shown that the total activity of the DM locus was unaffected in DM patients but that the levels of processed mutant transcripts were decreased (Krahe et al., 1995). In another analysis, Wang et al. (1995), have shown that the levels of DMK mRNAs were not significantly altered in total RNA preparations, whereas they were markedly reduced in poly-A$^+$ samples. Surprisingly, this decrease was observed for both the wild type and mutant DMK transcript. These authors have proposed that the expanded CTG repeat may act as a dominant-negative mutation that affects RNA processing in *trans*. However, using a polyclonal antibody that we have generated and have shown to react specifically to DMK isoforms, we have demonstrated that the levels and distribution of DMK protein are not significantly altered in congenital DM patients (Sabourin et al., submitted; see chapter IV), in contrast to other studies (Fu et al.,1993; Koga et al., 1994). These observations suggest that the observed reduction in DMK expression in DM patients does not lead to a decrease in DMK protein, supporting the hypothesis that the dominant inheritance pattern of DM may not be due to changes in DMK protein levels or activity but rather to be the result of a *cis* or *trans* dominant effect of the amplified repeat in the 3'UTR.

Numerous studies have defined major roles for mRNA 3' UTRs. Specific motifs found in the 3' UTR of several classes of transcripts have been shown to mediate post-transcriptional mRNA stability or have been demonstrated to be involved in the control of translation (reviewed in Belasco and Brawermann, 1993). Recently, novel functions

in the control of growth and diferentiation have been assigned to the 3' UTR of differentiation-specific mRNAs such as α-skeletal actin and tropomyosin. The over-expression of these UTRs have been shown, in a genetic complementation system, to rescue the mutant phenotype of differentiation defective myogenic mutant cell lines and to suppress their tumorigenicity (Rastinejad and Blau, 1993; Rastinejad et al., 1993). They have been proposed to act as *trans*-acting regulators in a feedback loop by promoting differentiation. Taken together, these results, and the developmental delay observed in muscle tissues from congenital DM cases, raise the interesting possibility that the DM phenotype may be mediated by the mutant 3' UTR. Supporting this is the recent observation that mutant DMK transcripts have been shown to accumulate in the nuclei of fibroblasts or muscle cells isolated from DM patients, suggesting that the expanded repeat may alter the normal processing or export of the mutant mRNA (Taneja et al., 1995).

We have previously reported that CTG expansion in the 3' UTR of the DM gene increases mutant DMK mRNA steady state levels in muscle tissues isolated from congenital DM patients (Sabourin et al., 1993; see chapter III). Combined with the recent findings that CTG repeat expansion may alter mRNA metabolism, these observations suggest that the expanding repeat may lead to an accumulation, or increased steady state levels, of the mutant message. Consequently, this may affect the normal processing of the DMK message and possibly of other transcripts.

In order to gain insight into the role of DMK during myogenesis and the

mechanism underlying DM, we decided to characterize DMK expression during the terminal differentiation of C2C12 myoblasts and to investigate the effect of DMK over-expression on myogenesis. Our results show that DMK is expressed in various myoblast cell lines and is slightly up-regulated during skeletal myogenesis *in vitro*. We report that DMK over-expression markedly inhibits myogenesis, a result consistent with previously reported findings of delay in muscle development in congenital DM. Remarkably, we demonstrate that this activity maps to the 3' UTR of the DMK mRNA as it does not appear to involve the DMK protein. In addition, the activity could also be conferred to a heterologous gene, suggesting that over-expression of the 3' UTR is sufficient to mediate the observed phenotype. These results suggest that over-expression of the 3' UTR may function in a feedback loop and may interfere with the expression or metabolism of muscle-specific mRNAs required for muscle terminal differentiation.

2. Methodology

Plasmid constructions.

DMK expression vectors consisted of full length or partial DMK cDNAs cloned into pcDNA3 (Invitrogen) using standard cloning protocols (Sambrook et al., 1989). Briefly, pCMV-DMK comprises a full length DMK cDNA bearing both 5' and 3' UTRs in pcDNA3. pDΔ35 was generated by cloning a PCR-amplified fragment corresponding to the coding portion of the full length DMK cDNA. pDΔ5 was generated by replacing

the 5' KpnI-XhoI portion of pCMV-DMK by the corresponding segment of pDΔ35. Plasmid pK100R was obtained by *in vitro* mutagenesis of pCMV-DMK using the Mutagene II kit (Bio-Rad) according to the manufacturer's instructions. A point mutation was introduced at amino acid residue 100, converting the conserved lysine residue of the ATP-binding site to an arginine. To construct HT-3' and HT-3'R, a 1.0 kb BamHI fragment encompassing the DMK 3' UTR was cloned in both orientation, into the BglII site downstream of the hygromycin-tk hybrid gene encoded by ptgCMV/Hy-tk (Lupton et al., 1991). The control plasmid HT-PGK was generated by cloning a 1.0 kb XhoI-HindIII fragment, comprising the mouse PGK 3' UTR, into the SalI-HindIII sites of ptgCMV/Hy-tk. In order to generate pHTΔR, pHT-3' was digested using PstI and SacII, the ends were filled in and the resulting vector was religated. pHTΔ5' and pHTΔ3' were constructed by cloning PCR products corresponding to sequences 3' and 5' to the CTG repeat region, respectively, dowstream of the Hy-tk hybrid gene (see figure 5-12).

Cell culture, Transfections and RNA analysis.

C3H10T1/2, C2C12 and NIH 3T3 cells were purchased from the American tissue type culture collection (ATCC). C3H10T1/2 and NIH 3T3 fibroblasts were maintained in α-MEM supplemented with 10% fetal bovine serum (FBS) and 2mM glutamine. C2C12 myoblasts were grown in α-MEM containing 15% FBS and glutamine. All cultures were maintained under 70-80% confluency and grown at 37°C in a humidified atmosphere containing 5% CO_2. For stable transfections, C2C12 or NIH 3T3 cells were

plated at 5 x 10^5 in 100 mm dishes 24 hours prior to transfections. The cells were transfected by standard calcium phosphate precipitation (Sambrook, 1989) for 8 hours and then refed with fresh growth medium. Following a 24 hour incubation, G418 (Geneticin; Gibco/BRL) or hygromycin B (Sigma) was added at final concentrations of 0.4mg/ml or 0.2mg/ml, respectively. After a 14-day incubation, resistant clones (or pools) were isolated using cloning rings and grown for RNA, protein and fusion analyses. For Northern blotting experiments, total RNA was extracted from confluent cultures of each individual clone and analysed as previously described (Sabourin et al., 1993). For analysis of DMK expression during differentiation, C2C12 cultures were grown to 80% confluency, then transferred to differentiation medium (α-MEM containing 10% horse serum) and RNA and proteins were analysed for DMK at various times as indicated. For myogenic conversion of 10T1/2 fibroblasts, 2 x 10^4 cells were plated in 35 mm dishes and grown for a further 24 hours. The cultures were then treated with 3 μM 5-aza-cytidine (Sigma) for 24 hours. The cells were then refed with fresh medium (day 0) and DMK expression was analysed by western blotting at different time points.

Transient transfections and CAT assays.

For transient transfections, C3H10T1/2 fibroblasts were plated at 2 x 10^5 in 60 mm dishes and transfected with plasmid DNA as above except that the cultures were switched to differentiation medium (α-MEM + 10% horse serum) 24 hours following

transfection. The calcium phosphate precipitate consisted of EMSV-MyoD (5 μg), the 4RtkCAT reporter construct (5 μg) and 5 μg of control vectors or expression vectors bearing the DMK 3' UTR or coding region. As a control for transfection efficiency, pSV-βGal (2 μg) was included in all transfections and following a 48 h incubation of the cultures in differentiation medium, the cells were washed with PBS and scraped in 0.25M tris (pH 7.8). The cell pellets were subjected to 3 freeze-thaw cycles and equivalent aliquots of lysates were assayed for CAT activity in a liquid scintillation based assay as described by the manufacturer (Promega). In parallel, similar portions of cell lysates were subjected to β-Galactosidase assays for normalization of transfection efficiency. All CAT activity values were standardized to β-Galactosidase activities for the same sample.

Immunofluorescence analysis of DMK.

For immunostaining, cultures of C2C12 myoblasts or myotubes in 35 mm plates were fixed with pre-chilled (-20°C) 100% methanol for 5 min and washed with standard phosphate buffered saline (PBS). Fixed cultures were incubated with a 1:500 dilution of anti-DMK-1 (Whiting et al., 1995) for 1 hour at room temperature. DMK was localized by incubation with a FITC conjugated goat anti-rabbit secondary antibody according to the manufacturer's specifications (Pierce). Stained cultures were viewed and photographed using an Olympus (Model BH2-RFCA) fluorescence microscope.

Fusion index determination

For fusion assays, C2C12 clones over-expressing the various DMK 3' UTR constructs, were plated at 4 x 10^5 cells in 35 mm dishes and incubated for an additional 16 to 20 hours. The resulting confluent cultures were then washed once with PBS and incubated for 48 hours in differentiation medium. The cultures were then washed with PBS and fixed in 100% methanol as above. Following myosin heavy chain staining using the monoclonal antibody MF20 (Bader et al., 1982) and peroxidase-conjugated anti-mouse antibody, the cultures were counterstained with Giemsa for visualization of the nuclei. In some assays, the fixed cultures were stained with Giemsa only, which was found to preferentially stain myotubes. The fusion index was calculated as the percent ratio of the number of nuclei in myotubes over the total number of nuclei in one field ((# nuclei in myotubes/total # nuclei) x 100). For each culture, at least 1500 nuclei were counted from several random fields and the fusion index was taken as the average±standard error from the mean (S.E.M) of all calculated indices for one culture. The indices for the other cultures were graphed as the average index with the standard error of the proportion. For each clone, the fusion index was determined in at least two separate experiments. The results shown are from one representative experiment.

Western blot analysis

For western blot analysis of DMK expression, confluent cultures (in 35 mm dishes) of DMK over-expressing clones, or 5-aza-cytidine-treated 10T1/2, were washed with PBS

and lysed in SDS sample loading buffer (Sambrook et al., 1989). The samples were boiled for 3 min and equivalent portions of total lysates were fractionated onto 10% SDS-PAGE gels and transfered to polyvinylidene difluoride membranes (PVDF; Immobilon-P). Electrophoresis and transfer were performed using a Bio-Rad Mini-Protean II system. The efficiency of transfer was evaluated by Ponceau S staining (0.2%) and DMK was detected as previously described (Whiting et al., 1995). Equivalent loading of protein samples was confirmed by Coomassie blue staining of replicate gels.

Proliferation assays

To measure the effect of the DMK 3' UTR on proliferation, hygromycin-resistant or control NIH 3T3 fibroblasts were plated in 96-well plates at 2500 cells/well in 100 µl. Following an overnight incubation at 37°C, 10 µl of a 5 mg/ml solution of 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT; Skidmore et al.,1975) was added to each well. The cells were incubated for an additional 4 hours and solubilized with 0.1 N HCl in isopropanol. The optical density (O.D.) of each well was then read at 570 nm in a Bio-Rad microtiter plate reader. The reading obtained from a blank with no cells was taken as background and subtracted from all other values. Each cell line was assayed in quadruplicate samples for each independent experiment. Similar readings were obtained for three independent experiments. For relative comparisons, the average reading for untransfected NIH 3T3 cells was arbitrarily set at 100±standard error from

the mean.

3.Results

Expression of DMK during myogenesis

Previous studies have demonstrated that DMK is highly expressed in smooth, skeletal and cardiac muscle tissues (Jansen et al., 1992b; Sabourin et al., 1993). This muscle specific pattern of expression and the primary involvement of skeletal muscles in DM, suggest a functional role for DMK in normal muscle function and development. To begin to investigate this role, several myogenic cell lines were grown and poly-A' RNA was prepared from undifferentiated cells. The analysed cell lines included the skeletal myoblast line C2C12 (Blau et al., 1983); BC3H1, a skeletal myoblast line defective for terminal differentiation (Taubman et al., 1989); the rat cardiac myoblast line H9C2 (Hescheler et al., 1991) and the multipotent embryocarcinoma cell line P19 (Rudnicki and McBurney, 1987). Following Northern blot analysis, DMK was found to be expressed at similar levels in the skeletal myoblast lines (Figure 5-1A). Interestingly, relative to the PGK control, DMK expression was found to be significantly higher in the cardiac myoblast cell line. In contrast, remarkably low levels of DMK were observed in the multipotent stem cell line P19, suggesting that DMK is significantly upregulated during myogenic lineage determination. Similarly, NIH 3T3 and

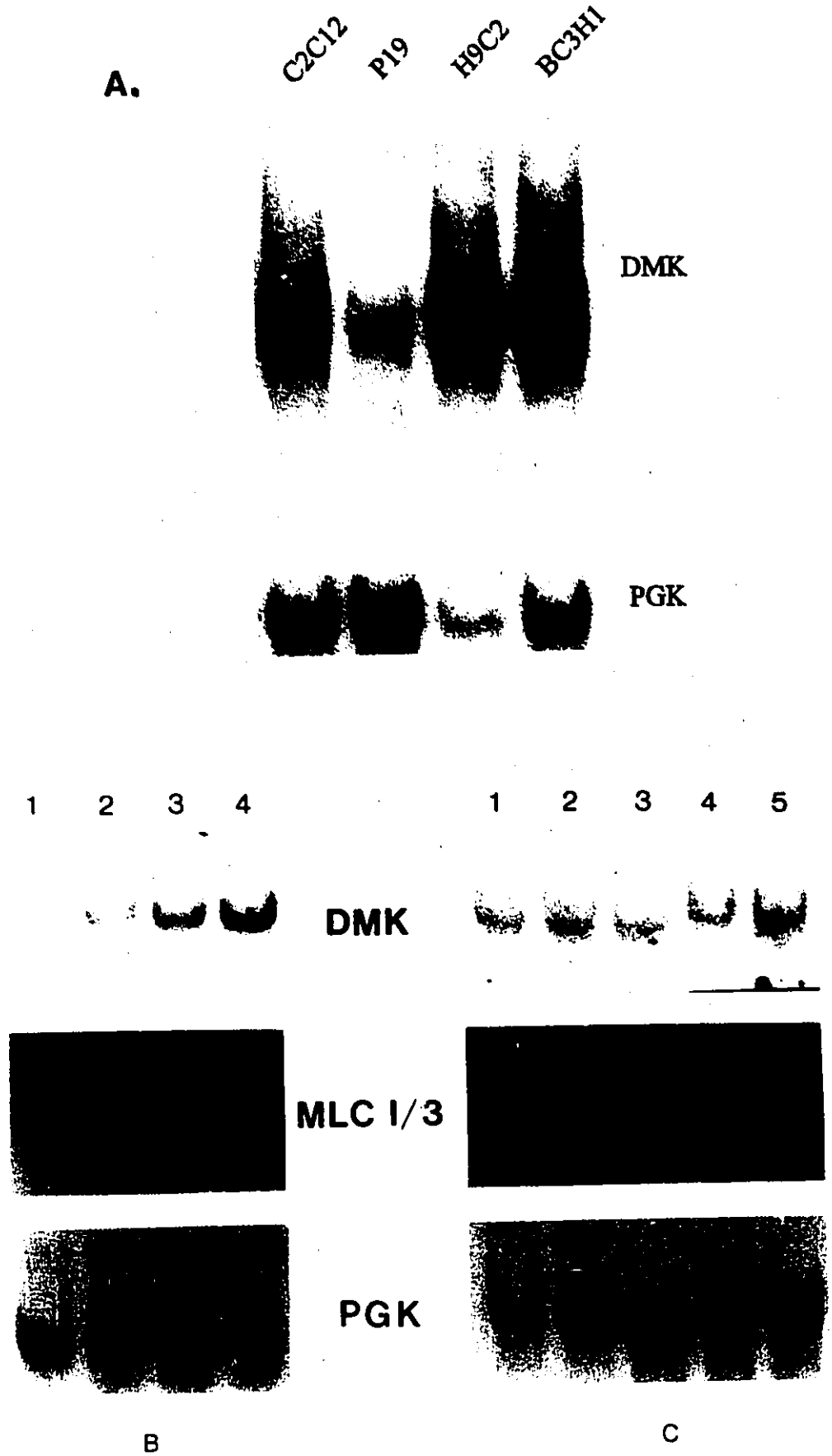

A.
C2C12
P19
H9C2
BC3H1
DMK
PGK
1 2 3 4
DMK
MLC I/3
PGK
B
1 2 3 4 5
C

Figure 5-1. Expression of DMK mRNA in various cell lines and during skeletal myogenesis.
(A) Poly-A^+ RNA form various cell lines was analysed by Northern blotting for the expression of DMK. Myogenic lines included the skeletal muscle lines C2C12 (1 μg) and BC3H1 (1 μg); the cardiac muscle line H9C2 (1 μg) and the EC cell line P19 (4 μg). DMK was found to be expressed a higher levels in all the myogenic cell lines relative to P19 stem cells. (B) Expression of DMK mRNA during differentiation of C2C12 myoblasts upon serum withdrawal. Total RNA was analysed after 0 (lane 1), 2 (lane 2), 3 (lane 3) and 4 days (lane 4) after transfer of the cultures to horse serum. (C) Expression of DMK during myogenesis of C2C12 in growth medium. The RNA samples analysed were growing myoblasts (lane 1); 1 day after plating (lane 2) and 3, 5 and 7 days after plating (lanes 3, 4 and 5, respectively). As an indicator of differentiation, a myosin light chains 1/3 probe (MLC 1/3) was re-hybridized to the filters. Equivalent RNA loading was confirmed using a mouse PGK-1 probe. Normalization to PGK-1 shows a 2-3-fold increase in the steady state levels of DMK during myogenesis *in vitro*.

C3H10T1/2 fibroblasts did not show any detectable expression of DMK (data not shown).

Skeletal myoblasts have been demonstrated to express lineage determination genes such as myf-5 or MyoD at high levels (reviewed in Olson and Klein, 1994; Weintraub et al., 1991). In marked contrast to C2C12, BC3H1 cells expresses myf-5 predominantly (Braun et al., 1989a; Davis et al., 1989). Interestingly, both skeletal myoblast cell lines expressed DMK at similar levels, implying that the control of DMK expression during myogenic cell determination may be achieved through either of the two regulators.

As a first step into the characterization of DMK expression during myogenesis, we determined DMK mRNA levels during the differentiation of C2C12 myoblasts. Cells were grown to 80% confluency and induced to differentiate in fusion promoting medium. Total RNA was isolated at various intervals and analysed for DMK expression. Northern blot analysis showed a 2 to 3-fold increase in the steady state levels of DMK transcripts following transfer of the cells to differentiation medium (Figure 5-1B). Interestingly, the major increase in DMK levels occured 72 to 96 hours following the induction of differentiation, suggesting a more predominant role for DMK in mature myotubes. Similarly, when the cells were allowed to differentiate following confluency in growth medium, DMK mRNA levels were found to increase approximately 2-fold during differentiation (Figure 5-1C). For both time courses, myoblast fusion, myosin light chains 1 and 3 (MLC 1/3) and α-skeletal actin levels

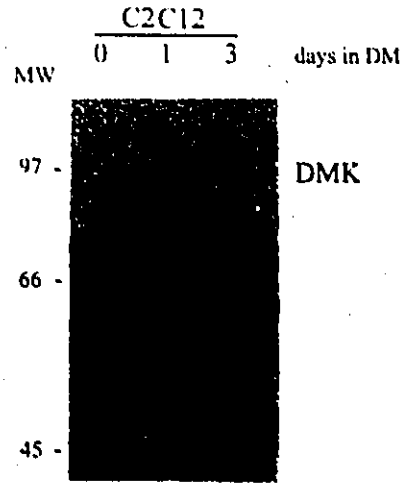
(data not shown) increased significantly, indicative of muscle cell terminal differentiation.

Having established the pattern of DMK mRNA expression in various myoblast lines and during skeletal myogenesis, we sought to examine the expression and distribution of the DMK protein in myoblasts and myotubes. For expression studies, C2C12 cells were induced to differentiate under low serum conditions. The cultures were harvested at specific times following the induction of differentiation and equivalent amounts of total protein lysate were analysed for DMK expression by western blotting using anti-DMK-1. The accumulation of the 72 kDa and 84 kDa DMK protein isoforms (Whiting et al., 1995) was found to parallel that of the mRNA during differentiation (Figure 5-2A).

Immunofluorescence analysis, using anti-DMK-1, shows a reticular pattern of staining (Figure 5-2B), similar to what has been observed for protein kinase C (PKC)-βI in fibroblasts (Mochly-Rosen, 1995). Confocal microscopy has more accurately localized DMK to the Golgi apparatus in COS-1 cells over-expressing DMK (Be Wieringa, Nijmegan, Netherlands, personal communication). Interestingly, upon differentiation and myoblast fusion, DMK appears to be redistributed uniformly in the myotubes (Figure 5-2C), suggesting that the induction of differentiation triggers its relocalization.

Our expression results with the various cell lines suggest that DMK may become upregulated during myogenic determination (see above). In order to investigate this

A.



B.

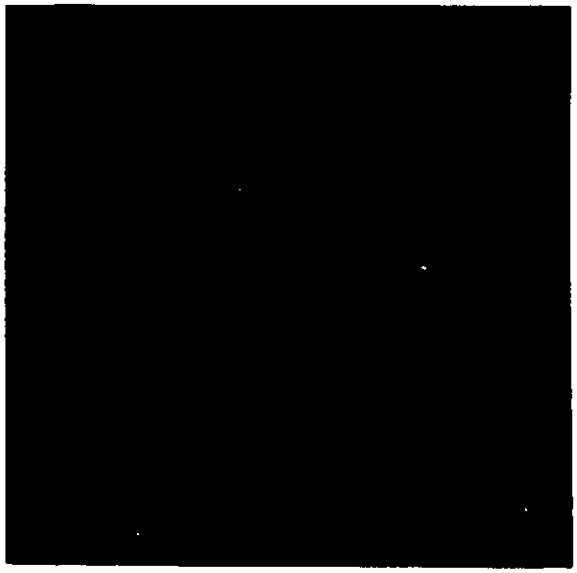
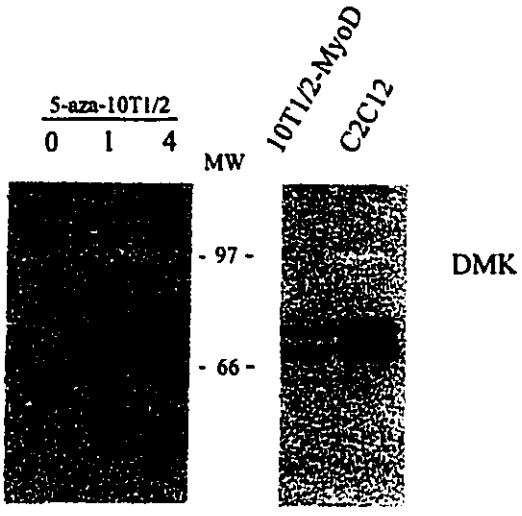


Figure 5-2. Expression and localization of DMK protein during myoblast differentiation and myogenic conversion. (A) Equivalent amounts of total protein lysate from control C2C12 or C2C12 cells incubated in differentiation medium (DM) for 1 and 3 days were western blotted and incubated with anti-DMK-1. The levels of DMK isoforms at 74 kDa and 82 kDa are found to increase slightly upon differentiation. Cross-reactive species (about 52 kDa) are detected with anti-DMK-1 but not anti-DMK-2 (see fig.2D). Immunolocalization using anti-DMK-1 in C2C12 myoblasts (B) and myotubes (C) shows punctate and homogeneous staining, respectively. (D) Expression of DMK protein in control 10T1/2 cells or following a brief treatment with 5-aza-cytidine. Lanes 0, 1 and 4 indicate the time (in days) at which the cultures were harvested following a 24 h treatment with 5-aza-cytidine. Upregulation of DMK was seen 4 days after exposure to 5-aza-cytidine and was more pronounced when 10T1/2 cells were transfected with a MyoD expression vector (10T1/2-MyoD). C2C12 was included as a control for DMK expression.

C.

D.



possibility. DMK expression was analysed in the mesodermal precursor cell line C3H10T1/2 which has been demonstrated to differentiate predominantly into myoblasts as well as chondrocytes and adipocytes following a brief treatment with 5-aza-cytidine (Konieczny and Emerson, 1984). As shown in figure 5-2D, the presence of the 82 kDa DMK isoform is apparent four days after treatment of 10T1/2 cells with 5-aza-cytidine. Similarly, DMK mRNA was found to be up-regulated following 5-aza-cytidine treatment and this induction coincided with an increase in MyoD mRNA in these cells (data not shown). The observed low level of DMK protein is presumably due to the fact that only 25% of the cells get converted to myoblasts under these conditions (Konieczny and Emerson, 1984). Myogenic conversion by transient transfection of a MyoD expression vector resulted in higher expression of DMK (Figure 5-2D), suggesting that the observed DMK expression in 5-aza-cytidine treated cultures is not likely to be due to the presence of the other cell types. Supporting this, neuromuscular junctions are the only structures to be stained by anti-DMK-1 in frozen skeletal muscle sections (Whiting et al., 1995). Therefore, our observations suggest that DMK is up-regulated during myogenic determination of precursor cells directly or indirectly through either myf-5 or MyoD.

Over-expression of DMK inhibits the terminal differentiation of cultured myoblasts

Previous histological studies have shown a marked delay of muscle cell terminal

differentiation and an abundance of satellite myoblasts in the developing skeletal muscle tissue of severely affected congenital DM patients (Sarnat and Silbert, 1976; Farkas-Bargeton et al., 1988). Furthermore, protein profile analysis on similar biopsies have shown that the expression of structural polypeptides, characteristic of mature muscles, was significantly reduced (Soussi-Yanicostas et al., 1991). Recently, our laboratory has shown that CTG expansion caused an elevation of DMK mRNA steady state levels in muscle tissues from congenital DM individuals (Sabourin et al., 1993). These data suggest that DMK over-expression could directly inhibit or delay myogenesis in these congenitally affected patients. Our working hypothesis, therefore, was that the dominant nature of the disease, and the ultimate effect on myogenesis, was due to the over-expression of the DM kinase gene and/or protein.

Having established that DMK expression is induced relatively late during the terminal differentiation of C2C12 myoblasts, we sought to investigate the effect of DMK over-expression on myoblast differentiation. To this end, an expression vector, carrying a full length human DMK cDNA was constructed. The cDNA, bearing 325 bp of 5' UTR, the coding region and the entire 3' UTR, with 11 CTG repeats (Mahadevan et al., 1993b), was introduced into the pcDNA3 expression vector (Invitrogen) to generate pCMV-DMK (Figure 5-3A). Transcription is under the control of the human cytomegalovirus promoter (CMV; Seed and Aruffo, 1987). Plasmid DNA was transfected into C2C12 and twelve G418-resistant clones were randomly isolated and analysed for DMK mRNA expression. When normalized to PGK, all the clones

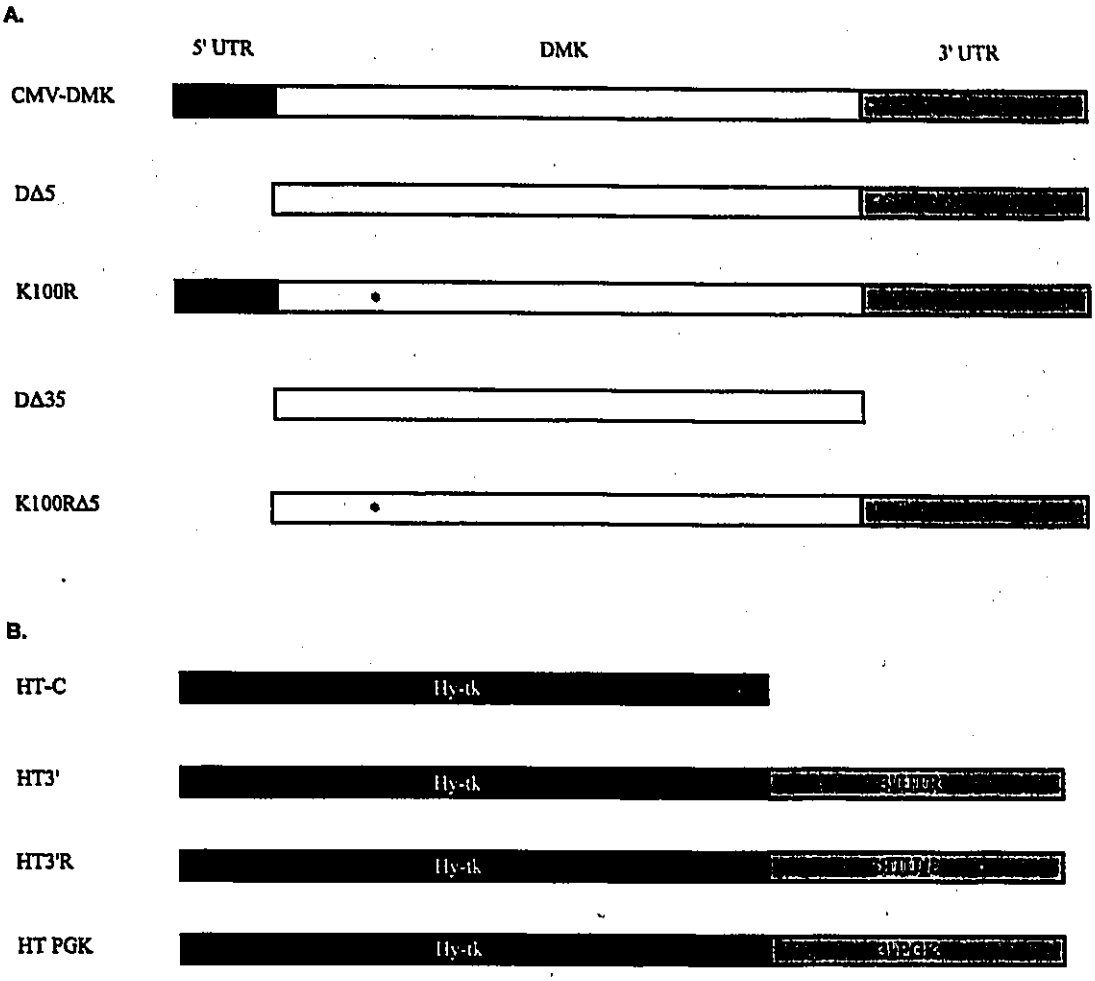

A.
5' UTR
DMK
3' UTR
CMV-DMK
DΔ5
K100R
DΔ35
K100RΔ5
B.
HT-C
Hy-tk
HT3'
Hy-tk
HT3'R
Hy-tk
HT PGK
Hy-tk

Figure 5-3. DM Kinase cDNA plasmid constructs. (A) DMK expression vectors carrying wild type or mutant full length DMK (CMV-DMK and K100R, respectively). Expression vectors in which the 5' UTR or both the 5' and the 3' UTRs were deleted are denoted by DΔ5 and DΔ35, respectively. K100RΔ5 represent the catalytic mutant version of DΔ5. The position of the point mutation is shown by the asterisk. (B) Hygromycin-tk hybrid control vector (HT-C) or vectors carrying the DMK 3' UTR in the sense (HT3') or antisense (HT3'R) orientation were used to analyse the effect of 3' UTR over-expression on myoblast differentiation. An additional vector bearing the PGK 3' UTR was constructed for use as a control (HTPGK).

surveyed displayed a 5 to 10-fold higher level of DMK mRNA relative to untransfected C2C12 myoblasts (for example, those clones designated B6 and C4 shown in figure 5-4A) and were morphologically similar to the parent cell line (data not shown). In parallel, these clones were subjected to western blot analysis and myoblast fusion assays under low serum conditions. Following myosin heavy chain (MHC) or Giemsa staining of the fixed cultures, the fusion index (# nuclei in myotubes/total # of nuclei) was calculated from several random fields and used as an indicator of terminal differentiation. Although the mRNA was highly expressed in all clones, DMK protein levels were found to be only slightly elevated or relatively unchanged compared to C2C12 (Figure 5-4B). This could be due to the presence of two other AUGs in the 5' UTR which may considerably reduce the translational efficiency or, alternatively, the 5' UTR could play a significant role in the control of DMK mRNA translation in myoblasts. However, COS-1 cells transfected with pCMV-DMK expressed extremely low levels of DMK protein, whereas high levels were observed when they were transfected with pDΔ5 (a DMK construct lacking the 5' UTR; Figure 5-3A), suggesting that the observed effect of the 5' UTR on translation is not restricted to tissue-specific control (Figure 5-4C). Surprisingly, when the differentiation potential was examined, the fusion index of all pCMV-DMK over-expressing myoblast clones was found to be consistently reduced by 3 to 5-fold (ranging from 5 to 14%) relative to wild type cells (displaying fusion indices of about 37% on average) or G-418-resistant C2C12 myoblasts transfected with pcDNA3 vector DNA (Figure 5-5A and 5-5B). Clones

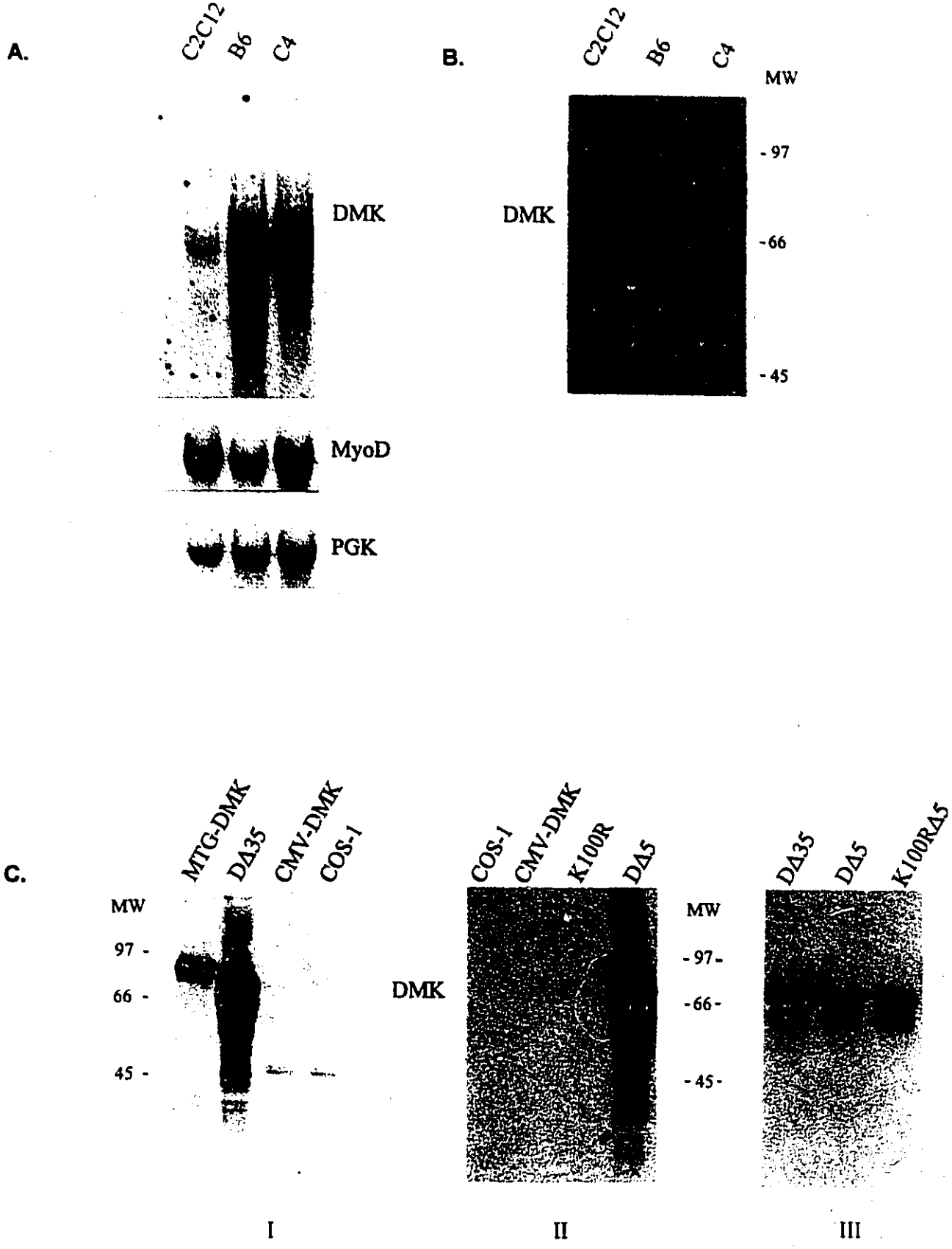

A.
C2C12
B6
C4
DMK
MyoD
PGK
B.
C2C12
B6
C4
MW
DMK
-97
-66
-45
C.
MTG-DMK
DΔ35
CMV-DMK
COS-1
MW
97 -
66 -
45 -
I
COS-1
CMV-DMK
K100R
DΔ5
DMK
MW
-97-
-66-
-45-
II
DΔ35
DΔ5
K100RΔ5
III

Figure 5-4. Expression of DMK mRNA and protein in myoblast clones and COS-1 cells. (A) Northern blot analysis of two independent myoblast clones (B6 and C4) isolated from CMV-DMK-transfected C2C12 cells. Normalization to MyoD and PGK showed a 10-fold over-expression of DMK for both clones. Exposure time was for 24 h. (B) Western blot analysis of the same B6 and C4 clones analysed in (A) using anti-DMK-2. Normalization to a parallel coomassie blue stained gel shows no difference in DMK protein levels between C2C12 and clones over-expressing DMK mRNA. (C) Anti-DMK-2 immunoblot analysis showing expression of the various DMK constructs in COS-1 cells. COS-1 cells were transiently transfected with the indicated vectors (see figure 3) and analysed for DMK expression 48 h following transfection. Panels I and II show the results of one experiment comparing the relative expression levels of CMV-DMK, K100R, DΔ35 and DΔ5. MTG-DMK represent a *myc* epitope-tagged DMK vector used in kinase activity assays. The relative expression levels for DΔ5, DΔ35 and K100RΔ5, obtained in an independent experiment, are shown in panel III. DMK was found not to be expressed from CMV-DMK or K100R but was observed to be expressed equally well from all the other constructs. In all panels, DMK was detected with anti-DMK-2.

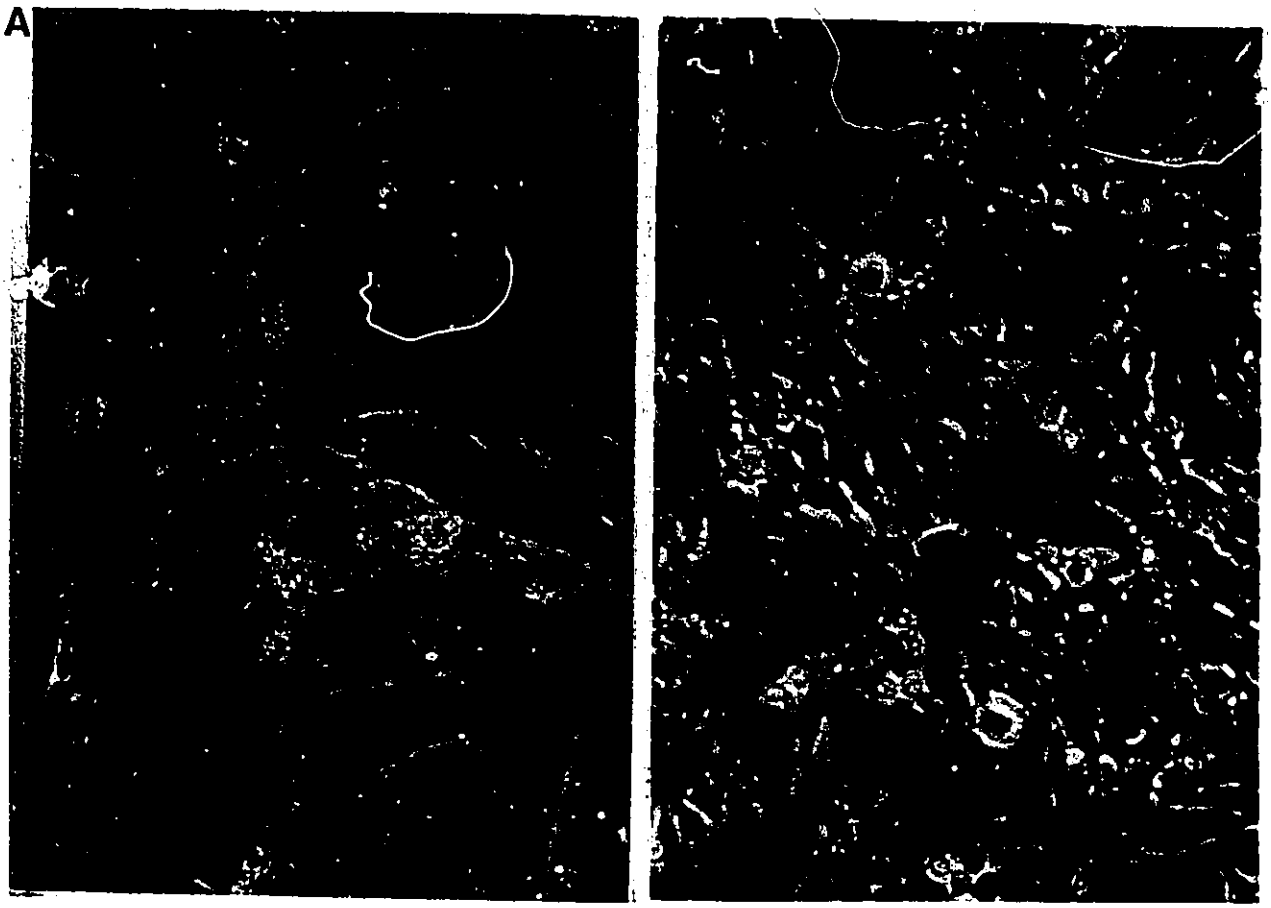
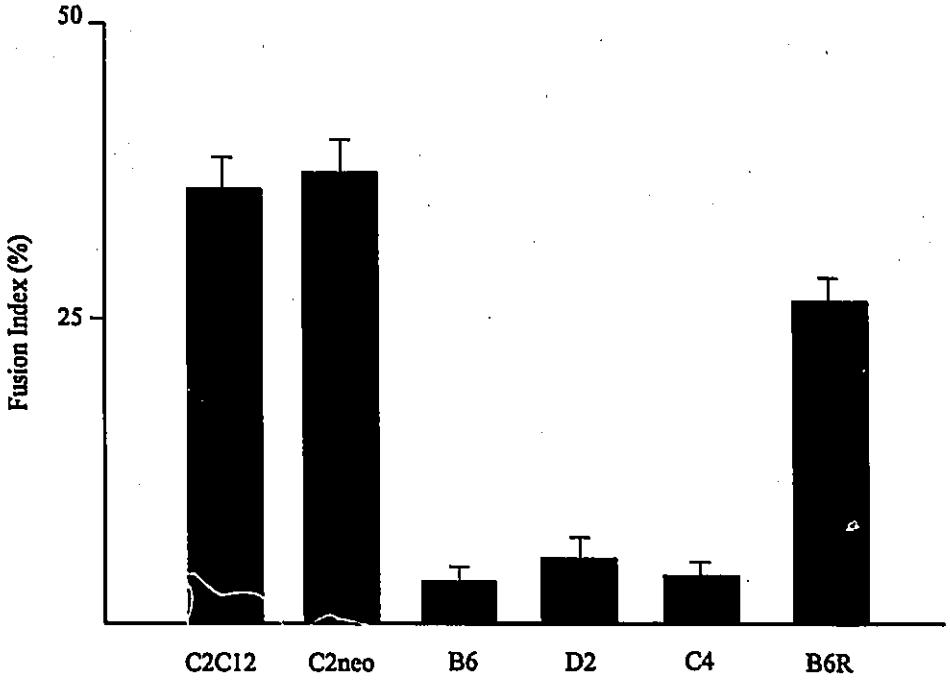

A

Figure 5-5. Effect of DMK over-expression on myoblast differentiation. (A) C2C12 (left) or B6 (right) cells were grown to confluency, induced to differentiate in 10% horse serum for 48 h and then stained for myosin heavy chain (MHC) using MF-20 (Bader et al., 1982). Cultures of C2C12 display several large myotubes whereas B6 cultures showed very few multinucleated cells. (B) Fusion index determination of DMK over-expressing clones. Following the induction of differentiation. C2C12 myoblasts or neomycin-resistant C2C12 clones displayed indices of about 35% whereas DMK (CMV-DMK) over-expressing clones B6, D2 and C4 showed fusion indices of about 6-10%.

B.



expressing a lower level of DMK mRNA were found to have higher myogenic indices, suggesting a correlation between DMK mRNA levels and the reduction in fusion index (data not shown). The fact that all the over-expressing clones tested exhibited a reduced fusion potential combined with the observation that differentiation defective clones spontaneously occur at a frequency of about 10^{-3} (Wright, 1984; Black and Hall, 1985) suggest that the observed phenotype is not due to the isolation of mutant clones. Supporting this, a revertant population isolated from the B6 clone (B6R; Figures 5-4 and 5-5), expressing only 2-fold higher DMK mRNA levels (not shown), was shown to fuse as readily as control cells (Figure 5-5B). Previous studies have demonstrated that impaired myoblast fusion could be attributed to a decrease or loss of MyoD expression (Brennan et al., 1990; Song et al., 1992). However, Northern blot analysis of pCMV-DMK clones did not show any significant differences in the levels of MyoD mRNA (Figure 5-4A), indicating that DMK exerts its effect dowstream of MyoD transcription.

The apparent absence of increased DMK protein levels in our differentiation defective clones over-expressing DMK mRNA, suggests that this phenotype is not mediated by the protein kinase itself but, rather through, perhaps, one or both untranslated regions. Alternatively, a slight increase in DMK protein levels and/or activity which may not be readily detectable by western blot analysis, or kinase activity assays, might be detrimental to myogenesis. In order to test these possibilities, we constructed additional DMK expression vectors and assayed differentiation in over-expressing clones. Initially, the conserved lysine of the ATP binding site (Hunter and

Sefton, 1991), at amino acid position 100, was converted to an arginine by *in vitro* mutagenesis (Smith, 1985) to generate pK100R (Figure 5-3A). Kinase inactivation was verified by the method of Dunne et al. (1994) using immunoprecipitation and histone III phosphorylation (data not shown) by comparing the kinase activity of DMK generated from *c-myc* epitope-tagged (Ellison and Hochstrasser, 1991) K100R and wild type DMK constructs expressed in COS-1 cells (Figure 5-4C). In addition, a DMK expression vector in which both UTRs have been deleted through PCR amplification of the coding region (pDΔ35; Figure 5-3A), was also tested. Both vectors were transfected into C2C12 myoblasts and 12 individual clones were analysed in each case, as described above. As for pCMV-DMK, all the clones isolated from pK100R-transfected cells expressed high levels (up to 10-fold) of DMK mRNA encoding the inactive kinase (for example those clones K5 and K6 shown in Figure 5-6A). Similarly, these clones, as well as transfected COS-1 cells, did not show any appreciable increase in DMK protein levels (Figure 5-4C and 5-6B). However, the fusion index of these clones was also significantly reduced compared to control cultures (Figure 5-6C). The use of a DMK catalytic mutant in these experiments indicates that active kinase is not required to confer the observed phenotype.

In marked contrast to pK100R-transfected clones, both myoblasts and transfected COS-1 cells showed high levels of DΔ35-specific mRNA (Figure 5-7A) and were found to express 3 to 5-fold higher levels of DMK protein when compared to C2C12 (Figure 5-7B). Interestingly, clones over-expressing DMK protein displayed fusion indices that

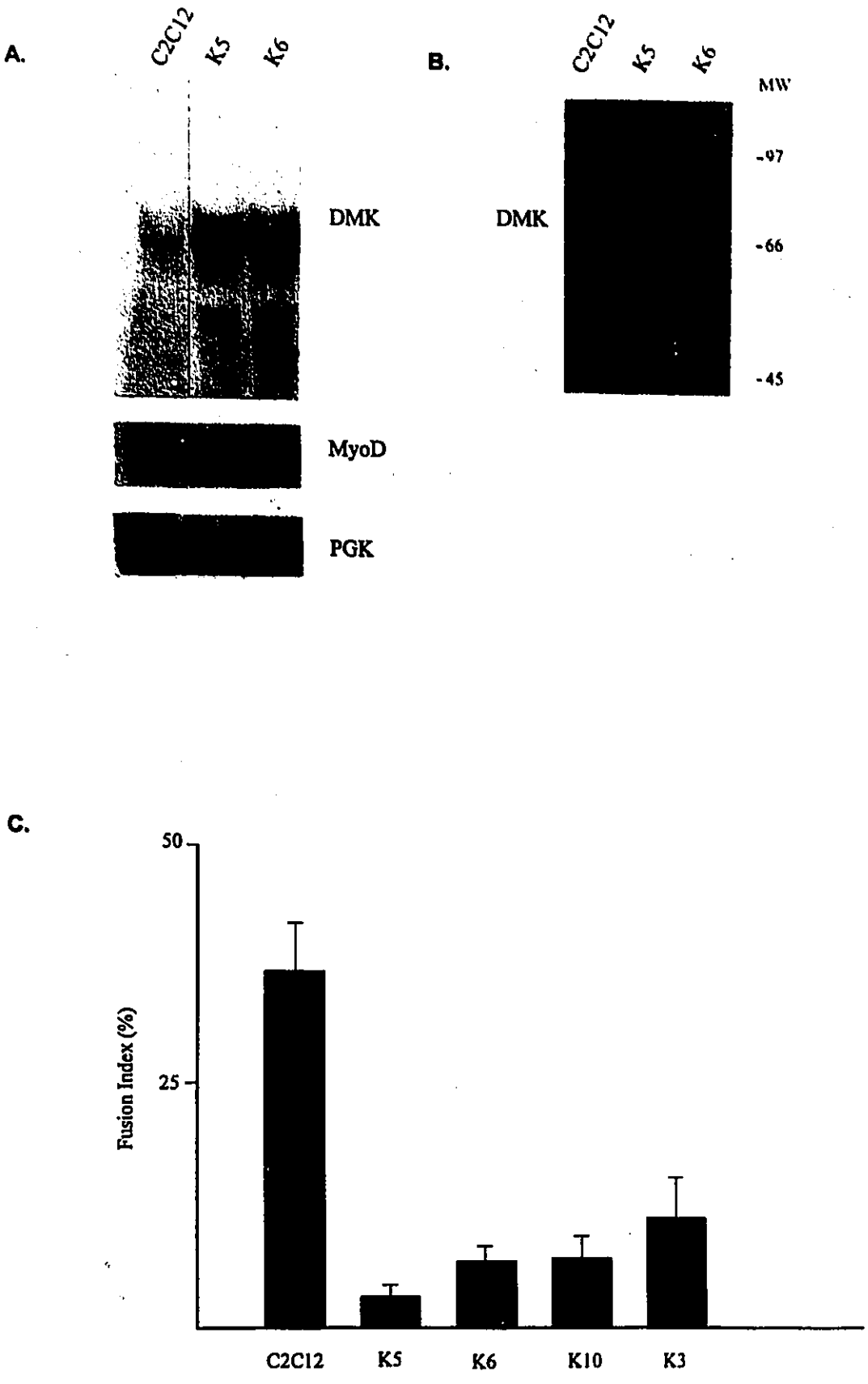

A.
C2C12
K5
K6
DMK
MyoD
PGK
B.
C2C12
K5
K6
MW
-97
-66
-45
DMK
C.
50
25
Fusion Index (%)
C2C12
K5
K6
K10
K3

Figure 5-6. Expression of DMK mRNA and protein in K100R-expressing myoblast clones.
(A) Northern blot analysis of DMK mRNA expressed from C2C12 cells or K100R-transfected clones, K5 and K6. DMK was found to be expressed about 8-10-fold higher than the endogenous DM kinase mRNA. Exposure was for 24 h. (B) Western blot analysis of DMK protein expression in K5 and K6 clones using anti-DMK-2. No significant differences were observed in the levels of DMK protein between control and transfected cells. (C) Fusion index determination for K100R-transfected clones. Following 48 h in differentiation medium, fusion indices were determined as described (see procedures) and compared to wild type C2C12 cells. In all the clones over-expressing mutant DMK mRNA, reduced fusion indices were observed.

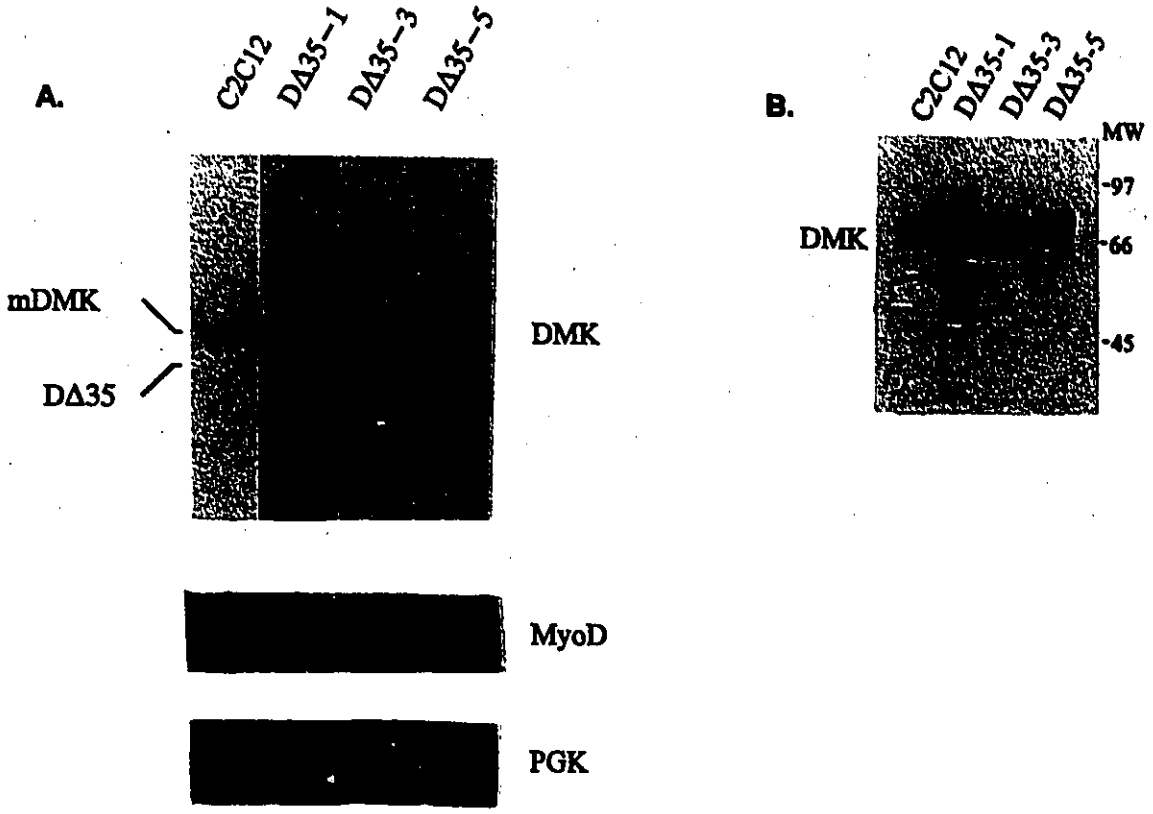
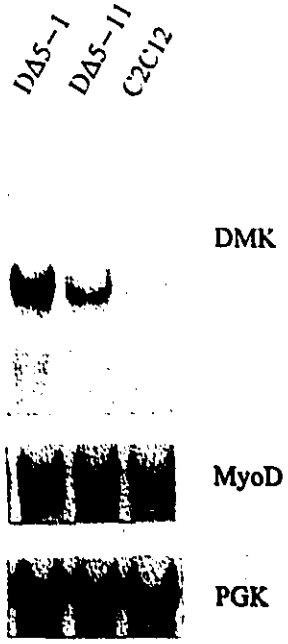
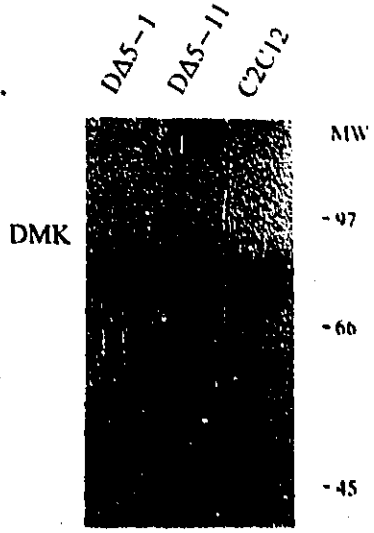

A.
C2C12
DΔ35-1
DΔ35-3
DΔ35-5
mDMK
DΔ35
DMK
MyoD
PGK
B.
C2C12
DΔ35-1
DΔ35-3
DΔ35-5
MW
97
66
45
DMK

Figure 5-7. Expression of DMK and differentiation potential of DΔ5 and DΔ35-transfected myoblasts. Expression of DMK mRNA (A) and protein (B) from pDΔ35-transfected clones. High levels of both DΔ35 mRNA and DMK protein were observed in three representative over-expressing clones (DΔ35-1, -3 and -5) relative to C2C12 control. Similarly, clones transfected with DΔ5 (-1 and -11) over-expressed DMK mRNA (C) but showed no significant increase in DMK protein expression (D). (E) Fusion index comparison between DΔ5, DΔ35 clones and C2C12 cells. DΔ35 clones over-expressing DMK mRNA and protein fused as well as C2C12 whereas DΔ5 clones showed reduced fusion potential.

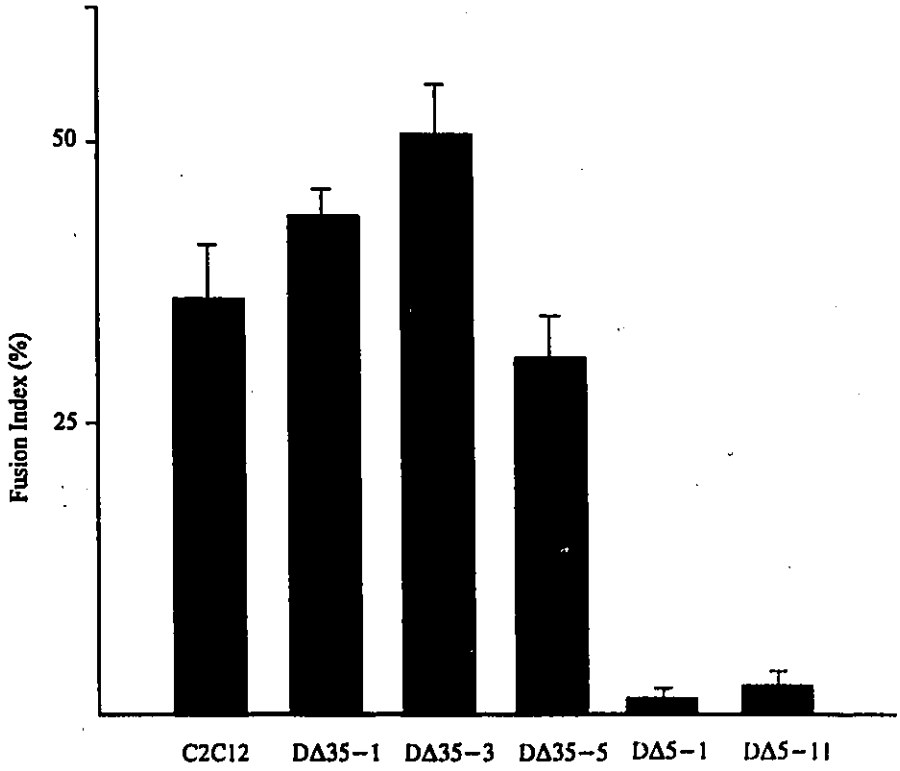
C.


DΔ5-1
DΔ5-11
C2C12
DMK
MyoD
PGK

D.


DΔ5-1
DΔ5-11
C2C12
MW
DMK
97
66
45

E.


Fusion Index (%)
50
25
C2C12
DΔ35-1
DΔ35-3
DΔ35-5
DΔ5-1
DΔ5-11

were similar to control cultures (Figure 5-7E). This is in marked contrast to what has been previously demonstrated for protein kinase A and protein kinase C over-expression in myoblasts, for which an inhibition of differentiation was observed (Li et al., 1992b; Li et al., 1992c). Taken together, our observations support the hypothesis that one or both non-coding UTR regions and not the protein, are responsible for the observed inhibition of differentiation.

The inhibition of myogenesis by DMK is mediated by the 3' UTR

The findings that the only DM mutation identified to date maps to the 3' UTR of the DMK transcript (reviewed in Wieringa, 1994) and the recent implication of specific 3' UTRs in the control of myoblast differentiation (Rastinejad and Blau, 1993; Rastinejad et al., 1993) prompted us to examine whether the DMK 3' UTR alone could function in our assay as an inhibitor of myoblast fusion. Therefore, an additional vector was constructed in which the 5' UTR was removed by replacing the 5' portion of pCMV-DMK with the corresponding 5' segment of pDΔ35, creating pDΔ5 (Figure 5-3A). As for the other constructs, G418-resistant clones were isolated and analysed for DMK expression. All DΔ5 clones expressed high levels of DMK mRNA (for example those shown in Figure 5-7C) but surprisingly, did not express DMK protein significantly higher than C2C12 (Figure 5-7B). However, pDΔ5-transfected COS-1 cells expressed high levels of DMK protein (Figure 5-4C), suggesting that the 3' UTR may

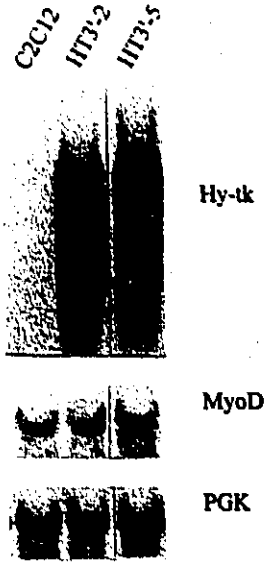
play an important role in the control of DMK translation in a tissue-specific manner. When assayed for differentiation, DΔ5 clones showed a more dramatic reduction in fusion index relative to C2C12 control myoblasts (ranging from 2 to 8%; Figure 5-7E) when compared to pCMV-DMK (Figure 5-5B) or pK100R-transfected cells (Figure 5-6C).

Overall, these results suggest that the over-expression of the 3' UTR is sufficient to mediate the observed phenotype in myoblasts and that the 5' UTR may have some negative effect on the inhibition of terminal differentiation conferred by the 3' UTR. If over-expression of the 3' UTR alone is sufficient to block differentiation, it should be able to do so when inserted downstream of a reporter gene construct. To test this, we used the ptgCMV/Hy-tk vector encoding a hybrid gene, under the control of the CMV promoter, conferring resistance to hygromycin B and sensitivity to gancyclovir (Lupton et al., 1991). The DMK 3' UTR, including 255 bp of coding region upstream of the termination codon, was cloned downstream of the hybrid Hy-tk gene creating pHT3' (Figure 5-3B). The expression of truncated DMK product from this vector is unlikely since no initiation codons are observed in the proper DMK reading frame and termination codons are present in the other two frames (data not shown). This hybrid vector was then introduced into C2C12 myoblasts and a total of 15 hygromycin-resistant clones were isolated and analysed for Hy-tk expression by Northern blotting. These clones were found to express the Hy-tk gene at levels that were considerably higher than DMK expressed from pCMV-DMK, or pK100R, and the endogenous DMK

(for example, Figure 5-8A and data not shown). When subjected to differentiation assays, similar to DΔ5 clones, cultures of HT3'-transfected cells exhibited a drastic reduction in their fusion index (Figure 5-8B) supporting the hypothesis that over-expression of the DMK 3' UTR alone is sufficient to cause a block of myogenesis. As a control, the same 3' UTR segment was cloned in the reverse orientation (pHT3'R; Figure 5-3B) and 24 hygromycin-resistant clones were assayed for fusion. All of these clones exhibited fusion indices that were comparable to control cultures consisting of wild type C2C12 myoblasts or hygromycin-resistant cells that had been transfected with vector DNA alone (Figure 5-8B). Furthermore, an important additional conclusion of these results is that the observed phenotype is dependent upon transcription of the 3' UTR and that it does not act as a DNA binding site competing for regulatory factors. As an additional control, the 3' UTR of the mouse phosphoglycerate kinase (PGK) cDNA (Adra et al., 1987) was also cloned into ptgCMV/Hy-tk to generate pHTPGK (Figure 5-3B). As for pHT3'R clones, hygromycin-resistant myoblast clones isolated following transfection with pHTPGK fused equally well when compared to control cutures (Figure 5-8B).

In addition to the selection of hygromycin-resistant clones over-expressing the 3' UTR, the use of the ptgCMV/Hy-tk vector allows for the selective killing of these clones by gancyclovir treatment. Consequently, clones that have lost the expression of the 3' UTR through loss of hygromycin B resistance can be isolated and assayed for differentiation. Two clones over-expressing the 3' UTR were grown in the presence of

A.


C2C12
HT3'-2
HT3'-5
Hy-tk
MyoD
PGK

B.

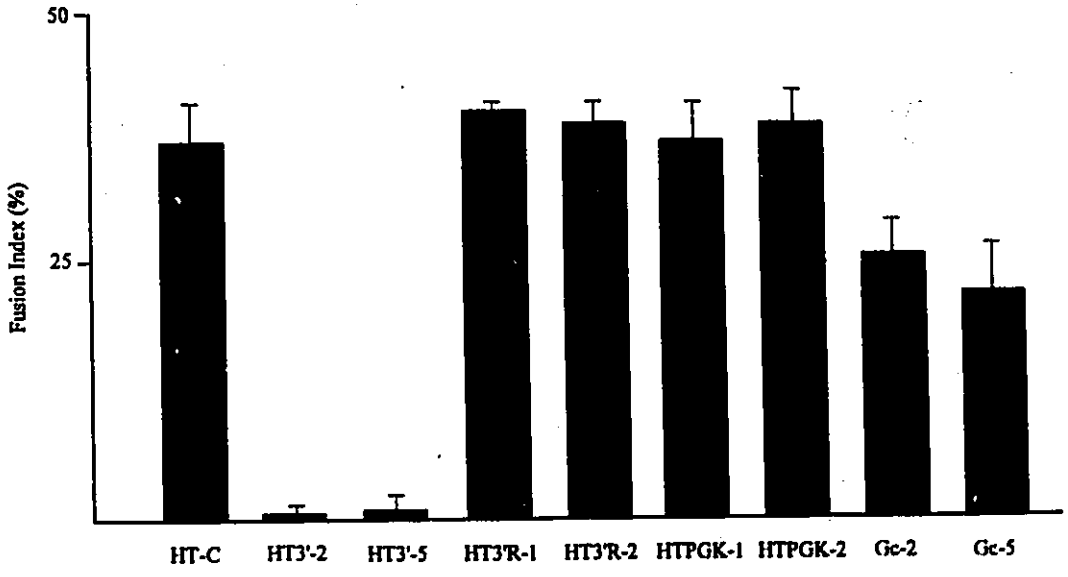

50
25
Fusion Index (%)
HT-C
HT3'-2
HT3'-5
HT3'R-1
HT3'R-2
HTPGK-1
HTPGK-2
Gc-2
Gc-5

Figure 5-8. Inhibition of myoblast differentiation by DMK 3' UTR. (A) Over-expression of Hy-tk hybrid mRNA carrying the DMK 3' UTR (HT3') in myoblast clones. The membrane was probed with a Hy-tk fragment and exposed to X-ray film for 7 h. Clones transfected with HT3'R or HT3'PGK expressed similar levels of Hy-tk hybrid mRNA (not shown). (B) Fusion index measurement on myoblast clones transfected with various Hy-tk constructs. Clones over-expressing the DMK 3' UTR in the sense orientation (HT3') showed a reduced fusion potential when compared to C2C12 cells, hygromycin-resistant C2C12 control clones (HT-C), or control HTPGK clones. Gancyclovir-resistant clones (Gc-2 and Gc-5) obtained through negative selection were found to fuse significantly more than the parent clones HT3'-2 and HT3'-5. (C) Photomicrographs of Giemsa-stained differentiated cultures of control C2C12 cells (100X), HT3'-1 clone (100X), a HT3'-R clone (40X) and a HT3'GK clone (phase contrast; 40X). All but HT3' clones fused as well as control cells.

C2C12

HT3'

HT3' R

HT PGK

gancyclovir for 21 days and resistant colonies were isolated. When assayed for differentiation, two individual resistant clones isolated from each gancyclovir-treated cultures of HT3'-2 and HT3'-5 (Figure 5-8A) displayed myogenic indices that were comparable to those of control cultures (Figure 5-8B). When re-plated in medium containing hygromycin B, resistant clones were isolated at a frequency of about 10^{-4}, suggesting that loss of the hygromycin B-resistance phenotype occurs at a relatively high frequency. These data strongly suggest that the DMK 3' UTR is sufficient to mediate the observed inhibition of myoblast terminal differentiation in transfected C2C12 cultures.

Proliferation and differentiation are hypothesized to be mutually exclusive (Olson et al., 1991). Therefore, the DMK 3' UTR may inhibit myogenesis by stimulating proliferation, thus preventing cell cycle exit, a pre-requisite for differentiation. Furthermore, previous studies have shown that, in addition to enhancing myogenesis in myoblasts, muscle-specific 3' UTRs could suppress the proliferation of fibroblasts (Rastinejad and Blau, 1993). We therefore sought to examine the effect of the DMK 3' UTR on the proliferation of NIH 3T3 fibroblasts and myoblasts. Pools of hygromycin-resistant NIH 3T3 cells and myoblast clones were subjected to proliferation assays using 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT;Skidmore et al., 1975). As shown in Figure 5-9, expression of the DMK 3' UTR did not have any significant effect on the proliferation of fibroblasts or myoblasts, suggesting that it does not increase their growth rate which would alter their capacity to arrest upon induction

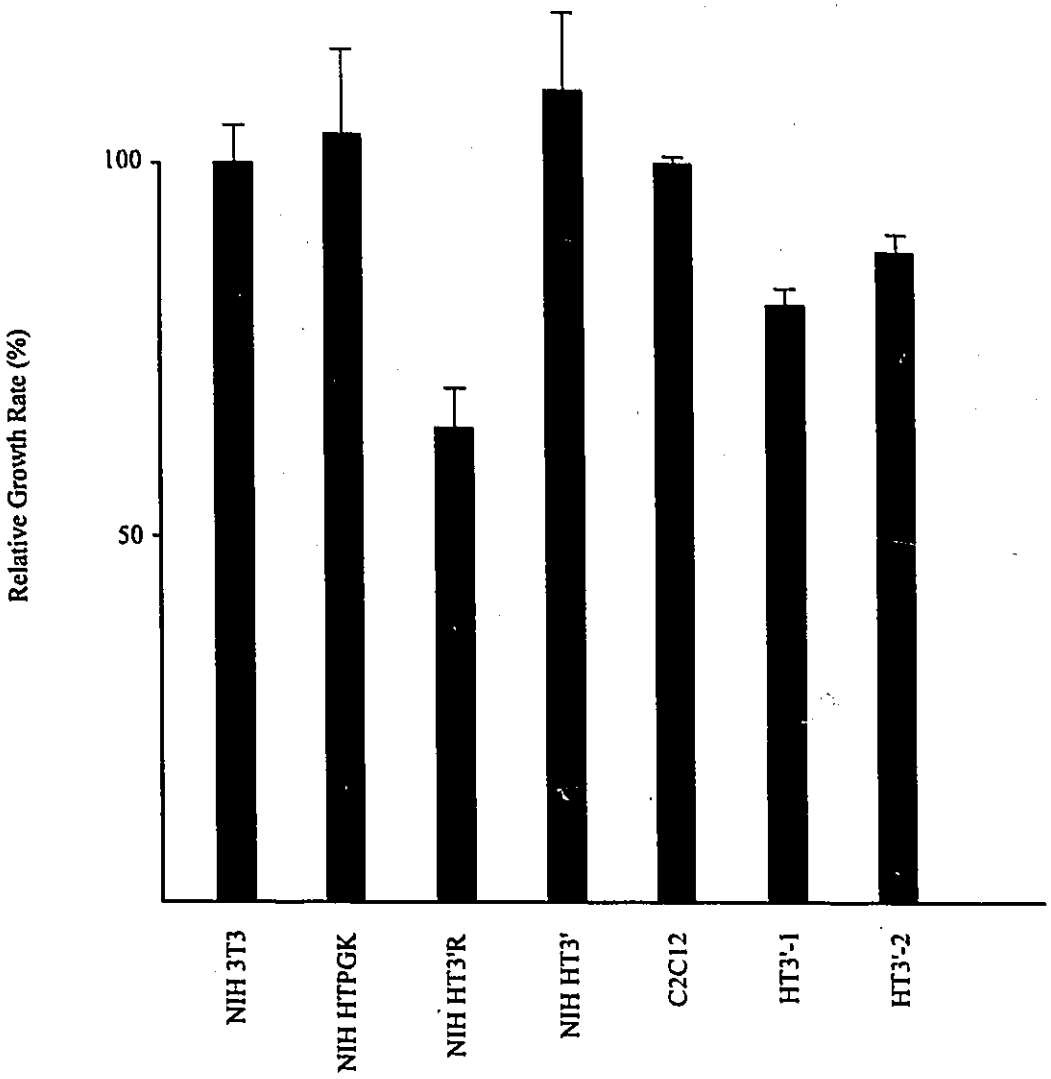

Relative Growth Rate (%)
100
50
NIH 3T3
NIH HTPGK
NIH HT3'R
NIH HT3'
C2C12
HT3'-1
HT3'-2

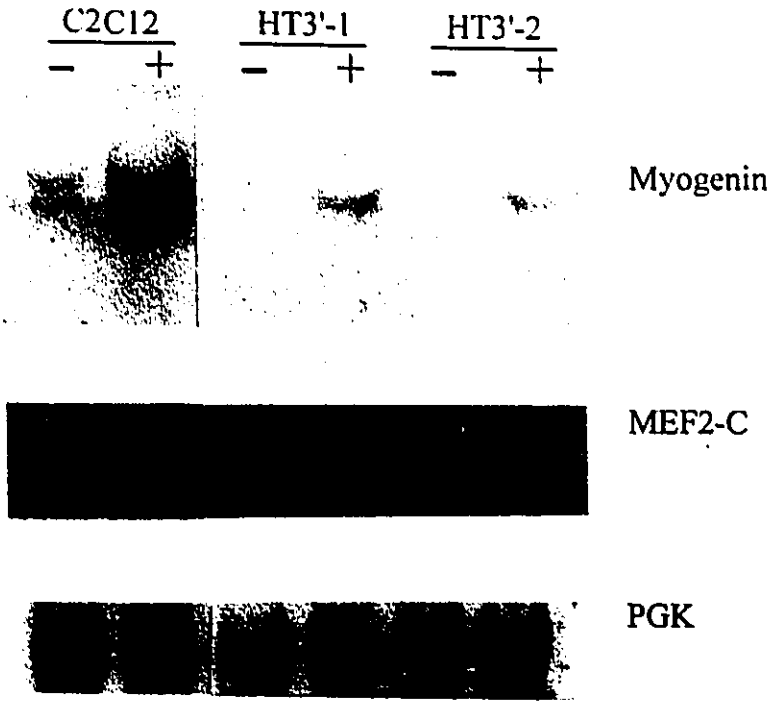
Figure 5-9. Effect of DMK 3' UTR over-expression on the growth rate of NIH 3T3 fibroblasts.
Subconfluent cultures of wild type NIH 3T3 cells or hygromycin-resistant pools transfected with the various Hy-tk constructs were pulsed with 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) and O.D. readings were used as an indicator of proliferation rates. The average absorbance value for NIH 3T3 control cultures was arbitrarily set at 100%. The DMK 3' UTR did not have any significant effect on the growth rate of these cells when compared to HTPGK or untransfected cells. Similarly, myoblast clones over-expressing the DMK 3' UTR did not show any significant differences in proliferation rate relative to C2C12.

of differentiation. Interestingly, fibroblasts expressing pHT3'R consistently showed a 20% reduction in their proliferation rate, however, the basis and significance of this decrease is unknown.

Over-expression of DMK 3' UTR inhibits myogenin, but not MEF-2C expression

The differentiation of myoblasts *in vitro* has been shown to be associated with a rapid induction of myogenin, a member of the basic helix-loop-helix (bHLH) family of transcriptional activators (Edmondson and Olson, 1989; Wright et al., 1989). Interestingly, myogenin is also required for the formation of myotubes and myofibers *in vivo* (Hasty et al., 1993; Nabeshima et al., 1993), suggesting that it plays a central role in the terminal differentiation of myoblasts. Therefore, we investigated the effect of DMK 3' UTR over-expression on myogenin gene expression following the induction of differentiation. Clones HT-3'-1 and HT-3'-2 were grown to 80% confluency and transferred to differentiation medium. Total RNA was prepared 48 hours later and analysed for myogenin expression. After normalization to PGK levels, Northern blot analysis demonstrated that myogenin mRNA levels were reduced by about 75% in clones over-expressing the 3' UTR (Figure 5-10A) compared to C2C12 cells. Similarly, pCMV-DMK clones showed a 50% reduction in myogenin and MLC 1/3 (data not shown) mRNA levels. The filter was subsequently probed for MEF-2C, another early regulator of myogenesis, and a member of the MADS-box family of proteins (reviewed

A.


C2C12
HT3'-1
HT3'-2
– + – + – +
Myogenin
MEF2-C
PGK

B.

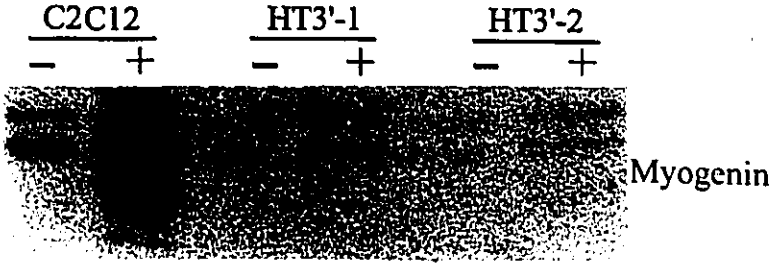

C2C12
HT3'-1
HT3'-2
– + – + – +
Myogenin

Figure 5-10. Inhibition of myogenin expression in clones over-expressing the DMK 3' UTR. (A) Northern blot analysis of total RNA from clones HT3'-1 and HT3'-2 following the induction of differentiation. The minus (-) and the plus (+) indicate the RNA samples obtained prior to, or 48 h after switching the cells to differentiation medium, respectively. A four-fold reduction in myogenin mRNA levels was measured relative to the PGK control. (B) Western blot analysis of total protein lysates of HT3'-1 and HT3'-2 clones following the same treatment described in (A).

in Olson and Klein, 1994). Interestingly, MEF-2C mRNA was found to be induced at levels that were similar to that of C2C12 control cultures (Figure 5-10A), indicating that the 3' UTR may exert its effect on specific pathways of terminal differentiation.

Surprisingly, when total lysates from similar HT3' cultures were analysed for myogenin protein expression, no significant differences were observed in the levels of myogenin protein prior to and after the induction of differentiation. Although the levels of myogenin mRNA between C2C12 and both HT-3' clones were found to be reduced by approximately four-fold, the corresponding levels for myogenin protein were shown to be about 20-fold less, suggesting that the DMK 3' UTR may ultimately alter the translational efficiency of myogenin mRNA (Figure 5-10B). One explanation may be that it prevents myogenin mRNA export or processing. Alternatively it may inhibit the expression of factors potentially involved in differentiation-specific control of translation.

One of the upstream regulators of myogenin expression is thought to be MyoD (Weintraub et al., 1991; Hollenberg et al., 1993). Through post-translational modification, MyoD is presumed to activate its downstream targets by direct DNA binding and transcriptional activation (Olson and Klein, 1994; Kim et al., 1992), making it a potential target for the DMK 3' UTR. To test whether MyoD activity was impaired by the 3' UTR, transient co-transfection experiments were performed in 10T1/2 fibroblasts using a MyoD expression vector along with a reporter construct (4R-tkCAT) and various plasmids expressing the DMK 3' UTR. Following transfection and transfer

of the cells to differentiation medium, chloramphenicol acetyl transferase (CAT) activity was determined. A slight decrease in CAT activity was observed (about 30%) when pHT-3' was co-transfected with MyoD, although this may not be significant as pHT3'R also showed a similar decrease in CAT activity (Figure 5-11). Similarly, pCMV-DMK and pD5 did not have any significant effect on the measured CAT activity, suggesting that the 3' UTR does not affect MyoD activity directly, but rather one or several co-activators of myogenesis.

The 3' UTR inhibitory activity maps upstream of the CTG repeat.

In order to define the sequence elements that mediate the inhibition of myoblast terminal differentiation, a series of three expression vectors carrying various portions of the 3' UTR (figure 5-12) were transfected into C2C12 cells and 12 hygromycin B resistant clones were isolated and tested for fusion potential. As shown in figure 5-13, clones that expressed a mutant 3' UTR that lacked the repeat region (pHTΔR) or a 239 bp element dowstream of the repeat (pHTΔ3') displayed reduced fusion potential similar to HT-3' clones. However, clones expressing a mRNA in which the 239 bp region upstream of the repeat had been deleted were found to fuse as well as control cultures. These data suggest that the inhibitory domain comprised in the DMK 3' UTR maps to a region of at most 239 bp 5' to the repeat. In contrast to the CTG repeat region, this portion of the 3' UTR shows a high degree of conservation between the murine and

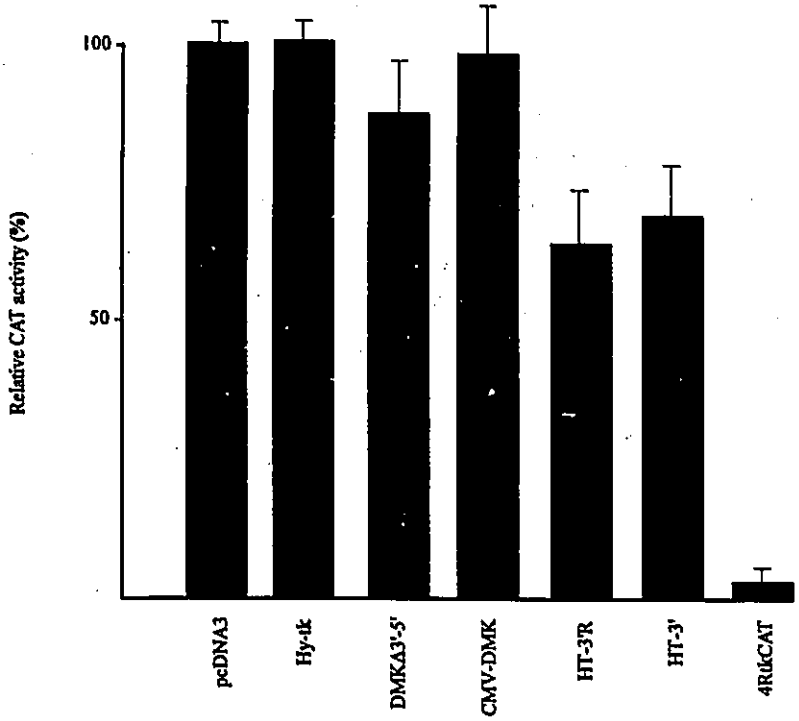

Relative CAT activity (%)
100
50
pcDNA3
Hy-tk
DMKΔ3'-5'
CMV-DMK
HT-3'R
HT-3'
4RtkCAT

Figure 5-11. Over-expression of the DMK 3' UTR does not affect MyoD activity in a transient differentiation system. Various plasmid constructs were co-transfected transiently in 10T1/2 cells, in each case with a MyoD expression vector along with a CAT reporter plasmid, 4RtkCAT, which contains four multimerized E-boxes upstream of the herpes simplex thymidine kinase minimal promoter. 48 h following the induction of differentiation, CAT activity was assayed for the each of the different combinations of plasmids. The CAT activity obtained with an empty expression vector (pcDNA3 or Hy-tk) was arbitrarily set at 100%. The values obtained for DMKΔ3'-5' and CMV-DMK were normalized to the activity determined with the pcDNA3 control whereas those determined for HT3'R and HT-3' were standardized to the Hy-tk control. Similar values were obtained in three independent experiments and the results of one such experiment are presented.

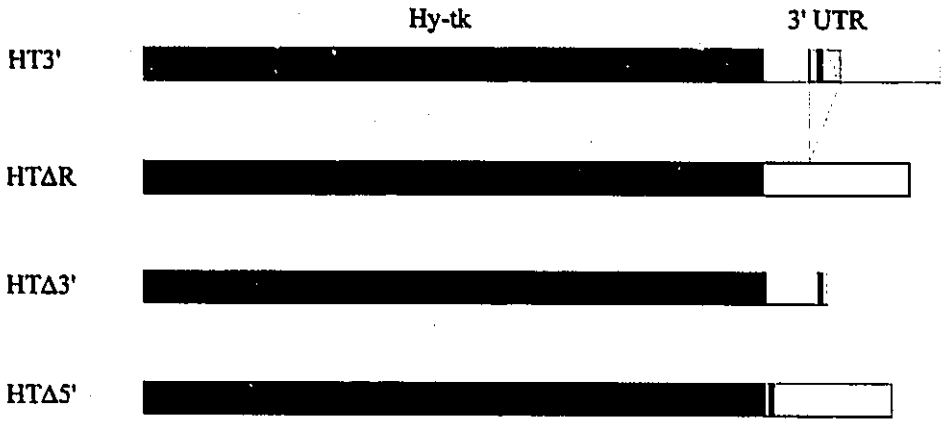

Hy-tk
3' UTR
HT3'
HTΔR
HTΔ3'
HTΔ5'

Figure 5-12. Schematic representation of the various 3' UTR deletion constructs. pHTΔR denotes a Hy-tk vector in which the repeat region was deleted through restriction endonuclease digestion. Vectors corresponding to the deletion of regions downstream and upstream of the repeat are represented by pHTΔ3' and pHTΔ5', respectively. The shaded area of HT3' represents the deleted region of HTΔR. The solid vertical line denotes the position of the CTG repeats.

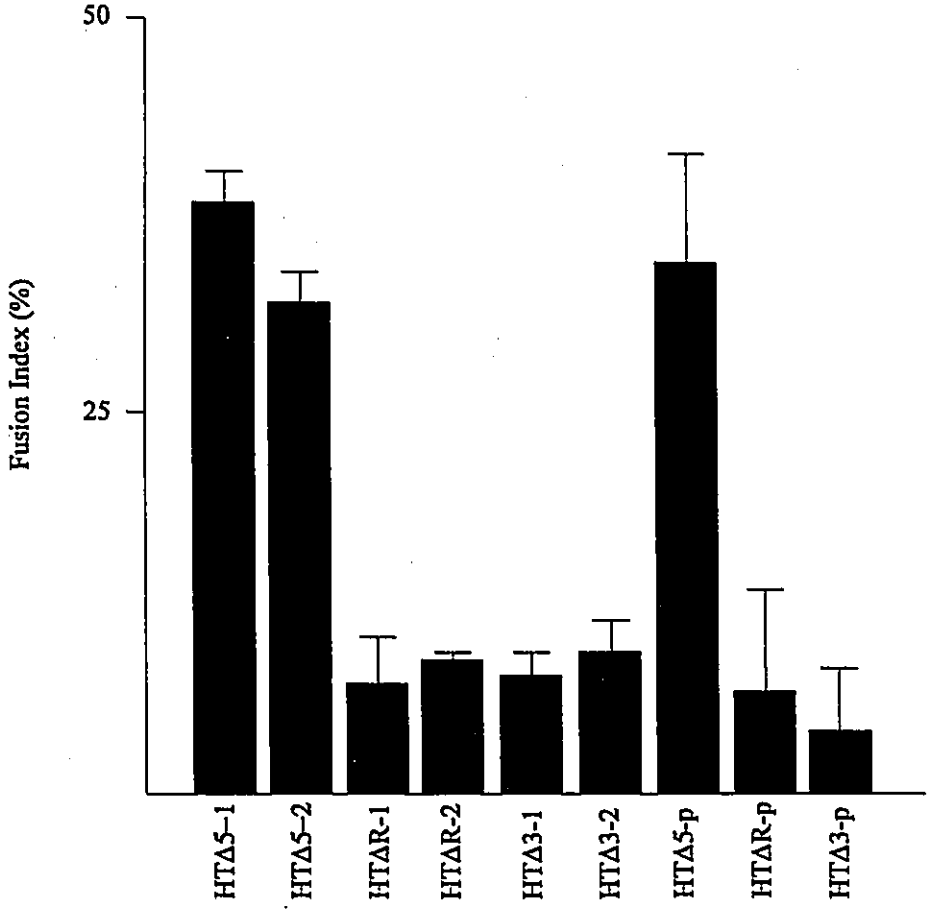

50
25
Fusion Index (%)
HTΔ5-1
HTΔ5-2
HTΔR-1
HTΔR-2
HTΔ3-1
HTΔ3-2
HTΔ5-p
HTΔR-p
HTΔ3-p

Figure 5-13. Fusion index determination of clones expressing various DMK 3' UTR regions. Clones expressing 3' UTRs lacking the repeat (HTΔR) or expressing a 239 bp region upstream of this repeat (HTΔ3') displayed reduced fusion indices in contrast to clones expressing the 3' UTR without the 239 bp most 5' region (HTΔ5'). These data indicate that the inhibitory activity maps to a 239 bp region 5' to the CTG repeat. The graph shows one representative fusion index experiment of two examples of clones expressing each of the vectors described in figure 5-12. The bars denoted by HTΔR-p, HTΔ5-p and HTΔ3-p represent the average fusion index±S.E.M obtained for one experiment for 10 independent clones.

human DMK cDNAs, suggesting that similar mechanisms may mediate the inhibition of differentiation.

Clearly, our data demonstrate that the inhibition of terminal differentiation in C2C12 myoblasts over-expressing a full length DMK cDNA is mediated by the 3' UTR of the mRNA. We have shown that the DMK 3' UTR alone is sufficient to block myogenesis and appears to do so, in part, by reducing myogenin expression. Similarly, other muscle-specific enhancers/promoters appear to be down-regulated by the DMK 3' UTR, which is likely to be a consequence of the inhibition of expression of upstream regulators. However the activity or transcriptional activation of other genes such as MyoD and MEF-2C, respectively, appears to be unaffected.

4. Discussion

As a first step into the characterization of DMK function during myogenesis, we have analysed its expression in various myoblast cell lines as well as a multipotent stem cell line. In addition, we have analysed DMK expression during the differentiation of C2C12 myoblasts in culture. Our results indicate that DMK is expressed in both skeletal and cardiac myoblasts but at very low levels in undifferentiated P19 cells and fibroblasts. Similar to its expression pattern in mature tissues (Sabourin et al., 1993; Jansen et al., 1992b), DMK was found to be expressed at relatively higher levels in cardiac myoblasts. Myoblast cell lines such as C2C12 and BC3H1 express primarily

MyoD and myf-5, respectively (Davis et al., 1988; Braun et al., 1989a). Furthermore, our laboratory has identified three conserved E-boxes in the first intron of the DMK gene and characterized the involvement of these E-boxes in the up-regulation of the expression of a reporter gene in myoblasts (C. Storbeck, unpublished data). These findings suggest that DMK expression in skeletal myoblasts may be achieved directly through either MyoD or myf-5. Supporting this hypothesis, we have found that DMK was up-regulated significantly in C3H10T1/2 mesodermal precursors treated with 5-aza-cytidine or transfected with a MyoD expression vector. Similarly, when P19 stem cells or normal human fibroblasts (as well as DM fibroblasts) are transfected with MyoD, up-regulation of DMK is observed (Skerjanc et al., 1994; Otten and Tapscott, 1995). Taken together, these data suggest that DMK may be an important marker of early myogenic lineages and can be directly up-regulated during myogenic determination by members of the muscle-specific bHLH family members.

Following the induction of differentiation, DMK mRNA and protein levels were observed to be increased by about 2 to 3-fold relative to uninduced C2C12 cells, although this increase is considerably less than what has been observed for other muscle-specific structural or regulatory genes. The observed increase in DMK levels during differentiation was found to occur 72 to 96 hours following the onset of differentiation, implying a role role for DMK in the process of myotube maturation. This pattern of expression is similar to that of MRF-4, a member of the bHLH family, postulated to have a significant role in the maturation of myotubes *in vitro* (Olson and

Klein, 1994; Rhodes and Konieczny, 1989; Hinterberger et al., 1991).

Immunofluorescence studies on C2C12 myoblasts shows a reticular pattern of anti-DMK staining whereas myotubes were found to stain homogeneously. Whether DMK is actively involved in processes such as protein transport through the Golgi in unclear. Alternatively, it could be anchored to this sub-cellular site, acting as a mediator of signal transduction. Another possibility is that it may be kept inactive in a protein complex localized at the Golgi membranes and subsequently activated and redistributed to the cytoplasm in myotubes. Interestingly, DMK was shown to localize to the postsynaptic side of neuromuscular junctions of mature skeletal muscles and to co-localize with acetylcholine receptor (AchR) clusters in cultures of primary rat myotubes (Whiting et al., 1995; Van der Ven et al., 1993). These observations suggest that, *in vivo*, DMK may be more specifically re-localized to these sites where it may be involved in the control of the excitation-contraction coupling mechanism (Catterall, 1991). The finding that DMK shows a cytoplasmic distribution in C2C12 myotubes suggest that these cultured cells lack some component necessary for the aggregation of DMK in differentiated multinucleated cells.

We have previously shown that DMK mRNA steady state levels were increased in muscle tissues from severely affected congenital DM patients (Sabourin et al., 1993). In addition, others have reported that skeletal muscles from these individuals show an abundance of satellite cells and immature myofibers (Harper, 1989). Furthermore,

contractile protein profiles indicative of immature skeletal muscles have been observed in congenital DM muscles, suggesting a delay of muscle terminal differentiation (Sarnat and Silbert, 1976, Farkas-Bargeton et al., 1988 Soussi-Yanicostas et al., 1991). Therefore, to gain insight into the molecular mechanisms underlying DM, we have investigated the effect of DMK over-expression in C2C12 myoblasts. Our data show that over-expression of a full length DMK cDNA in myoblasts significantly reduces the fusion potential of C2C12 cells, a result consistent with the *in vivo* observation of delayed muscle differentiation muscle cross-sections of congenital DM patients. Surprisingly, we determined that this activity mapped to the 3' UTR of DMK, as elevated DMK protein levels or active kinase were not required. Furthermore, this activity could be conferred to a heterologous gene using chimeric drug resistance-DMK 3' UTR constructs. This inhibitory activity was mapped to a 239 bp region upstream of the CTG repeat which is highly conserved between human and mouse.

Significantly, CTG amplification, the only DM mutation identified to date, occurs in the 3' UTR of DMK, suggesting that CTG expansion may result in the over-expression of the DMK mRNA, but not the DMK protein, in congenital DM patients, interfering with muscle differentiation.

The 3' UTR of several growth-associated and developmentally regulated mRNAs has been shown to play an important role in the sub-cellular localization of these transcripts (reviewed by Belasco and Brawerman, 1993; Jackson, 1993, Izaurralde and Mattaj, 1995; St-Johnston, 1995). The 3' UTR's appear to bind factors directly involved

in the transport of these mRNAs through the nuclear pore complex and to localized sites of translation. The nuclear export of several classes of RNAs is saturable, that is, a given RNA can competitively inhibit the export of some RNAs but not of others (Jarmolowski et al., 1994). Therefore, it is possible that the accumulation of mutant DMK mRNAs in the nucleus, through aberrant processing, may titrate away processing factors that interact with RNA sequences adjacent to the repeat region. If they are saturable, the titration of these factors may potentially affect the normal metabolism of other mRNAs. Also, the presence of a large repeat in the 3' UTR of the DMK message may interfere with the binding of export protein factors to adjacent regions, altering the transport of mutant DMK mRNA. Alternatively, the repeat may enhance or disrupt the binding of saturable factors involved in the normal control of stability or processing of DMK mRNA, leading to its accumulation in muscle cells of congenital DM patients. This would then result in increased steady state levels of the 3' UTR, mediating the delay of muscle terminal differentiation in these individuals.

Recently, *in situ* hybridization studies have demonstrated that the mutant DMK mRNA accumulates in the nuclei of fibroblasts and muscle cells isolated from adult DM patients (Taneja et al., 1995). These studies, however, were not able to specifically distinguish between altered pre-mRNA processing or transport of the mutant DMK mRNA. Nevertheless, these observations support the hypothesis that the expanded repeat may alter the normal metabolism of the mutant transcript. If the expanded CTG interferes with normal DMK mRNA processing or transport, its accumulation in the

nuclei of these cells may lead to a competition for 3' UTR binding sites involved in the export or processing of differentiation-specific mRNAs. Expression of very high levels of a wild type 3' UTR such as the one used in the present study could produce a similar effect in cultured myoblasts.

The mRNAs of neuromuscular junction-specific protein have been shown to be expressed by synaptic nuclei (Burden, 1993). Therefore, considering its sub-cellular localization in mature muscle, over-expression of DMK in these nuclei may affect the processing or expression of such mRNAs, leading to altered physiological responses in the muscles of DM patients (Harper, 1989).

Four major bHLH proteins have been demonstrated to play central roles in the control of myoblast determination and differentiation (Olson and Klein, 1994). MyoD is thought to be an important regulator of myoblast determination and to be involved in the onset of differentiation. Myogenin has been demonstrated to be more directly involved in the control of secondary myotube formation (Venuti et al., 1995). Our results show that over-expression of the 3' UTR interferes with maximal myogenin induction but it did not inhibit the induction of MEF-2C mRNA (Martin et al., 1993; Leifer et al., 1993; Cserjesi and Olson, 1991), indicating that it may affect specific pathways of differentiation. This is similar to previously reported results for myogenin-mutant mice in which MEF-2C expression was shown to be unaltered (Venuti et al., 1995). In addition, the 3'.UTR did not have any significant effect on the activity of

MyoD when co-transfected in 10T1/2 cells, suggesting that it may affect other regulators of differentiation. However, this does not exclude the possibility that the 3' UTR interferes with the ability of other factors to activate MyoD through post-translational modification (Kim et al., 1992).

At the protein level, myogenin was not induced following the induction of differentiation in clones over-expressing the DMK 3' UTR, suggesting that the 3' UTR may affect the normal metabolism of other mRNAs *in trans*. Previous studies have shown that MEF-2C appears to be required for optimal myogenin induction *in vivo* (Cheng et al., 1993). One possible explanation for the observed reduction in myogenin expression is that the export of MEF-2C mRNAs to the cytoplasm is impaired by the 3' UTR, leading to a decrease in MEF-2C protein, resulting in reduced myogenin expression. However, further studies will be required in order to verify this possibility. Finally, over-expression of the 3' UTR did not have any significant effect on the growth rate of fibroblasts or myoblasts, suggesting that it does not inhibit terminal differentiation by preventing cell cycle arrest.

We have previously reported that mutant DMK transcripts were over-expressed in a congenital DM patient (Sabourin et al., 1993). Combined with the recent findings that the expanded CTG repeat may alter the processing or export of the mutant mRNA, these data suggest that the amplified repeat may disrupt the normal metabolism of the mutant transcript. Therefore, the CTG repeat may lead to the accumulation of mutant

DMK mRNAs and/or full length mutant heteronuclear RNAs and this could exert an effect on the disease process such as the delay of muscle maturation in congenital DM patients. Although the levels of DMK protein isoforms do not appear to be dramatically altered in DM patients (Sabourin et al., submitted; see chapter IV), a slight change in cellular DM kinase levels (or activity), which may not be readily detectable by western blot analysis, may have profound effects.

Overall, our data indicate that the DMK 3' UTR alone is sufficient to inhibit myogenesis in over-expressing myoblasts, a result consistent with the *in vivo* results showing a delay of muscle differentiation in congenital DM patients. Mapping of the specific elements in the 3' UTR that mediate this effect and the identification of potential RNA binding protein(s) will provide further clues as to the molecular mechanisms underlying the pathophysiology of DM.

Chapter 6: General Discussion

Myotonic dystrophy (DM) is the most common form of inherited neuromuscular disorders in adults, affecting 1 in 8000 individuals globally (Harper, 1989). DM is an autosomal dominant disease characterized mainly by myotonia and progressive muscle wasting. In addition, central nervous system, endocrine, cardiovascular and occular manifestations frequently occur, reflecting the multisystemic nature of the disease. Clinically, affected individuals present with highly varible phenotypes ranging from an assymptomatic condition to a severe congenital form. Furthermore, DM has been shown, within pedigrees, to display genetic anticipation, an increase in disease severity in successive generations (Howeler et al., 1990).

The molecular basis underlying DM has been identified as the amplification of a CTG trinucleotide repeat in the 3' UTR of a serine/threonine protein kinase family member (Brook et al., 1992; Fu et al., 1992; Mahadevan et al., 1992; Buxton et al., 1992; Harley et al., 1992a). The size of this expansion has been directly correlated with disease severity (Harley et al., 1992b; Tsilfidis et al., 1992; Barcelo et al., 1993). Furthermore, within families, the succesive increase in size of the CTG repeat during transmission has helped to explain the phenomenon of genetic anticipation observed in DM pedigrees.

The objectives of this thesis were to analyse the effects of CTG repeat amplification, the only DM mutation reported thus far, on the expression of the DMK

gene and protein products in affected individuals and to examine the consequences of DMK over-expression on the terminal differentiation of cultured C2C12 murine myoblasts.

The presence of the DM mutation in the 3' non-coding region of the mutant allele excludes the possibility that the mutant mRNA encodes an aberrant form of the DMK protein. Furthermore, in all cases of DM, CTG repeat amplification is the only mutation reported to date, suggesting that CTG repeat expansion mediates the DM phenotype through a different mechanism. Therefore, as a first step, we have analyzed the expression of DMK in total RNA samples prepared from muscle tissues sampled from a congenital DM patient. Our results showed that the mutant DMK transcript was expressed in congenital DM muscle tissues carrying very large CTG repeat expansions. Furthermore, we observed that the levels of the mutant DMK mRNA were significantly increased in these tissues compared to control tissues, suggesting that the mutation may increase the steady state levels of the mutant transcript. Whether the observed increase in DMK mRNA levels are due to a change in mRNA stability or altered transcription rate remains to be determined. In contrast to previous reports (Hoffman-Radvayani et al., 1993; Carango et al., 1993; Fu et al., 1993), we have demonstrated that the mutant DMK mRNA was expressed in fibroblasts derived from a DM patient and that the levels of the wild type transcript were not affected. These results suggested that the dominant inheritance pattern of DM could be mediated by over-expression of the protein kinase. Interestingly, over-expression of two growth regulated kinases, PKA and

PKC, have been demonstrated to inhibit myoblast terminal differentiation (Li et al., 1992b; Li et al., 1992c), suggesting that PKC, PKA and possibly DMK over-expression may have profound effects on myogenesis. Significantly, a delay of muscle maturation has been observed in skeletal muscle tissue sections from congenital DM patients, implying an inhibition of muscle terminal differentiation in these individuals (Soussi-Yanicostas et al., 1991; Sarnat and Silbert, 1976 ; Farkas-Bargeton et al., 1988). Therefore DMK over-expression in these patients may result in the observed delay of muscle development.

In order to further investigate the effect of CTG amplification on DMK expression in DM patients, we developed polyclonal anti-DMK antibodies reacting with authentic human, rat and murine DMK. Surprisingly, when muscle tissue samples from DM individuals were analysed by western blotting for total DMK protein expression, no significant differences were observed in the levels of both DMKα and DMKβ isoforms relative to control samples when normalized to the levels of α-skeletal actin. This is in marked contrast to what had been observed previously by Fu et al., (1993) and Koga et al., (1994) who reported a reduction in the levels of a 54 kDa protein. However, this 54 kDa species has been subsequently demonstrated not to be a product of the DMK gene (Sabourin et al., 1995; Be Wieringa, personal communication)

The adult onset of DM has been characterized by an atrophy of type I fibers. Significantly, DMK was shown to be expressed preferentially in type I fibers (van der Ven et al., 1993 ; H.F. Epstein, personal communication), suggesting that abberant

expression of DMK leads to the specific loss of these fibers. In agreement with this, the adult DM muscle sample was found to be dramatically depleted of type I myofibers when anti-MHC I antibodies were used to normalize the protein samples (Harper, 1989; see chapter IV). Interestingly, normalization to MHC-I content showed that DMK protein levels were slightly elevated in DM skeletal muscle samples. However, care should be taken in the interpretation of these data because appropriately matched controls with respect to fiber type distribution and atrophy are difficult to obtain. In addition, protein quantitation by western blot analysis is not trivial. Regardless, our results showed that the levels and distribution of DMK protein were not significantly altered in muscle tissue and primary myoblast cultures of various DM individuals, suggesting that the DM phenotype may be mediated *in trans* at the RNA level and may not involve the DMK protein.

Our initial working hypothesis was that the over-expression of DMK was sufficient to mediate the DM phenotype. However, the observation that DM muscle tissues expressed DMK protein at levels that were comparable to control samples suggested that the DM phenotype did not involve over-expression of the DMK protein. In order to test this hypothesis, we constructed a series of DMK expression vectors and over-expressed them in the continuous murine myoblast cell line C2C12. The observation that congenital DM patients display a delay of muscle maturation was used as a criterion in a myoblast differentiation assay to examine the effect of DMK mRNA and protein over-expression. Our results demonstrated that over-expression of a wild

type DMK mRNA caused a marked delay of myoblast terminal differentiation *in vitro*, as observed *in vivo* in cases of congenital DM (see chapter V). Interestingly, over-expression of a DMK mRNA carrying a dominant mutation in the catalytic kinase domain had a similar effect on myoblast differentiation, suggesting that active kinase was not required to mediate the observed phenotype. Furthermore, over-expression of DMK protein in these myoblasts had no effect on their differentiation potential, indicating that one or both non-coding regions were sufficient to inhibit myoblast differentiation. Further investigations lead to the findings that the 3' UTR of the DMK mRNA was sufficient to cause a delay in the terminal differentiation of C2C12 myoblasts over-expressing the 3' UTR. We found that over-expression of the 3' UTR had no effect on the activity of proteins involved in the onset of myogenesis such as MyoD (Davis et al., 1987) but had a dramatic effect on the expression of early myogenic regulators such as myogenin (Edmondston and Olson, 1989; Wright et al., 1989). Interestingly, the 3' UTR of other muscle-specific mRNAs have been demonstrated to stimulate myogenesis and to inhibit proliferation (Rastinejad and Blau, 1993; Rastinejad et al., 1993). They have been proposed to act in a feedback loop regulating growth and differentiation. However, the DMK 3' UTR had no effect on the growth rate of fibroblasts, suggesting that its effect is restricted to differentiation. Further analysis of the 3' UTR mapped the elements responsible for the observed phenotype to a conserved 239 bp sequence upstream of the CTG repeat in the 3' UTR, suggesting that the inhibition of differentiation is not mediated directly by the repeat but

rather through adjacent sequences.

Recently, Taneja et al., (1995) reported that the mutant DMK mRNA accumulated in the nuclei of fibroblasts and muscle cells isolated from DM patients suggesting that the CTG repeat alters the normal processing or metabolism of the mutant DMK mRNA. These results are consistent with our previous observations on the over-expression of mutant DMK transcripts in DM tissues (Sabourin et al., 1993). Therefore, cellular processes such as nuclear mRNA export may be significantly impaired by the presence of the amplified repeat. Furthermore, the presence of multiple DMK mRNA foci in the nuclei of affected cells (Taneja et al., 1995) suggest that the repeat may directly (or indirectly) confer increased stability to the mutant mRNA through the protective binding of nuclear export factors or through novel interactions between the CTG repeat and nuclear retention factors. Therefore, based on our observations, one possible mechanism is that the expanded repeat alters nuclear export of the mutant DMK mRNA leading to its accumulation in these nuclei. This increase in copy number of the mutant DMK transcript may lead to competitive interactions or a titration of protein factors involved in the expression or metabolism of differentiation-specific mRNAs through regions adjacent to the repeat. The expression of high levels of a wild type 3' UTR in our system may be similar, in part, to the nuclear accumulation of mutant DMK transcripts observed *in vivo*, thereby altering differentiation.

In myofibers, the nuclei located below the neuromuscular junctions have been

observed to specifically transcribe genes whose products are components of these junctions (Burden, 1993). Combined with the observation that DMK localizes to neuromuscular junctions (van der Ven et al., 1993; Whiting et al., 1995), the accumulation of mutant DMK mRNA in these nuclei may be sufficient to alter the normal excitation-contraction mechanism (Caterall, 1991) or may affect the metabolism of other mRNAs through competitive interactions with nuclear factors leading to altered physiological responses in DM muscles (Harper, 1989).

Concluding Remarks

The data presented in this thesis documents that one of the effects of CTG trinucleotide repeat expansion in the DMK gene is to increase the steady state levels of the mutant DMK transcript in muscle tissues of a severely affected patients. However, protein analysis of similar muscle samples showed that DMK protein levels remained relatively unchanged. Therefore, the dominant inheritance pattern of DM may be mediated *in trans* at the mRNA level, not at the protein level.

Consistent with this hypothesis, over-expression of the DMK 3' UTR alone, independently of the coding region, in murine myoblasts inhibits the terminal differentiation of these cells *in vitro*. The sequences that mediate this phenotype were found to map upstream of the CTG repeat in the 3' UTR, suggesting that the repeat may directly or indirectly alter the stability of the mutant DMK mRNA and that the

over-expression of adjacent elements may be detrimental to the cells.

Our data strongly suggest that the DM phenotype may be mediated *in trans* by the 3' UTR of the gene. However, several immediate questions remain to be answered with respect to the mechanism underlying the pathophysiology of DM. One important aspect is whether the repeat prevents processing, transport or stability of the mutant transcript and whether the CTG repeat, or adjacent elements, compete with other mRNAs for the binding of specific factors involved in distinct functions of mRNA metabolism. Finally, it will be of importance to determine whether CTG amplification disrupts the specific binding of nuclear export factors or induces novel interactions with nuclear components. The observation that the DM phenotype appears to be mediated at the RNA level and the availability of *in vitro* muscle differentiation systems will help to answer these questions and to delineate the mechanism underlying myotonic dystrophy.

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