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EXPRESSION OF THE HUMAN MYOTONIC DYSTROPHY KINASE (DMK) GENE IN TRANSGENIC MICE

by

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ABSTRACT

Myotonic dystrophy (DM) is a phenotypically variable, neuromuscular disorder with a number of multisystemic effects. This autosomal dominant disease is associated with the expansion of a triplet (CTG) repeat, located in the 3'-untranslated region of the DM kinase (DMK) gene. The magnitude of CTG expansion in successive generations corresponds with increasing severity of DM (genetic anticipation). However, the identification of the mutation associated with DM has done little to resolve the molecular effect of CTG expansion on DMK expression or disease determination. To clarify the effect of triplet expansion, DMK protein levels were assessed by immunoblot analysis in DM patient skeletal muscle samples and normal matched controls using a polyclonal antibody generated in our laboratory. Protein levels were found to reflect a role of over-expression of DMK transcript and protein as a mechanism of disease. As a consequence, transgenic mice over-expressing the human DMK gene ("DMK transgenics") and a DMK minigene construct with 100 CTG repeats ("CTG transgenics") were generated in the presence of matrix attachment region (MAR) sequences.

DMK transgenic mice show substantial over-expression of human DMK transcript and protein in brain, skeletal muscle, tongue and eye, tissues typically affected in DM. Cryostat sections of skeletal muscle from DMK transgenic animals revealed diagnostic hallmarks of DM including sarcoplasmic masses, increased centronucleation and type I fiber atrophy. Additionally, primary myoblasts established from these mice showed reduced fusion potential indicating a delay or defect in myoblast differentiation.

Furthermore, similar to other effects documented in DM patients, retinal and behavioural alterations were also observed in DMK transgenics. CTG transgenic mice showed intergenerational trinucleotide repeat instability, the degree of which correlated with increasing DM-like pathology in skeletal muscle with each successive generation. These results indicate that over-expression of DMK in mice confers a pathology similar to that observed in DM suggesting that over-expression at the RNA and/or protein level plays a role in the human disease. Furthermore, trinucleotide instability in mice may allow for reproduction of the effects of genetic anticipation.

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LIST OF ABBREVIATIONS

α	Alpha
β	Beta
bp	Base pair
°C	Degree Celsius
CAT	Chloramphenicol transferase
cDNA	Complementary DNA
cm	Centimetre
CMV	Cytomegalavirus
dATP	Deoxyadenosine triphosphate
DM	Myotonic dystrophy
DMK	Myotonic dystrophy kinase
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylenediaminetetraacetic acid
γ	Gamma
kb	Kilobase
M	Molar
MAR	Matrix attachment region
min	Minute
mg	Milligram
MHC	Myosin heavy chain
mm	Millimetre
mM	Millimolar
mRNA	messenger RNA
ng	Nanogram
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
%	Percent
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SSC	Standard saline citrate
sec	Second
TBE	Tris-borate EDTA buffer
TNR	Trinucleotide repeat
μg	Microgram
μl	Microlitre
μm	Micrometre
UTR	Untranslated region
v/v	Volume per unit volume
w/v	Weight per unit volume

CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

The identification of a genetic defect facilitates the molecular diagnosis of the associated human hereditary disease. Studies involving the pathogenesis of a mutation on gene expression, regulation and development of disease increases our understanding of the biology of complex systems and ultimately leads to the assessment of therapeutic strategies. However, studies such as these are limited by the availability of patient and normal control tissues. Alternatively, *in vitro* results often do not faithfully represent *in vivo* conditions and effects. Clearly, an *in vivo* system which allows observation of an effect that can be extrapolated to the clinical and biological aspects of human disease would be invaluable. Such a system is possible by the creation of a transgenic animal model by the experimental introduction of foreign DNA into the germline such that the introduced gene or mutation is subject to the complete developmental program of the organism. A particular challenge, documented in this thesis, is the creation of a transgenic model for myotonic muscular dystrophy (DM), a common, phenotypically variable, neuromuscular disease caused by a novel class of mutation, an expanding trinucleotide repeat (TNR).

1.1 CLINICAL ASPECTS OF MYOTONIC DYSTROPHY:

Myotonic Dystrophy (DM), the most common form of a muscular dystrophy affecting approximately 1 in 8000 adults, is an autosomal dominant multisytemic disorder characterized by a complex and progressive clinical phenotype (Harper, 1989). DM shows a marked variability in expression of disease, even within families. Furthermore,

in successive generations, increasing severity of disease and earlier age of onset, termed genetic anticipation, has been observed (Howeler *et al.*, 1989). Nevertheless, two primary forms of DM have been documented.

The classical form of the disease manifests in early adult life as impaired muscle relaxation (myotonia) followed by progressive muscle weakness and wasting (Carpenter and Karpati, 1984; Harper, 1989). The dystrophic muscle changes occur predominately in distal limb muscles but also affect facial and neck muscles resulting in a characteristic myopathic or droopy facial configuration (Korenyi-Both, 1983). Along with muscular wasting and weakness, there is a loss of subcutaneous fat imparting a haggard appearance in DM patients (Korenyi-Both, 1983). In some cases, a mild phenotype may develop in middle age consisting of cataracts only with little to no muscle involvement (Harper, 1989). In addition, adult onset DM patients often suffer from other complications including cardiac conduction defects, smooth muscle malfunction (primarily of pulmonary and gastrointestinal muscles), endocrine dysfunction, retinal degeneration, ovarian and testicular atrophy, hypersomnia, sensory loss and mental and cognitive impairment (Adams, 1975; Harper, 1989; Korenyi-Both, 1983). Typically, adult DM patients survive to 50 to 60 years of age with death occurring as a result of pulmonary infection or cardiac arrest (Korenyi-Both, 1983).

Congenital DM is a severe form of the disease with such a different clinical presentation that it was once considered a different disorder all together until it was recognized that affected infants were invariably born to mothers with DM (Vanier, 1960). This form of DM is characterized by extreme, generalized muscular hypotonia and

weakness, feeding difficulties due to weak jaw muscles and a high neonatal mortality due to respiratory distress (Harper, 1989; Vanier, 1960). Additionally, congenital DM patients suffer from diaphragmatic paralysis, decreased gastrointestinal motility, electrocardiographic abnormalities, facial displegia and thinness of the ribs. Patients that survive the neonatal period exhibit a delay in muscle development, mental retardation and the classical form of the disease in adolescence (Harper, 1989; Korenyi-Both, 1983). The congenital form of DM is almost exclusively maternally inherited (Harper, 1989). The identification of the mutation has helped to explain at least some of these observations.

1.2 IDENTIFICATION OF THE DM MUTATION:

The genetic basis of DM was mapped using a traditional positional cloning approach to an unstable genomic fragment located within a genetically and physically defined candidate region on chromosome 19q13.3 (Aslandis *et al.*, 1992; Buxton *et al.*, 1992; Harley *et al.*, 1992; Jansen *et al.*, 1992a; Shutler *et al.*, 1992). This fragment was found to increase in size in DM patients only. Moreover, the increase in size correlated with severity of disease. Sequence analysis of this genomic fragment revealed a trinucleotide (CTG) repeat located in the 3'UTR of a gene predicted to encode a serine-threonine kinase (Brooke *et al.*, 1992; Fu *et al.*, 1992; Mahadevan *et al.*, 1992). The size variation of this fragment is due to changes in the number of CTG repeats which forms the mutational basis for manifestation of DM. The gene product has come to be known as myotonin protein kinase (Fu *et al.*, 1992), DM-protein kinase (DMPK; Jansen *et al.*, 1993; Shaw *et al.*, 1993) or for the purposes of this thesis, DM kinase (DMK; Mahadevan

et al., 1993; Sabourin *et al.*, 1993).

1.3 CTG EXPANSION AND DISEASE:

The magnitude of amplification of the CTG repeat has been shown to correspond to severity of DM (Harley *et al.*, 1993; Hunter *et al.*, 1992; Tsilfidis *et al.*, 1992). Furthermore, expansion was found to increase in successive generations, leading to the term "dynamic mutation". When correlated with earlier age of onset and increased severity of disease, CTG instability proved to be the molecular basis for genetic anticipation (Brooke *et al.*, 1992; Fu *et al.*, 1992; Howeler *et al.*, 1989; Mahadevan *et al.*, 1992).

In the normal population, the CTG repeat is polymorphic and varies from 5 to 35 repeats (Brunner *et al.*, 1992; Harley *et al.*, 1993; Hunter *et al.*, 1992; Tsilfidis *et al.*, 1992). Allele sizes in the range of 40 to 80 CTG repeats are associated with an asymptomatic to mild state whereby pre-senile cataract is usually the only indicator of disease (Harley *et al.*, 1992; Redman *et al.*, 1993). Moreover, these allele sizes are stably transmitted for a number of generations (Barcelo *et al.*, 1993). Unstable CTG transmission is obvious above 80 repeats. Patients with the classical form of DM typically carry 100 to 1000 CTG repeats whereas congenital DM patients have larger expansions in the range of 500-2000 repeats (Harley *et al.*, 1993; Hunter *et al.*, 1992; Tsilfidis *et al.*, 1992). There is significant overlap between repeat sizes making an absolute correlation between disease state and CTG repeat size unreliable (Brunner *et al.*, 1993; Novelli *et al.*, 1993; Thibault *et al.*, 1993). However, reported cases of repeat

contractions have resulted in inherited milder DM phenotypes as a consequence of smaller CTG size (Brunner *et al.*, 1993; O'Hoy *et al.*, 1993; Shelbourne *et al.*, 1992).

Unstable CTG transmission from an affected parent to offspring (intergenerational or meiotic instability) can result in a 20-fold trinucleotide repeat (TNR) expansion in a single generation (Harley *et al.*, 1993). Maternal transmission is particularly associated with large CTG expansions perhaps accounting for the observation that congenital DM is inherited primarily from affected mothers (Harley *et al.*, 1993; Lavedan *et al.*, 1993; Mulley *et al.*, 1993; Tsilfidis *et al.*, 1992). In contrast, paternal transmission results in a larger percentage of repeat contractions (Brunner *et al.*, 1992; Shelbourne *et al.*, 1992). Moreover, intergenerational expansion of alleles smaller than 100 CTG repeats is more likely a result of paternal transmission (Brunner *et al.*, 1992).

Instability does not occur only during meiosis. Heterogeneity of the CTG repeat was first observed upon genetic analysis of the expanding genomic fragment in affected individuals (Aslandis *et al.*, 1992; Buxton *et al.*, 1992; Harley *et al.*, 1992). This phenomenon was observed in a variety of tissues reflecting mitotic or somatic instability. CTG expansions are consistently larger in muscle than in blood although there is no correlation between repeat size in muscle and disease severity (Anvret *et al.*, 1993; Ashizawa *et al.*, 1993; Thornton *et al.*, 1994; Zatz *et al.*, 1995). However, somatic instability may contribute to the variable phenotypic expression of DM (Wieringa, 1994). Continued CTG expansions have been detected in blood as DM patients age (Martorell *et al.*, 1995; Wong *et al.*, 1995). Significantly, CTG repeat sizes are often much larger in the blood of affected offspring than in the sperm of the affected father confirming the

continued somatic instability during development (Wieringa, 1994). In contrast, CTG expansions do not appear to continue in muscle past early adulthood suggesting that most somatic instability in terminally differentiated cell types occurs early, perhaps in embryogenesis (Thornton *et al.*, 1994 ; Kinoshita *et al.*, 1996)

1.4 CHARACTERIZATION OF THE DM LOCUS:

The DMK gene is comprised of 15 exons covering an area of approximately 14 kilobases (kb) (Figure 4-1). The cDNA encodes a 629 amino acid protein with a predicted weight of approximately 69 kDa. The predicted DMK protein was found to be unique containing a leucine-rich motif in exon 1 and domains of homology to: the serine-threonine family of protein kinases from exons 2-8, the α-helical coil forming segment of filamentous proteins within exons 9-12 and a hydrophobic sequence predicted to a act as a transmembrane anchor in exon 15 . Serine-threonine kinase activity has since been demonstrated *in vitro* using histone H1 as a substrate (Dunne *et al.*, 1994). Since the identification of DMK, kinases that appear to belong to the same class as DMK have been isolated. These include human kinase PK428, Rho-binding kinases Rokα and β, *C. elegans* rho-kinase homologue LET-502, Cot-1 from *Neurospora crassa* and warts from *Drosphila* (Justice *et al.*, 1995; Leung *et al.*, 1996; Wissman *et al.*, 1997; Yarden *et al.*, 1992). Some of these homologous kinases have been characterized. Warts appears to function as a tumor suppressor, Cot-1 is a temperature sensitive growth mutant and Rho-binding kinases modulate smooth muscle contractions and stress-fiber formation. The function of PK428, as well as DMK protein, is still unknown.

The DMK gene is highly conserved between human and mouse with 90% identity at the nucleotide level (Jansen *et al.*, 1992b). Although there is some homology in 3'UTR sequences shared by both of these species, the CTG repeat in the mouse is only 5 repeats long, composed of three CTG repeats and two CAG repeats, and is not polymorphic between mouse strains. Spliced isoforms of DMK from human and mouse cDNA libraries have been isolated (Fu *et al.*, 1992; Jansen *et al.*, 1992b; Mahadevan *et al.*, 1992). cDNAs corresponding to the splicing of exon 1 (Fu *et al.*, 1992) and exons 13-14 (Mahadevan *et al.*, 1992) have been predominantly identified in human. However, only the full-length transcript has been detected by northern blot (Brooke *et al.*, 1992; Jansen *et al.*, 1992b; Sabourin *et al.*, 1993).

The cloning of the DM locus (Mahadevan *et al.*, 1992; Shaw *et al.*, 1993) has resulted in the identification of two close flanking genes, DMR-N9 (or 59; Jansen *et al.*, 1995) and DMAHP (Boucher *et al.*, 1995). DMR-N9 lies immediately upstream of DMK. It is composed of 5 exons, encoding a 68 kDa protein with unknown function. Due to its expression in brain and testes, it was thought that it too may be affected by expansion of the CTG repeat and thereby contribute to the multisystemic effects of DM (Jansen *et al.*, 1995). However, no differences in expression have been documented in DM patients. DMAHP or DM locus-associated homeobox domain protein lies downstream of the DMK gene (Boucher *et al.*, 1995). It is composed of 3 exons predicted, by homology, to encode a homeodomain protein. Using RT-PCR, DMAHP transcripts were detected in skeletal muscle, heart and brain. Due to its location with respect to DMK, the CTG repeat is likely to lie within its 5'UTR. However, no

differences in DMAHP expression have been reported in DM patients. Isolation of a cDNA clone is still pending.

1.5 TNR DISEASES:

It was not long ago when the first description of an expanding TNR was attributed as being the cause of phenotypically variable disorders: fragile X (FRAXA) and spinal and bulbar muscular atrophy (SBMA), (Fu *et al.*, 1991; LaSpada *et al.*, 1991; Verkerk *et al.*, 1991). The dynamic nature of the TNRs was evident because of a detectable increase in the magnitude of expansion of the trinucleotide repeats in successive generations. These expanding trinucleotide repeats have now been associated with an ever-increasing list of neurodegenerative genetic diseases (Table 1-1). Fragile X mental retardation (FRAXA and FRAXE), spinal and bulbar muscular atrophy or Kennedy's disease (SBMA), myotonic dystrophy (DM), Huntington's disease (HD), the spinocerebellar ataxias (SCA1, 2 and 3), dentatorubral pallidolysian atrophy (DRPLA) and Machado-Joseph disease (MJD) all result from the expansion of a CG-rich trinucleotide repeat that is present in the mature transcript (Brooke *et al.*, 1992; Fu *et al.*, 1991; Fu *et al.*, 1992; Ikeda *et al.*, 1996; Imbert *et al.*, 1996; ; Knight *et al.*, 1993; Koide *et al.*, 1994; LaSpada *et al.*, 1991; Mahadevan *et al.*, 1992; Nagafuchi *et al.*, 1994; Orr *et al.*, 1993; Pulst *et al.*, 1996; Sanpei *et al.*, 1996; The Huntington's Disease Collaborative Group, 1993; Verkerk *et al.*, 1991). Whereas somatic and intergenerational instability of the TNR is observed in affected individuals, the triplet repeat remains stable and polymorphic in length among normal individuals. Furthermore, the degree of amplification of this mutation has been

Table 1-1: Human Disorders Caused by an Unstable Trinucleotide Repeat (TNR)

Disorder	Trinucleotide Repeat (TNR)	Repeat Location	TNR Size	
			Normal Range	Disease Range
Fragile X Syndrome (FRAXA)	CGG	5'UTR	6-54	200-1000
Fragile XE (FRAXE)	CGG	5'UTR	5-45	200-1000
Friedreich's Ataxia (FRDA)	GAA	Intron 1	10-21	200-900
Kennedy's Disease (SBMA)	CAG	Coding	13-30	30-62
Spinocerebellar Ataxia (SCA1-3)	CAG	Coding	25-36	43-81
Huntington's Disease (HD)	CAG	Coding	9-37	37-121
Dentatorubral-pallidoluysian Atrophy (DRPLA)	CAG	Coding	10-26	35-78
Machado-Joseph Disease (MJD)	CAG	Coding	10-31	42-90
Myotonic Dystrophy (DM)	CTG	3'UTR	5-37	50-3000

demonstrated to be correlated to severity of the disorder and age of onset. Expansion also provides the molecular basis of observed anticipation in these disorders whereby an increase in pathogenesis of disease is observed in successive generations. An exception to this is the recently identified GAA repeat responsible for Friedreich's ataxia (FRDA), a autosomal recessive neurodegenerative disease that involves the central and peripheral nervous systems and heart. This TNR is found within the first intron of the frataxin gene and dramatic genetic anticipation is not observed (Campuzano *et al.*, 1996).

Five of these diseases, SBMA, HD, SCA1-3, DRPLA and MJD are all neurodegenerative disorders affecting the central nervous system and all result from the expansion of a TNR within the coding region. In each case, the repeat in question is a CAG triplet that forms a polyglutamine tract (Ikeda *et al.*, 1996; Imbert *et al.*, 1996; Kawaguchi *et al.*, 1994; Koide *et al.*, 1994; LaSpada *et al.*, 1991; Nagafuchi *et al.*, 1994; Orr *et al.*, 1993; Pulst *et al.*, 1996; Sanpei *et al.*, 1996; The Huntington's Disease Collaborative Research Group, 1993). This tract has since been shown to induce apoptosis or cell death in cultured neurons (Ikeda *et al.*, 1996; Nasir *et al.*, 1996). In SBMA, expansion of the TNR results in disruption in the normal function of the androgen receptor (AR) protein causing disease (LaSpada *et al.*, 1991). A similar situation is postulated for HD, SCA1-3, DRPLA and MJD but as these respective proteins are unique and share no homology with other known proteins, the effect of amplification of the CAG is still unknown.

Interestingly, TNR expansion for these disorders appears to be limited to between 42-100 repeats, possibly due to intolerance and lethality of resulting protein inactivation.

Similarly, in the normal population, alleles up to 35 repeats have been reported for these diseases which indicates the extent of expansion that does not affect protein function. The degree of amplification and intergenerational instability of the TNR has been clearly demonstrated to be correlated to the severity of the disorder and age of onset for SBMA, HD, SCA1-3, DRPLA and MJD (Andrew *et al.*, 1993; Ikeda *et al.*, 1996; Imbert *et al.*, 1996; Kawaguchi *et al.*, 1994; Koide *et al.*, 1994; LaSpada *et al.*, 1991; Nagafuchi *et al.*, 1994; Orr *et al.*, 1993; Pulst *et al.*, 1996; Sanpei *et al.*, 1996). This suggests that the amplification of the TNR represents the molecular mechanism of anticipation observed with these particular disorders. Knock-out mice made nullizygous for the HD gene were found to show significant loss of neuronal cells in the subthalmic nucleus of the brain indicating the importance of the HD gene product, Huntingtin (Nasir *et al.*, 1995). However, these mice showed no signs of chorea, implying that the knock-out was not an authentic model of the human disease. Recently, transgenic models over-expressing mutant gene products for SCA1 and HD have resulted in replication of disease in mouse (Burright *et al.*, 1995; Mangiarini *et al.*, 1996). Furthermore, instability of the over-expressed TNR has been observed in this HD model. Neither disease nor instability was detected in a transgenic model for SBMA (Bingham *et al.*, 1995).

The other category of TNR associated diseases are expansions that are located in non-coding sequences. FRAXA (the most commonly inherited form of mental retardation) and DM (the most common form of inherited neuromuscular disease in adults) are associated with amplification of a TNR in the 5' untranslated region (UTR) and 3'UTR, respectively (Brook *et al.*, 1992; Fu *et al.*, 1991; Fu *et al.*, 1992; Harper,

1989; Mahadevan *et al.*, 1992; Verkerk *et al.*, 1991). Each disorder is characterized clinically by great phenotypic variability. Likewise, the extent of amplification in affected individuals ranges from intermediate allele sizes associated with carrier-like or mildly affected states (known as pre- or proto-mutation) that are stably transmitted to highly variable alleles sizes over 1000 repeats associated with severely affected states (Harley *et al.*, 1993; Hunter *et al.*, 1992; Redman *et al.*, 1993; Tsilfidis *et al.*, 1992). As a consequence, anticipation is observed whereby amplification of the TNR manifests itself as an increase in number of affected individuals in successive generations in FRAXA and increased severity of disease, such as earlier age of onset in DM (Harley *et al.*, 1993; Hunter *et al.*, 1992; Oberle *et al.*, 1991; Tsilfidis *et al.*, 1992). Mechanistically, amplification of the TNR in FRAXA appears to result in variable transcriptional repression of the FMR-1 gene due to abnormal hypermethylation of a CpG island proximal to the expanded triplet (Bell *et al.*, 1991; Oberle *et al.*, 1991). The FMR-1 gene product has since been shown to be a RNA binding protein that associates with ribosomes (Ashley *et al.*, 1993; Eberhart *et al.*, 1996). FMR-1 deficient mice were found to exhibit a number of changes similar to FRAXA including learning deficits and macroorchidism (The Dutch-Belgium Fragile X Consortium, 1994). For FRDA, the intronic expansion of a TNR is thought to prevent expression of the frataxin gene (Campuzano *et al.*, 1996). FRDA patients have been found to have little if any frataxin message. A point mutation, proposed to result in a truncated product, has also been identified in some patients indicating that a reduced level of frataxin is responsible for disease. In contrast, the disease phenotype in DM is likely to be mediated by a different mechanism since the

unstable CTG repeat lies in the 3' UTR of a serine-threonine kinase.

1.6 EFFECT OF THE DM MUTATION ON DMK EXPRESSION

1.6.1 DMK TRANSCRIPT LEVELS:

Consistent with the primary involvement of muscle tissues in DM, DMK transcript levels were found by northern blot analysis to be most highly expressed in heart and skeletal muscle (Brook *et al.*, 1992; Jansen *et al.*, 1992b; Sabourin *et al.*, 1993). Lower levels were found in brain, liver, kidney, spleen and in tissues containing smooth muscle such as intestine, lung, stomach and bladder. Using *in situ* hybridization, DMK transcript was first detected on day 10.5 in the myotome restricted region of the somites in mouse embryo (Jansen *et al.*, 1996). By day 14.5 of embryogenesis, DMK is expressed in all smooth and striated muscle cells and its expression remains high throughout maturation. The first signs of expression in the central nervous system (CNS; retina, cerebellum and hippocampus) are not observed until 14 days post-natal.

In DM patients, DMK expression at the transcriptional level has been found to be altered although the exact effect is still controversial. Using RT-PCR based methods, reduced expression of mutant DMK transcript has been shown in total RNA samples from adult patients (Fu *et al.*, 1993). Furthermore, the effect of the normal allele, in *trans*, was found to be negligible as a somatic cell hybrid containing chromosome 19 from an adult patient was found to express reduced levels of mutant DMK (Carango *et al.*, 1993). By RT-PCR and northern blot analysis of total RNA, reduced expression of mutant and normal transcripts was observed in congenital DM patient samples (Hoffman-

Radvanyi *et al.*, 1993). The reduction in normal transcript levels suggested that the mutant transcript can exhibit an effect in *trans*. DMK transcript levels were found to be reduced in total RNA samples isolated from adult DM patient muscle samples and myopathic control samples suggesting that the reduction was due to disease pathogenesis rather than a result of CTG expansion (Wang *et al.*, 1995). However, a significant reduction in normal and mutant poly A+ DMK transcripts was observed in patient samples compared to controls. Reduction in the normal poly A+ fraction presented further evidence for an effect of the mutant transcript on normal DMK transcript levels in *trans* (Wang *et al.*, 1995). Furthermore, in a survey of adult and congenital samples, no change was observed in unprocessed normal and mutant transcripts (Bhagwati *et al.*, 1996; Krahe *et al.*, 1995). However, processed mutant transcript was reduced in patient samples (Krahe *et al.*, 1995). This consistent reduction has led to the proposal of haploinsufficiency as a mechanism of disease (Fu *et al.*, 1993).

In contrast to these reports, an increase in total transcript levels was detected in a variety of tissues from congenital DM patients using slot-blot analysis and competitive PCR (Sabourin *et al.*, 1993). Moreover, mutant transcripts were detected by northern blot. As no change was observed in normal transcript levels from DM patients and normal controls, the increase in total transcript levels was inferred to represent an overall increase in mutant DMK transcript levels. Mutant DMK transcripts have also been shown to be expressed by *in situ* hybridization techniques (Taneja *et al.*, 1995). Additionally, they were found to accumulate in discrete foci in the nuclei of fibroblasts and muscle biopsies isolated from DM patients. However, the cytoplasmic distribution of

DMK transcripts appeared to be identical between normal and patient samples. These investigators suggested that the presence of an expanded CTG repeat in the mutant transcript leads to its increased accumulation or over-expression due to enhanced transcription or stability or to impaired RNA processing or export (Sabourin *et al.*, 1993; Taneja *et al.*, 1995). Furthermore, over-expression, particularly in the nuclear fraction, could result in a gain in function leading to the onset of DM.

Certain inconsistencies observed surrounding the effect of CTG expansion on DMK transcript expression may be attributed to a number of factors. First, techniques used to assay transcript levels were different. Early reports relied on quantifying transcript levels using RT-PCR through or close to the repeat sequence in order to differentiate mutant and normal alleles (Fu *et al.*, 1993; Carango *et al.*, 1993; Hoffman-Radvanyi *et al.*, 1993). Inefficient polymerization through a region prone to the formation of secondary structures is reasonable to expect and may have led to a reduced yield in PCR product. For northern blot, the ability to isolate large transcripts is hampered by the techniques used and the condition of samples from which RNA is to be isolated (Roses, 1994). Tissue samples are mostly obtained from patients who die as a result of DM and the condition of these samples can affect results. Furthermore, the inability in obtaining suitable controls can also be a source of discrepancy. This was most evident with the results of Wang *et al* (1995a) who reported reduced DMK transcript levels in DM tissues as well as in myopathic control samples suggesting that myopathy and not DM is causing this difference in DMK message levels. Moreover, heterogeneity of mutant transcripts predicted by the mitotic instability of the genomic

sequence would decrease the resolution of these transcripts below the point of detection (Sabourin *et al.*, 1993). The type of tissue assayed may affect results. Comparing levels of DMK transcript in tissues typically affected by DM, such as skeletal muscle and cardiac tissues, rather than somatic cell hybrids, may present more reliable indicators of an effect of CTG expansion on DMK expression. Finally, differences in DMK transcript levels between adult and congenital patient samples may reflect different mechanisms for these two forms of disease.

Nevertheless, many of these results can be consolidated to propose a model in agreement with the dominant nature of DM. The *in situ* experiments clearly demonstrate that the mutant allele is expressed (Taneja *et al.*, 1995). The reduced levels of processed mutant transcript (Krahe *et al.*, 1995) and normal and poly A+ transcripts (Wang *et al.*, 1995) that are observed may be due to the accumulation of the mutant transcript in the nucleus. The reduction of normal unprocessed DMK transcript (Hoffman-Radvanyi *et al.*, 1993) and normal processed DMK transcripts (Wang *et al.*, 1995) suggests that the presence of a CTG expansion on a DMK transcript may elicit an effect in *trans*, representing a gain of function. If so, DMK mutant transcripts may also affect other transcripts which may lead to the multisystemic effects associated with DM. Dependence of this observed DMK transcript over-expression or accumulation on CTG repeat size would ultimately explain its correlation with disease severity. The relevance of haploinsufficiency itself on disease state requires determination of the DMK protein levels in DM affected tissues.

1.6.2 DMK PROTEIN LEVELS:

A number of antisera against DMK protein, have been generated independently. The first antibodies used to detect DMK protein were developed against peptides derived from different regions of the protein. Authenticity was suggested by the detection of species ranging between 42-62 kDa primarily in skeletal muscle and heart (Fu *et al.*, 1993; Koga *et al.*, 1994, Tachi *et al.*, 1995; van der Ven *et al.*, 1993). However, although the size of the major species detected by these peptide antisera were similar, they were considerably smaller than that predicted by primary structure. The difference in size was suggested to be due to alternative initiation or splicing (Fu *et al.*, 1993; Timchenko *et al.*, 1995). In the interim, our laboratory generated a polyclonal antiserum against a C-terminal fragment of the DMK protein (Whiting *et al.*, 1995). Two species (74 and 82 kDa) were detected with preferential expression of the 82 kDa species in skeletal muscle and predominance of the 74 kDa species in cardiac and brain. Using a monoclonal antibody generated against full-length DMK protein, Dunne *et al.* (1996) reported DMK species of 64 kDa in muscle and 79 kDa in brain. These DMK isoforms are larger than predicted suggesting that they are derived by alternative splicing and/or post-translational modification (Maeda *et al.*, 1995; Waring *et al.*, 1996).

A number of these antibodies have been used for localization studies of DMK protein in various tissues. Although the antisera used were generated by a variety of means and detected different sized species by immunoblot analysis, the immunofluorescent distribution patterns were quite similar. In skeletal muscle, prominent staining was observed in immature myotubes (van der Ven *et al.*, 1993; Tachi

et al., 1995), in type I fibers of mature muscle (van der Ven *et al.*, 1993; Dunne *et al.*, 1996), in the I-band which is composed of thin actin filaments (Dunne *et al.*, 1996; Neville, 1979) and in the post-synaptic neuromuscular junction (Tachi *et al.*, 1995; van der Ven *et al.*, 1993; Whiting *et al.*, 1995). In cardiac muscle, DMK protein was localized to intercalated discs (van der Ven *et al.*, 1995; Whiting *et al.*, 1995). DMK protein, in brain, was found in most neurons (van der Ven *et al.*, 1993), ventricular ependymal and choroid plexus cells and discrete neural populations in the hippocampus, cerebellum and medulla (Whiting *et al.*, 1995). These data suggest that DMK protein is involved in synaptic transmission and/or cell structure integrity.

In DM patients, DMK protein was found to be relocalized to sarcoplasmic masses, a histological hallmark of DM, in type I fibers of skeletal muscle (Dunne *et al.*, 1996). Another study surveying affected tissues observed no difference in the distribution or intensity of DMK protein staining by immunfluorescence (van der Ven *et al.*, 1993). By immunoblot analysis, reduced levels (Fu *et al.*, 1993; Koga *et al.*, 1994; Maeda *et al.*, 1995), unaltered (Bhagwati *et al.*, 1996) and slightly enhanced levels (Dunne *et al.*, 1996) of DMK protein candidate species have been reported. However, at least one of these candidate species has since been found to exhibit no kinase activity (Fu *et al.*, 1993; Timchenko *et al.*, 1995). Another failed to display the appropriate pattern of expression in DMK deficient and transgenic mice over-expressing human DMK (van der Ven *et al.*, 1993; Jansen *et al.*, 1996). Aside from difficulties due to questions of authenticity of antisera, studies assessing DMK protein levels in DM patients are complicated by the variable destructive effects of disease on affected tissues (Roses, 1994). Future studies

with the appropriate controls will be required to validate antisera in order to determine the effect of CTG expansion on DMK protein levels in patients. Resolving this effect is a necessary step to understanding disease pathophysiology.

1.7 OTHER EFFECTS OF CTG EXPANSION:

1.7.1 CHROMATIN STRUCTURE:

Fragile X, caused by the expansion of a CGG repeat in the 5'UTR of FMR-1, occurs as a result of transcriptional repression due to hypermethylation of a CpG island proximal to the expanded TNR (Bell *et al.*, 1991; Oberle *et al.*, 1991). The CTG repeat in DM also lies close to a CpG island but no evidence of abnormal methylation patterns in this region has been detected in affected individuals (Shaw *et al.*, 1993). However, CTG expansion does lead to increased resistance to DNase I treatment directed at a hypersensitive site downstream of the repeat suggesting that a change is induced in chromatin structure (Otten and Tapscott, 1995). Furthermore, CTG repeats have been demonstrated to be the strongest enhancers of nucleosome assembly in a repeat size dependent manner *in vitro*, providing further evidence of an effect of CTG expansion on chromatin structure (Wang and Griffith, 1995). Although altered expression of the neighbouring genes DMR-N9 (or 59) and DMAHP has not yet been found, a change in chromatin structure could affect expression of these and/or other genes in the region (Boucher *et al.*, 1995; Jansen *et al.*, 1995; Otten and Tapscott, 1995).

1.7.2 ROLE OF 3'UTR:

To date, DM remains the only TNR disease associated with a dynamic mutation in the 3'UTR of a gene. A number of regulatory roles have been attributed to 3'UTR sequences including: mRNA stability, processing, translation, localization and export (Curtis *et al.*, 1995; Jackson *et al.*, 1995). Furthermore, cellular differentiation processes mediated by *cis*-acting elements located in the 3'UTR have been described. These elements may act on their own or function by binding protein factors or short antisense mRNAs (Rastinejad and Blau, 1993; Rastinejad *et al.*, 1993). Ablation or creation of any one of these functions, by an expanded CTG repeat, could be responsible for disease. It has been demonstrated that TNRs have the capacity to form hairpin structures that could increase message stability (Gacy *et al.*, 1995). Moreover, a novel heterogeneous ribonucleoprotein (hnRNP) has been identified which binds CUG repeats (Timchenko *et al.*, 1996). This protein is localized primarily in the nucleus and may play a role similar to other hnRNPs in export, translation and turnover of mRNAs (Pinol-Roma and Drefuss, 1992). In DM patients, it has been postulated that CTG expansion could result in increased binding of this protein (Timchenko *et al.*, 1996). As a consequence, this novel CUG binding hnRNP could become sequestered by the triplet repeat, preventing its normal cellular function and/or may cause increased stability or accumulation of DMK transcripts.

1.7.3 SKELETAL MUSCLE DIFFERENTIATION:

Skeletal muscle is composed of various fiber types with different contractile

properties. These fibers are classified as type I (slow twitch) or type II (A, B, X; fast twitch) based on myosin heavy chain (MHC) isoform content and their usage of oxidative enzymes (Dubowitz, 1985; Harper, 1989). Muscle fibers arise from the fusion of mononucleated myoblasts in response to the activation of the myogenic regulators MyoD, myogenin, myf5 and MRF4 (Braun *et al.*, 1989; Davis *et al.*, 1987; Edmonson and Olson, 1989; Miner and Wold, 1990; Rhodes and Konieczny, 1989; Wright *et al.*, 1989). MyoD family members share significant homology with each other (Lassar *et al.*, 1989; Lassar *et al.*, 1991; Olson and Klein, 1994; Thayer and Weintraub, 1993). Importantly, they also share homology to the basic helix-loop-helix (bHLH) domain of *c-myc* that is necessary for binding to DNA following activation by heterodimerization via the HLH domain. These regulators bind to a consensus DNA sequence known as an E box (CANNTG) found in most muscle-specific genes including myosin heavy chain (MHC; Olson and Klein, 1994; Weintraub *et al.*, 1991). When expressed ectopically in nonmuscle cells, each of these regulators is capable of inducing myogenesis (Weintraub *et al.*, 1989). Furthermore, it has been shown that MyoD mediates myoblast growth arrest which is a prerequisite to terminal differentiation (Crescenzi *et al.*, 1990).

Determining the various expression patterns of these myogenic regulators has done little in determining their specific roles. In cell culture, MyoD or myf5 is expressed in myoblasts (Weintraub *et al.*, 1993). Upon induction of differentiation, myogenin is up-regulated and continues to be expressed in myotubes followed by MRF4 expression 2-3 days later (Edmondson and Olson, 1989; Rhodes and Konieczny, 1989). *In vivo*, the pattern of expression in mouse is quite different. The first regulator to be expressed is

myf5 at embryonic day (*e*day) 8 in the somites, a cell lineage that leads to the eventual formation of skeletal muscle, dermis and cartilage. Myogenin is detected in the somites at day 9.5 of embryogenesis. MyoD is expressed beginning at *e*day 10.5 throughout development while MRF4 is transiently expressed from *e*day 10 to 12 and re-expressed at *e*day 16 to maturity (Olson and Klein, 1994). The differential expression of each of these factors suggests that each has a specific role in myogenesis.

The generation of mice deficient for the members of the MyoD family has led to a better understanding of the specific function of each of them. Most surprisingly, MyoD deficient mice show no obvious abnormality. However, a significant increase in myf5 expression is seen indicating a redundancy in function between these two factors and negative regulation of myf5 by MyoD (Rudnicki *et al.*, 1992). Myf5 deficient mice have normal muscle but die at birth as a result of a rib defect (Braun *et al.*, 1992). MyoD/myf5 deficient mice lack muscle completely (Rudnicki *et al.*, 1993). Myogenin null mice have very few myofibers and as a consequence do not survive past birth (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993). Finally, MRF4 deficient mice have normal muscle, some rib abnormalities and express myogenin at high levels suggesting that MRF4 negatively regulates myogenin (Zhang *et al.*, 1995). These results suggest that: MyoD or myf5 is necessary for activation of myoblast differentiation, myogenin is required for myoblast fusion and MRF4 is important for myofiber maturation.

In DM, myopathic-specific manifestations have been thought to be due to a developmental delay or immaturity of skeletal muscle fibers in contrast to the degenerative/regenerative processes associated with Duchenne muscular dystrophy

(Carpenter and Karpati, 1984; Dubowitz, 1985; Iannacconne *et al.*, 1986; Sarnat and Silbert *et al.*, 1976). A number of *in vitro* studies have indicated that DMK expression has a role in myogenesis. Recently, it has been found that P19 stem cells (a mouse pluripotent embryonic carcinoma cell line) that express MyoD also express DMK *de novo* (Skerjanc *et al.*, 1994). Moreover, E boxes located within the first intron of the DMK gene have been shown to be necessary for DMK expression in myoblasts (Storbeck *et al.*, 1997). Fibroblast BC3H1 cells stably expressing DMK exhibit up-regulation of the skeletal muscle specific proteins β-tropomyosin, myogenin and muscle-type creatine kinase (Bush *et al.*, 1996). Activation of these skeletal muscle components may explain the observed myoblast-like morphology of these cells. Finally, over-expression of DMK in mouse myoblast C2C12 cells results in inhibition of terminal differentiation (Sabourin, 1996). Deletion analysis indicates that over-expression of only the 3'UTR of DMK is sufficient to prevent myoblast fusion. Interestingly, the 3'UTR of other muscle specific genes have been shown to stimulate myogenesis and to inhibit proliferation (Rastinejad and Blau, 1993; Rastinejad *et al.*, 1993). These observations suggest that the expression of DMK in skeletal muscle appears to be relevant as it may play a role in myogenesis and perhaps ultimately in disease.

1.8 ANIMAL MODELS:

Although naturally occurring mutations resulting in myotonia have been identified in goat and mouse, neither model appears to be the result of a defect in an orthologous DMK gene showing a similar pattern of inheritance with similar progressive dystrophic

features (Burns et al., 1965; Harper, 1989; Kolb, 1938; Wischmeyer *et al.*, 1993). A mutant (LWC) strain of Japanese quail (*Coturnix coturnix japonica*) however, appears to share a number of features with DM patients including an autosomal dominant mode of disease inheritance, myotonia and muscle weakness (Braga *et al.*, 1995). Moreover, histological changes of skeletal muscle characteristic of DM were noted including ringed fibers, sarcoplasmic masses and an increase in centronucleation. However, investigators did not observe a reduction in size (or atrophy) of type I muscle fibers. They did observe testicular atrophy and cataracts consistent with the multisystemic effects of DM. However, the precise genetic defect in the quail model remains unknown.

Transgenic and gene targeting technologies have been utilized to better understand the role of DMK in DM pathogenesis. Recently, transgenic mice expressing the human DMK gene have been reported (Jansen *et al.*, 1996). No skeletal muscle myopathy was observed nor were changes in MHC composition or myotonia. However, transgenic mice with the highest levels of human DMK expression developed a cardiac myopathy but did not exhibit fatty infiltration or fibrosis that normally accompanies heart defects in DM patients.

In addition, two knock-out mouse models for DMK have been produced, both of which were generated by disrupting the kinase domain of the endogenous mouse DMK gene (Jansen *et al.*, 1996; Reddy *et al.*, 1996). In one of these models, MHC composition, fiber type distribution and size and myotonic profiles of homozygous DMK null mutant mice were no different from wild-type controls (Jansen *et al.*, 1996). Only inconsistent and minor changes in fiber size were observed in neck and head muscles in

older mice. No changes were detected in other organs normally affected by DM. Alternatively, in adult homozygous null mouse muscle derived from the other model, minor changes to fiber size, increased fiber degeneration, fibrosis and changes to muscle ultrastructure were observed (Reddy *et al.*, 1996). In agreement with fiber degeneration/regeneration processes, MyoD and embryonic myosin levels were elevated. However, DMK deficient mice that do show signs of mild myopathy do not have the characteristic skeletal muscle histological changes, nor other systemic involvement associated with DM (Harper, 1989; Korenyi-Both, 1983; Roses, 1979).

The first reports of TNR instability in transgenic mice have recently been documented. Transgenic mice were generated containing 55 (Gourdon *et al.*, 1997) and 162 CTG repeats (Monckton *et al.*, 1997) and in each case, instability was observed in a number of independent lines. Somatic instability was observed in these models even though the genomic context in which the repeats are integrated differ (Gourdon *et al.*, 1997; Monckton *et al.*, 1997). Furthermore, intergenerational instability was observed by Gourdon *et al* (1997) in mice containing 55 CTG repeats in the context of a human genomic fragment containing the entire DMK gene, DMR-N9 (or 59) and DMAHP. CTG expansions and contractions were detected in both models although the magnitude of instability was small in comparison to the corresponding human disease. This difference may represent an intolerance for greater instability of TNRs in mice as compared to humans.

Interestingly, somatic instability was detected in mice harbouring only CTG arrays in the absence of any coding sequence (Monckton *et al.*, 1997). This contrasts

with previous beliefs that instability of a TNR only occurs in expressed sequences (Richards and Sutherland, 1992). In addition, in a transgenic mouse model of HD, instability is only detected in transgenic lines that express the HD transgene enforcing the belief that expansion and pathogenesis of TNR requires transcription of this mutation (Mangiarini *et al.*, 1997). Whether or not these CTG arrays have integrated into genes and therefore are transcribed cryptically will be an interesting question as to the importance of transcription for CTG instability (Monckton *et al.*, 1997). Monckton *et al* (1997) also report increased preference of CTG expansion in paternal transmissions and contractions in maternal transmissions. This observation indicates that there are sex-specific differences in the degree of instability in mouse as reported in DM patients. However in humans, the majority of CTG contractions occur as a result of paternal inheritance (Brunner *et al.*, 1992; Harley *et al.*, 1993; Lavedan *et al.*, 1993; Mulley *et al.*, 1993; Shelbourne *et al.*, 1992; Tsilfidis *et al.*, 1992).

The detection of instability of a TNR in transgenic mice is most exciting in terms of detecting a phenotype associated with expansion. Unfortunately for the DM models (Gourdon *et al*, 1997; Monckton *et al.*, 1997), no phenotype and subsequently no genetic anticipation has yet been observed. These results are quite disappointing in light of the controversy surrounding the effect of the CTG expansion. Clinically, 55 repeats represents a protomutation size of repeat that has been observed to be stably transmitted without symptoms of disease. It may take a number of generations or the use of a greater initial repeat size before any signs of DM become apparent in mouse (Gourdon *et al.*, 1997).

The question remains as to why expansion was detected in these models and not previously. The size of TNR repeat appears not to be significant as 55 repeats were found to be unstable (Gourdon *et al*, 1997). Furthermore, genomic context appears to have little influence on TNR instability as instability was observed in a number of different lines (Gourdon *et al.*, 1997; Monckton *et al.*, 1997). Finally, expression levels may prove not to be significant since non-coding CTG arrays, integrated into the mouse genome, showed instability (Monckton *et al*, 1997). Perhaps the answer lies in the genetic background used to generate these mouse models. Variations in genetic background has been shown to effect phenotype and this factor may influence the mechanism of TNR expansion (Hsaio *et al.*, 1996; Rozmahal *et al.*, 1996). Nevertheless, the establishment of instability in transgenic mice should prove to be an important step in understanding how this novel mutation is generated and how it affects disease state.

1.9 THESIS OBJECTIVE AND OUTLINE:

The identification of the mutation associated with DM has done little to resolve the effect of CTG expansion on DMK expression. The studies described in this thesis represent an effort to determine whether DMK over-expression in transgenic mice is causative of disease. As a prerequisite to the development of animal models, the levels of DMK protein in congenital and adult tissues were examined with a number of controls using a previously characterized antibody generated in our laboratory (Chapter 3). These data indicated that DMK protein levels reflect a role for over-expression of DMK transcript and protein in determining these two clinically distinct forms of DM.

To examine the effect of over-expression of DMK *in vivo*, I generated two transgenic mouse models by co-microinjection of the DMK gene with chicken matrix attachment region sequences (MAR). MAR sequences, normally located at the boundaries of functional transcription units, have been shown to enhance tissue-specific expression in a copy number-dependent and position-independent manner in transgenic mice by reducing the effect of flanking chromatin (McKnight *et al.*, 1992; Phi-Van *et al.*, 1990; Stief *et al.*, 1989). The first transgenic model over-expressed a 14 kb genomic fragment containing the entire human DMK gene with 22 repeats (Chapter 4). This model was analyzed for phenotypic similarities to the clinical presentation of DM and was found to exhibit DM-like features, dependent on transgene expression levels, in skeletal muscle (Chapter 4), eye (Chapter 5) and brain (Appendix I).

The second mouse model was developed by over-expressing a DMK minigene construct with 5 and 100 CTG repeats. Transgenic mice were analyzed for TNR instability and DM-associated pathology (Chapter 6). This mouse model provides further evidence that TNR instability, to the degree observed in DM patients, can be faithfully reproduced in mice. In addition, analysis of these mice revealed an increasing DM-like pathology in skeletal muscle intergenerationally, which may be representative of anticipation. These results demonstrate that over-expression of DMK has a role in determining DM pathogenesis. Furthermore, the usefulness of both of these models in determining the mechanisms of disease is discussed.

CHAPTER 2: GENERAL MATERIALS AND METHODS

2..1 DNA Preparation and Analysis

2.1.1 Genomic DNA Isolation from Mouse Tail:

Approximately 1 cm of mouse tail was placed in a 1.5 ml Eppendorf tube containing 500 µl of tail prep solution (50 mM Tris-HCl pH 7.5, 50 mM EDTA pH 7.5, 5% SDS), 50 µl of proteinase K (20 mg/ml stock) and 5 µl of RNase A (20 mg/ml stock) and incubated overnight at 56 °C. The supernatant was transferred to a new tube following centrifugation for 2 minutes at maximum speed. An equal volume of phenol/chloroform/isoamyl alcohol (24:24:1) was added to the supernatant and samples were shaken briefly. Tubes were spun at maximum speed for 6 minutes. The upper aqueous phase was transferred to a new tube and extracted with phenol/chloroform/isoamyl alcohol as before. The aqueous phase was mixed gently with 1/10 volume of 3 M sodium acetate pH 4.8 and 1 ml of absolute ethanol. The precipitated DNA was spooled onto a small pipette tip, transferred to a tube containing 30 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and incubated overnight at room temperature before use.

2.1.2 Rapid Extraction of Plasmid DNA by Boiling:

Approximately 1 ml of an overnight bacterial culture grown in LB medium (1% bactotryptone, 0.5% yeast extract and 1% NaCl) with 50 µg/ml ampicillin, was transferred to a 1.5 ml Eppendorf tube. Bacterial cells were collected by centrifugation for 2 minutes at maximum speed. The cell pellet was resuspended in 100 µl of STET

buffer (10 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, with 5% v/v Triton X-100 and 8% w/v sucrose) and 25 µl of a 10 mg/ml freshly prepared solution of lysozyme. Lysis was accomplished by transferring the tubes to a boiling water bath for 45 seconds. Lysate was collected by centrifugation for 5 minutes at maximum speed. The gelatinous cellular debris was removed using a toothpick. Plasmid DNA was recovered from the supernatant by adding an equal volume of isopropanol and centrifuged for 10 minutes at maximum speed. The precipitate was washed with 70% ethanol and collected by centrifugation for 5 minutes. The plasmid DNA precipitate was resuspended in 50 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).

2.1.3 Large Scale Isolation of Plasmid DNA:

An overnight culture, grown in LB medium (1% bactotryptone, 0.5% yeast extract, 1% NaCl) containing 50 µg/ml of ampicillin, was diluted 1:100 into 250 ml of LB and grown to stationary phase with vigorous shaking at 37 °C. The cells were harvested by centrifugation for 10 minutes at 6000 rpm. The supernatant was discarded and the cells were resuspended in 15 ml of cold TS buffer (50 mM Tris-HCl pH 8.0 with 25% w/v sucrose) and 5 ml of 0.25 M EDTA pH 8.0. To the cell suspension, 2.5 ml of a 10 mg/ml lysozyme solution were added. The resuspended cells were mixed gently and incubated on ice for 10 minutes. The cells were lysed following the addition of 4 ml of Triton X-100 lytic solution (50 mM Tris-HCl pH 8.0, 60 mM EDTA pH 8.0 and 2% v/v Triton X-100) and incubated at 65 °C for 5 minutes. The lysate was centrifuged for 60

minutes at 15000 rpm. DNA was precipitated from the supernatant using 1/3 volume of 40% PEG-NaCl (2 M NaCl with 40% w/v PEG 6000/8000) at 4 °C overnight.

The precipitate was collected by centrifugation at 6000 rpm for 10 minutes. The supernatant was discarded and the tubes were drained for 5 minutes. To the precipitate, 3 ml of TE (10 mM Tris-HCl, 1 mM EDTA pH 7.5), 4.0 g of CsCl and 0.8 ml of 10 mg/ml ethidium bromide were added followed by a 15 minute incubation on ice. Beckman quickseal ultracentrifuge tubes were filled with solution, layered with mineral oil and sealed. The samples were centrifuged using a Beckman TL-100 ultracentrifuge at 60000 rpm for 16 hours at 18 °C.

Using a long-wave ultraviolet lamp, the DNA bands were visualized. The lower band, corresponding to covalently closed circular plasmid DNA. was retrieved by side puncture of the tube with a 21 gauge needle and transferred to a disposable culture tube. Ethidium bromide was removed from the plasmid fraction by extracting thrice with 2 volumes of CsCl-saturated isopropanol. The DNA was dialyzed overnight against 1 litre of sterile TE buffer at 4 °C. Following dialysis, plasmid DNA was isolated by ethanol precipitation using siliconized tubes. The precipitate was pelleted by centrifugation at 7000 rpm for 60 minutes. The supernatant was discarded and the precipitate was air dried. The plasmid DNA precipitate was redissolved in 100 µl of TE buffer.

2.1.4 Southern Analysis:

Genomic DNA (5 µg) was digested with the appropriate restriction enzyme overnight at 37 °C. Samples were resolved on 0.8% agarose gels in 1x TBE buffer (89

mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA, pH 8.2) and transferred to nylon membrane (Amersham Hybond-N) using a standard Southern protocol (Sambrook et al., 1989). DNA was fixed to membranes by UV cross-linking (Fisher Scientific Co.).

2.2 RNA Preparation and Analysis

2.2.1 Isolation of Total RNA:

Total RNA was isolated from tissues by a modified guanidinium thiocyanate-phenol-chloroform method (Puissant and Houdebine, 1990). RNA was obtained from cultured cells, by collection of cells in 1x PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM sodium phosphate, 1.4 mM potassium phosphate) and spun at maximum speed for 5 seconds. After the supernatant was removed, cells were resuspended in 0.4 ml of RES-1 buffer (20 mM sodium citrate, 0.5 M LiCl, 1 M urea, 5 mM EDTA, 1% SDS, pH 6.8) and sonicated for 15 seconds. Lysates were incubated at 50 °C for 30 minutes in the presence of 100 μg/ml proteinase K. Following ethanol precipitation, the precipitate was resuspended in RES-1 buffer as before and re-digested with proteinase K at 50 °C for 30 minutes. The lysate was subjected to phenol/chloroform and chloroform extraction and the RNA was ethanol precipitated. The total RNA precipitate was resuspended in CES buffer (2 mM sodium citrate, 1 mM EDTA, 0.1% SDS, pH 6.8) and stored at -80 °C. A 1.2 kb *Xho* I fragment derived from the DMK cDNA was used as a probe for DMK transcripts.

2.2.2 Northern Blot Analysis:

Total RNA (10 μg) was diluted with half a volume of formamide and half a volume of a formaldehyde mixture (50% formaldehyde, 47% formamide, 33.3 mM sodium phosphate) and denatured by incubation at 55 °C for 20 minutes. Samples were cooled on ice for 2 minutes prior to loading onto 1.2% agarose gels (Hoeffer SE400 vertical slab unit) containing 0.6% formaldehyde in 20 mM sodium phosphate and 1 mM EDTA. Following electrophoresis, resolved RNA was transferred to Hybond-N membrane (Amersham) by vacuum blotting (LKB) and fixed by UV cross-linking (Fisher Scientific Co.).

2.3 DNA probes:

DNA probes were isolated following resolution of fragments generated by restriction enzyme digestion of various plasmids on 0.6% low melt agarose gels (Gibco/BRL). In some cases, probes were further purified using Geneclean (Bio 101). Probes were labeled by random priming (Rediprime; Amersham) using [α-^{32}P]dCTP (Amersham).

2.4 Cloning:

Approximately 1 μg of CsCl purified plasmid DNA was digested in a 20 μl volume using 5 units of restriction enzyme for 1 hour at the suggested temperature. Fragments were gel purified using Geneclean (Bio 101) and were ligated into vector using 1 unit of T4 DNA ligase per 10 μl reaction volume at 14 °C overnight. Half of the

ligation mix was used per transformation of *E. coli* DH5α. Positive clones were identified by restriction enzyme mapping of plasmid DNA.

2.5 Transgene Preparation for Microinjection:

Fragments containing transgenes were isolated following restriction enzyme digestion and resolution by agarose gel electrophoresis. The fragments were gel purified by electroelution or by Geneclean (Bio 101) and were resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) to a final concentration of 100 ng/μl.

2.6 Transgenic Mice:

The purified genomic fragment (2 ng) was co-microinjected with 2 ng of purified chicken MAR fragment into fertilized mouse oocytes obtained from the mating of CBA/C57BL6 F_1 mice. This procedure was done by the technical staff at the NCE core transgenic facility (director-Dr. Frank Jirik, UBC). I identified transgenic mice by transgene-specific PCR using upstream primer (A), 5'-AGCCACAGGCAGCCTTAAGC-3', located in intron 10 of human DMK and downstream primer (B), 5'-TCCGGGGAAGGGGACACATGA-3', located in intron 12 of human DMK or human DMK exon 8 specific primers (C), 5'-GAGCACCTCTCTCTGCCGCTG-3' and (E), 5"-CATGGCAGTGAGCCCGTCCTC-3'. Genomic DNA (0.1-0.5 μg), prepared from mouse tail tissue, was amplified using a commercial DNA amplification kit (Perkin-Elmer/Cetus) with 50 ng of each primer at an initial denaturation of 96 °C for 3 min followed by 35 cycles of 96 °C for 1 min, 60 °C

for 1.5 min (65 °C for 1.5 min for primers C and E) and 72 °C for 1.5 min, resulting in the amplification of a 427 bp fragment by primers A and B (or 148 bp fragment by primers C and E).

The intact transgene was verified by Southern blots of *Bam*HI or *Eco*RI digested tail DNA (5 μg), probed with a 2.6 kb *Sac* I genomic fragment from the 5' end of the human DMK gene, a 2.2 kb *Bam*HI genomic fragment derived from the 3' end of the human DMK gene, and 1.2 kb *Xho* I cDNA fragment derived from the 5' end of the cDNA. Co-integration of MAR sequences were verified by Southern analysis of *Pst* I digested tail DNA (5 μg) probed with a 1.4 kb *Bam*HI genomic fragment derived from the 3' end of the human DMK gene or *Xho* I digested genomic DNA (5 μg) probed with a 980 bp *Nde* I/*Bsp*HI fragment derived from the 5' end of the DMK repeat construct. All blots were washed with 0.2x SSC containing 0.1% SDS at 60 °C and exposed to x-ray film overnight at -80 °C.

2.7 Copy Number Estimation:

Human and mouse DMK sequence was amplified from transgenic genomic DNA (0.1 μg) using exon 8-specific primers: 5'-GAGCACCTCTCTCTGCCGCTG-3' (upstream (C); human specific), 5'-GAACACTTGTCGCTGCCGCTG-3' (upstream (D); mouse specific) and 5'-CATGGCAGTGAGCCCGTCCTC-3' (downstream (E); mouse and human specific). Test PCR was performed to determine the linear range of amplification by removing aliquots between 18 and 28 cycles from a PCR reaction containing 0.1 μg of genomic DNA. Aliquots were resolved on 2% agarose gels,

transferred to nylon membrane and hybridized to the end-labeled downstream primer. Audioradiographs were scanned by laser densitometry. The number of cycles was plotted against density. A total of 22 cycles was found to be the mid-range value in the linear portion of exponential amplification and was used for subsequent experiments.

Two PCR reactions, one containing primers C, D and E, and one containing only primers D and E were set up from each transgenic genomic DNA sample. A 248 bp fragment was obtained from a 25 µl reaction containing 50 ng of each primer carried out for 22 cycles using the following conditions: 96 °C for 1 min, 63 °C for 1 min and 72 °C for 1 min. Exactly 5 µl of each PCR product was digested with *Rsa* I, an enzyme that only restricts the mouse DMK product. Digests were resolved by gel electrophoresis using 2% agarose, transferred to nylon membrane and hybridized to the end-labeled downstream primer. Membranes were washed with 6x SSC containing 0.1% SDS at 40 °C and exposed to x-ray film overnight at -80 °C. Autoradiographs were scanned by laser densitometry to estimate the transgene copy number by comparison to the single copy mouse DMK product.

2.8 Preparation of Protein Extracts:

Tissue was homogenized in 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 2 mM PMSF, 6 µg/ml aprotinin and 1 µg/ml leupeptin. Homogenates were adjusted to 1% NP40 and incubated on ice for 15 min. Samples were spun at 3200 rpm for 10 min and protein concentration was determined from the supernatants using a Micro BSA protein assay kit (Pierce).

2.9 Immunoblot Analysis:

Protein extracts (30 μg for muscle, heart, tongue and 40 μg for brain and eye) were resolved by discontinuous SDS-PAGE (10% SDS-polyacrylamide gels) and transferred to nitrocellulose membrane using BioRad Transblot cells or a BioRad electroblotter. The membrane was blocked with 5% dry milk for 1 hour and then incubated for 1 hour with a primary antibody. Immune complexes were detected using horseradish peroxidase-coupled secondary antibody (Amersham) and ECL kit as recommended (Amersham).

2.10 Sectioning Tissues:

Mice were euthanized by an overdose of somnitol (MTC pharmaceuticals). Isolated tissues were frozen in isopentane cooled by liquid nitrogen. Cryostat longitudinal and cross-sections (12 μm) were cut at -25 °C and thaw mounted onto superfrost glass slides (Baxter). Slides were stored at -80 °C.

2.11 Histological Examination of Tissue Sections:

Frozen sections were stained with freshly filtered Harris modified haematoxylin with acetic acid (BDH) for 5 minutes. Slides were rinsed in tap water for 2 minutes and dipped 3 times in 1% acid alcohol (1% HCl in absolute ethanol). Following 2 quick rinses in warm tap water, slides were dipped in freshly made 1% lithium chloride until the sections appeared blue (4 to 5 times). The slides were then incubated in tap water for 10 to 15 minutes followed by treatment in 50% ethanol for 1 minute. Slides were

counterstained with eosin Y (Sigma, 0.25% eosin Y, 0.5% acetic acid in 80% ethanol) for 1 minute and then rinsed in tap water for 30 seconds. Sections were subjected to consecutive step-wise dehydration from 70% to absolute ethanol for 1 to 2 minutes each, followed by 2 incubations in xylene substitute (EM Diagnostic Systems) for 5 minutes each. Coverslips were mounted using permount (Fisher Scientific Co.) and slides were stored at room temperature.

2.12 Photography of Haematoxylin and Eosin Stained Sections:

- Cross-sectioned stained muscle fibers were photographed with Kodak Tmax 100 film within 10 randomly selected areas containing approximately 100 fibers at 20x magnification using a Zeiss Axiophot microscope. From these photographs, the total number of centronucleated fibers were counted from which the proportion of fibers with centronucleation was determined. In this manner, a total of 1000 muscle fibers per sample were analyzed.

CHAPTER 3: EXPRESSION LEVELS OF MYOTONIC DYSTROPHY (DM) KINASE PROTEIN IN CONGENITAL AND ADULT DM PATIENTS

3.1 INTRODUCTION:

Determining the effect of the CTG expansion on DMK expression is a necessary step to understanding and modeling DM pathogenesis. However, conflicting observations have been reported using DM patient tissues. Various studies have shown reduced DMK expression at the transcriptional level leading to the proposal of haploinsufficiency as a mechanism of disease (Carango *et al.*, 1993; Fu *et al.*, 1993; Hoffman-Radvanyi *et al.*, 1993). Conversely, over-expression of mutant DMK transcript has been observed by northern and dot blot analysis (Sabourin *et al.*, 1993). A recent report has also shown by *in situ* hybridization that not only is a mutant DMK transcript expressed but that it is preferentially accumulated in the nuclei of fibroblasts and muscle biopsies from DM patients (Taneja *et al.*, 1995). Similarly, at the protein level, reduced (Fu *et al.*, 1993; Koga *et al.*, 1994; Maeda *et al.*, 1995) and unaltered (van der Ven *et al.*, 1993; Bhagwati *et al.*, 1996) or slightly enhanced levels (Dunne *et al.*, 1996) of DMK have been reported. However, a number of these findings have been confounded by small sample sizes consisting of one or two samples, variability of clinical material and by antisera whose authenticity has not been substantiated.

To resolve these discrepancies, DMK protein levels were quantified in a number of congenital and adult patients and matched normal controls using a polyclonal antiserum generated in our laboratory. Previous characterization of this antiserum revealed detection of two DMK species (74 and 82 kDa) (Whiting *et al.*, 1995), authenticity of which has been confirmed in DMK deficient mice (Jansen *et al.*, 1996)

and in transgenic mice over-expressing the human DMK gene (Chapter 4). Additionally, DMK protein levels were normalized to various skeletal muscle proteins in order to account for muscle damage due to disease and sample preparation. Levels of DMK protein were found to be reduced in congenital patients and elevated in adult onset DM patients. How this may effect the mechanism of pathogenesis is discussed.

3.2 MATERIALS AND METHODS

3.2.1 Tissue Source:

Skeletal muscle tissue (forearm) from four congenital DM patients was retrieved post-mortem. Congenital DM patients from post-natal day 2, 3, 5 and 6 all died from complications due to respiratory distress (designated cDM1 to cDM4, respectively). As controls, post-mortem skeletal muscle samples were retrieved from 4 newborns from post-natal day 2, 3, 6 and 8 (designated n1 to n4, respectively). Cause of death for these neonates was listed as diaphragmatic hernia and congenital heart disease.

Adult skeletal muscle samples (forearm) were obtained during routine diagnostic biopsy. Four patients with DM were examined, a 37 year old male (DM1), a 53 year old male (DM2), a 56 year old female (DM3) and a 58 year old male (DM4). As well, four non-DM skeletal muscle samples were used as controls in this study. All were male, 32, 48, 52 and 60 years old at the time of biopsy (designated N1 to N4, respectively). As well, all DM patients suffered from progressive muscle weakness and diffuse myotonia. Muscle samples were quickly frozen in isopentane cooled in liquid nitrogen and stored at -80 °C until assayed.

3.2.2 Preparation of Protein Extracts and Immunoblot Analysis:

Samples were prepared and analyzed as outlined in Chapter 2.8 and 2.9. Membranes were incubated for 1 hour with one of the following primary antibodies: anti-DMK polyclonal antibody at 1:2500, anti-α-sarcomeric actin monoclonal antibody (5C5; Sigma) at 1:5000, anti-total myosin monoclonal hybridoma supernatant (clone MF20; courtesy of Dr. P. Merrifield, University of Western Ontario) at 1:10 and anti-type I myosin heavy chain (MHC) monoclonal hybridoma supernatant (clone 4A9; courtesy of Dr. P. Merrifield, University of Western Ontario) at 1:10. Immunoreactive bands were visualized using horseradish peroxidase secondary antibody (Amersham) at 1:3000 and enhanced chemiluminescent detection (ECL; Amersham). Signals were detected following 1-5 minute exposure to Hyperfilm ECL film (Amersham). All audioradiograms were subjected to laser densitometry (Bio-Rad GS-670 imaging densitometer). Each sample was normalized for α-sarcomeric actin, total myosin and type 1 MHC.

3.3 RESULTS:

A characterized DMK-specific polyclonal antibody (Jansen *et al.*, 1996; Maeda *et al.*, 1995; Whiting *et al.*, 1995) was used to determine expression levels of human DMK protein in adult and congenital DM patients. As shown previously, the larger protein species (82 kDa) is predominant in skeletal muscle samples (Figure 3-1 and 3-2; Whiting *et al.*, 1995). A comparison of DMK protein levels in skeletal muscle samples from four congenital DM patients with four normal neonate controls revealed no difference (Figure 3-1A). DMK protein levels were normalized to all three skeletal

Figure 3-1: Immunoblot analysis of congenital DM skeletal muscle (cDM1 to 4) and normal controls (n1 to 4) using antibodies against (A) DMK, (B) α-sarcomeric actin, (C) total myosin and, (D) type I myosin heavy chain (MHC). No difference was observed in DMK expression levels between congenital and normal samples. Total myosin levels appeared to be slightly elevated in congenital DM samples

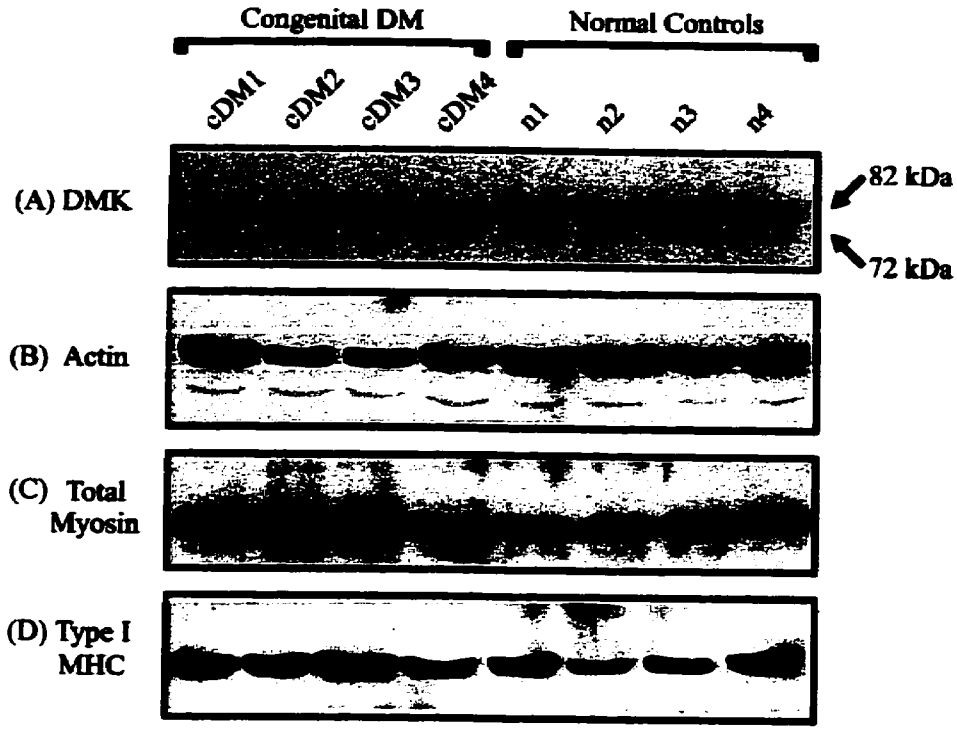
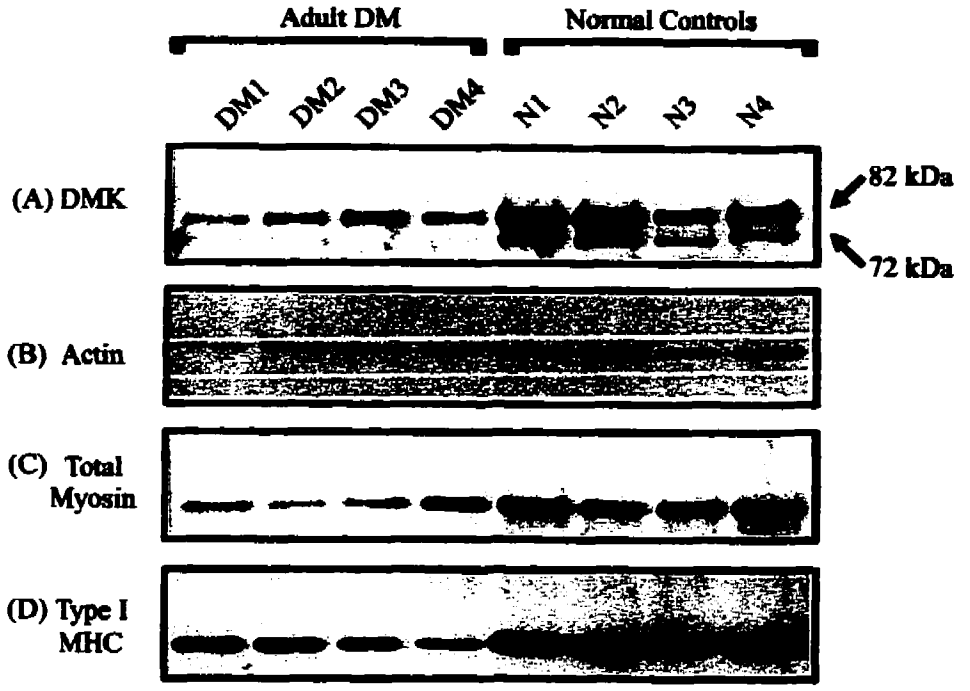

Congenital DM
Normal Controls
cDM1
cDM2
cDM3
cDM4
n1
n2
n3
n4
(A) DMK
82 kDa
72 kDa
(B) Actin
(C) Total Myosin
(D) Type I MHC

Figure 3-2: Immunoblot analysis of adult DM skeletal muscle (DM1 to 4) and normal controls (N1 to 4) using antibodies against (A) DMK, (B) α-sarcomeric actin. (C) total myosin and, (D) type I myosin heavy chain (MHC). A significant deficit of DMK protein, total myosin and type I MHC was found in adult DM patients compared to normal controls (Student's 2-tailed t-test, $p<0.005$).


Adult DM
Normal Controls
DM1
DM2
DM3
DM4
N1
N2
N3
N4
(A) DMK
82 kDa
72 kDa
(B) Actin
(C) Total Myosin
(D) Type I MHC

muscle constituents, α-sarcomeric actin, total myosin and type I-MHC (Figure 3-1B to D). Upon normalization, DMK protein levels in congenital patients were found to be slightly lower compared to normal controls (Table 3-1). In adult samples, a significant deficit of DMK protein was detected in DM patients (Figure 3-2A; Student's 2-tailed t-test, $p<0.005$). However, although no significant difference was observed in α-sarcomeric actin levels between adult DM patients and normal controls, the amount of total myosin and type I-MHC was depleted in adult DM patient samples indicative of the effects of myopathy in these samples (Figure 3-2B to D; Student's 2-tailed t-test, $p<0.005$). As a result, following normalization, the ratio of DMK protein levels was consistently found, on average, to be in the order of 1.6 fold higher in adult DM patients compared to normal controls (Table 3-1).

3.4 DISCUSSION:

There are two clinically distinct forms of myotonic dystrophy. In adults, DM is a highly variable, multisystemic disease characterized by cataracts, myotonia, progressive muscle weakness and wasting (Harper, 1989). A more severe form of disease presents in congenital cases where DM manifests as generalized muscular hypotonia that is responsible for a high neonatal mortality due to respiratory distress. The congenitally affected DM offspring that survive the neonatal period exhibit mental retardation and the classical form of disease in adolescence. Importantly, the molecular basis for severity of DM is determined by the size of the CTG repeat. From the data presented here, there appears to be a difference in DMK protein levels in congenital and

Table 3-1: Ratio of DMK Levels in Skeletal Muscle Samples from DM Patients Compared to Normal Controls

Normalized (*No*/*Yes*)	DMK Protein Levels in DM Patients/Normal Controls							
	Congenital DM Patients				Adult DM Patients			
	1	2	3	4	1	2	3	4
N	1.00	1.01	1.09	1.03	0.21	0.30	0.32	0.25
Y	0.79	0.84	0.94	0.92	1.48	1.62	1.51	1.83

DMK protein levels are reported using laser densitometry, as a ratio of DMK levels in DM patients to the normal matched control following normalization to sarcomeric actin, total myosin and type I myosin heav chain (MHC).

adult DM patients as well.

Taneja *et al.* (1995) have shown that mutant transcripts accumulate in the nuclei of DM patients fibroblasts and myoblasts. Furthermore, the extent of accumulation is dependent of the size of the CTG repeat. The detection of lower levels of DMK protein in the congenital patients of the present study is in keeping with the previous findings. However, the decreased availability of DMK protein is not likely the cause of congenital DM but rather an indicator of this accumulation. This statement is supported by the fact that there is an observed lack of a DM-like phenotype in DMK deficient mice (Jansen *et al.*, 1996; Reddy *et al.*, 1996). More likely, DMK transcript accumulation leads to a gain in function. Regulatory roles have been shown to be fulfilled by 3'UTRs in *trans* (Rastinejad and Blau, 1993). In DM patients the observation of decreased mutant and wild-type poly A+ DMK transcripts has lead to the suggestion that the mutant allele, in *trans*, affects wild-type DMK levels and in doing so, may affect other transcripts. Alternatively, sequestering a recently identified ribonucleoprotein constituent which binds CUG repeats may be central to disease (Timchenko *et al.*, 1996). These possible effects of transcript accumulation, occurring early in development, may explain the severity and generalized phenotype of congenital DM.

Although transcript accumulation may also have a role in adult onset DM. the multisystemic and progressive nature of this form of disease may alternatively be caused by over-expression of DMK protein. DMK protein belongs to a family of kinases known to regulate, by a cascade of enzymatic events, members of the myofibrillar and cytoskeleton protein families (Leung *et al.*, 1996). Dunne and co-workers (1996) have

also reported increased accumulation of DMK protein in severely affected DM muscle with respect to type I MHC. In the samples studied here, the amount of DMK protein is consistently elevated, although not by more than 1.6 fold. Although the function of DMK protein is still unknown, slight but chronic perturbations in kinase levels may be relevant in skeletal muscle contraction responses or cardiac conduction.

Part of the ongoing controversy surrounding the effect of the CTG expansion on DMK expression can be attributed to the samples themselves. Tissue damage and recovery procedure can influence quantitation (Roses, 1994). From Table 1, it is clear that in the absence of normalization to various skeletal muscle constituents, DMK protein levels are drastically reduced in all adult DM muscle samples examined here. However, in consideration of the characteristic skeletal muscle wasting and type I fiber atrophy associated with DM, this result is not unexpected. In fact, the importance of controlling for sample integrity is confirmed by a striking reduction of total myosin and type I-MHC levels in adult DM patients compared to normal controls (Figure 3-2).

Without the proper controls, the changes in DMK expression levels in DM patients can be substantially over or underestimated. This, in part, has lead to the proposal that CTG repeat expansion in DM patients, affects genes other than DMK (Harris *et al.*, 1996; Nawrotski *et al.*, 1996). However, to date, no change in expression levels of surrounding genes has been reported in DM patients (Jansen *et al.*, 1994; Boucher *et al.*, 1995). From the results presented here, DMK protein expression is altered in congenital and adult DM patients. Moreover, the opposing pattern of DMK protein expression in congenital and adult onset DM may represent the differences in

these two clinically distinct disease states. Whether the disease results from the increased accumulation of transcript or DMK protein, both are likely to be influenced by CTG size. Studies concerned with over-expressing DMK transcript and protein in mice will therefore be useful in delineating the mechanism(s) underlying the pathogenesis of DM.

CHAPTER 4: OVER-EXPRESSION OF THE HUMAN MYOTONIC DYSTROPHY (DM) KINASE GENE IN TRANSGENIC MICE CAUSES A DM-LIKE MYOPATHY

4.1 INTRODUCTION:

Myotonic dystrophy (DM) is a common, autosomal dominant disorder that classically manifests as impaired muscle relaxation (myotonia), progressive muscle weakness and wasting (Harper, 1989). Although this disease shows marked variability, even within families, myotonia is often an early symptom in the adult form of DM, shown by a patient's inability to release a grasped hand (Dubowitz, 1985). Electromyograms (EMGs) provide a means of monitoring such prolonged contractions following brief electrical or mechanical stimulation, typically to the hands, face or tongue (Adams, 1975). As DM progresses, muscle weakness and wasting become the predominant problems affecting distal rather than proximal limb muscles, calf, abdominal and facial muscles, eye lids and the sternomastoids (Dubowitz, 1985; Korenyi-Both, 1983). This weakness is thought to be the cause for the ensuing respiratory problem and cardiac arrhythmia associated with DM (Dubowitz, 1985).

DM also presents as a severe congenital form resulting in generalized hypotonia rather than myotonia and a high neonatal mortality due to respiratory distress presumed to be caused by a weakened thorax and diaphragm. Children surviving respiratory difficulties appear to be delayed in muscle development but eventually assume the adult form of the disease (Korenyi-Both, 1983). Nevertheless, histological changes in the skeletal muscle of DM patients are characteristic and similar for both forms of the disease

and have been considered a reliable indicator for diagnosis (Korenyi-Both, 1983). One of the earliest indicators of DM is a disparity between type I and II fiber size, with a characteristic reduction in type I fiber size (atrophy) which is accompanied in some cases, by type II fiber hypertrophy (Harper, 1989). The most striking feature of skeletal muscle from DM patients is an abundance of centronucleated fibers (Brooke, 1986; Harper, 1989; Korenyi-Both, 1983). On longitudinal sections, the centrally located nuclei are arranged in a chain or a row (Astrom and Adams, 1979; Dubowitz, 1985; Korenyi-Both, 1983). Furthermore, in DM affected muscle, nuclei are variable in shape, size and intensity of staining (Korenyi-Both, 1983). As the disease progresses, dystrophic changes become increasingly predominant and include the presence of sarcoplasmic masses which are homogeneous areas of sarcoplasm containing tubule aggregates, myofilaments and free ribosomes, and ringed fibers which are cross-sectioned muscle fibers encircled by perpendicularly arranged muscle fiber (Carpenter and Karpati, 1984; Harper, 1989; Korenyi-Both, 1983).

Recently, transgenic mice expressing the human DMK gene have been reported although no skeletal muscle myopathy was observed (Jansen *et al.*, 1996). In addition, two knock-out mouse models for DMK have been produced, both of which were generated by disrupting the kinase domain of the DMK gene (Jansen *et al.*, 1996; Reddy *et al.*, 1996). In adult mouse muscle derived from one of these models, minor changes in fiber size, increased fiber degeneration, fibrosis and changes to muscle ultrastructure were observed (Reddy *et al.*, 1996), while no significant change was observed in the other

model (Jansen *et al.*, 1996). Importantly, the DMK deficient mice that do show signs of mild myopathy have not had characteristic skeletal muscle histological changes described previously, myotonia or other systemic effects associated with DM (Harper, 1989; Korenyi-Both, 1983; Roses *et al.*, 1979).

As an alternative approach, I have developed transgenic mice carrying a genomic fragment containing the entire human DMK gene with chicken matrix attachment region (MAR) sequences in order to facilitate copy-number dependent DMK expression. The resulting transgenics, described here, express human DMK in tissues affected by DM indicating that tissue-specific regulatory elements are present in the transgene. Substantial over-expression of human DMK transcript and protein was found in skeletal muscle, brain, eye and tongue. In this study, transgenic skeletal muscle was examined for the existence of histological diagnostic hallmarks associated with DM patient muscle. I present evidence that over-expression of DMK in mice leads to a muscle pathology similar to DM suggesting that this mechanism plays a role in the human disease.

4.2 MATERIALS AND METHODS

4.2.1 Transgenic Mice:

A 14 kb genomic fragment containing the human DMK gene was isolated from cosmid clone YA100263 (Aslandis *et al.*, 1992) following digestion with *Nhe* I (Figure 4-1). Generation of transgenic mice and their analysis was done according to protocols in Chapter 2.5-2.12.

4.2.2 FISH Analysis (performed by Dr. Evica Rajcan-Separovic, Solange Gauthier Karsh Molecular Genetics Laboratory, Children's Hospital of Eastern Ontario):

Transgenic mice were injected subcutaneously with 0.1ml of a 10 μg/μl solution of colcemid 2 hours before sacrifice. Femur and tibia were removed and the bone marrow was washed out using a syringe and α-MEM tissue culture medium. The bone marrow suspension was centrifuged for 10 minutes at 1000 rpm. The cell pellet was resuspended in 0.075M KCl hypotonic solution and left for 30 min at 37 °C. It was then centrifuged for 10 minutes at 1000 rpm and washed 3 times in Carnoy fixative. Slides were kept at -20 °C.

The cosmid YA100263 from the DM region was labeled by random priming (Bioprime DNA labelling kit). The hybridization mixture was composed of 50-100 ng labeled probe, 2 μg dried cot-1 human DNA, 1 μg herring sperm DNA in 50% formamide/10% dextran sulphate/2x SSC in a volume of 10 μl. The probe was denatured for 10 minutes at 70 °C and then applied to slides previously dehydrated in three cold ethanol washes and denatured for 2 minutes in 70% formamide/2x SSC. Hybridization took place at 37 °C for 16 hours and was followed by washing at 42 °C in 50% formamide/2x SSC for 10 minutes, 2xSSC for 10 minutes and 5% Triton X-100/4x SSC for 5 minutes. After probe hybridization and washing, FITC avidin and antiavidin antibodies (Sigma) were used to visualize the hybridization signals. The chromosomes were counterstained with propidium iodide.

4.2.3 Immunohistology:

Gastrocnemius muscle, removed from 6 month old transgenic mice and wild-type littermates, was frozen in isopentane cooled by liquid nitrogen. Cryostat cross-sections (12 μm) from the midpoint of the muscle were cut at -25 °C and thaw mounted onto superfrost glass slides (Baxter). On adjacent cryostat sections, immunohistochemical discrimination of muscle fiber types was performed using goat anti-mouse monoclonal antibodies to fiber type I, type IIa and type IIb (courtesy of Dr. D. Parry, University of Ottawa). Detection of primary antibodies was performed using horse-radish peroxidase (Kirkegaard and Perry Laboratories) and 3,3'-diaminobenzidine as recommended (Sigma). After the reaction, slides were washed in distilled water, dehydrated in graded alcohols and xylene, and mounted. The muscle fiber area of type I, type IIa and type IIb was determined using a calibrated Leitze Laborlux II projection microscope with a Houston Instrument Highpad and digitizer interfaced with SigmaStat (Jandel). Each sample was scored blind with 1000 randomly chosen fibers from which the relative area of each fiber type was determined.

4.2.4 Myoblast Fusion Assay:

To establish primary myoblast cell cultures, gastrocnemius muscle was removed from 6 month old transgenic mice and wild-type littermates and minced finely in cold phosphate buffered saline (PBS). The minced tissue was incubated in trypsinization solution (0.25% trypsin, 50 mM EDTA pH 7.5, 10 mM glucose) at 37 °C for 15 min

while being shaken. Debris was allowed to settle and the supernatant was transferred into α-MEM medium supplemented with 15% fetal bovine serum (Gibco/BRL). The remaining precipitate was re-trypsinized as before. The supernatants were pooled and centrifuged at 1500 rpm for 10 min. The pellet was resuspended in fresh medium, plated and maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

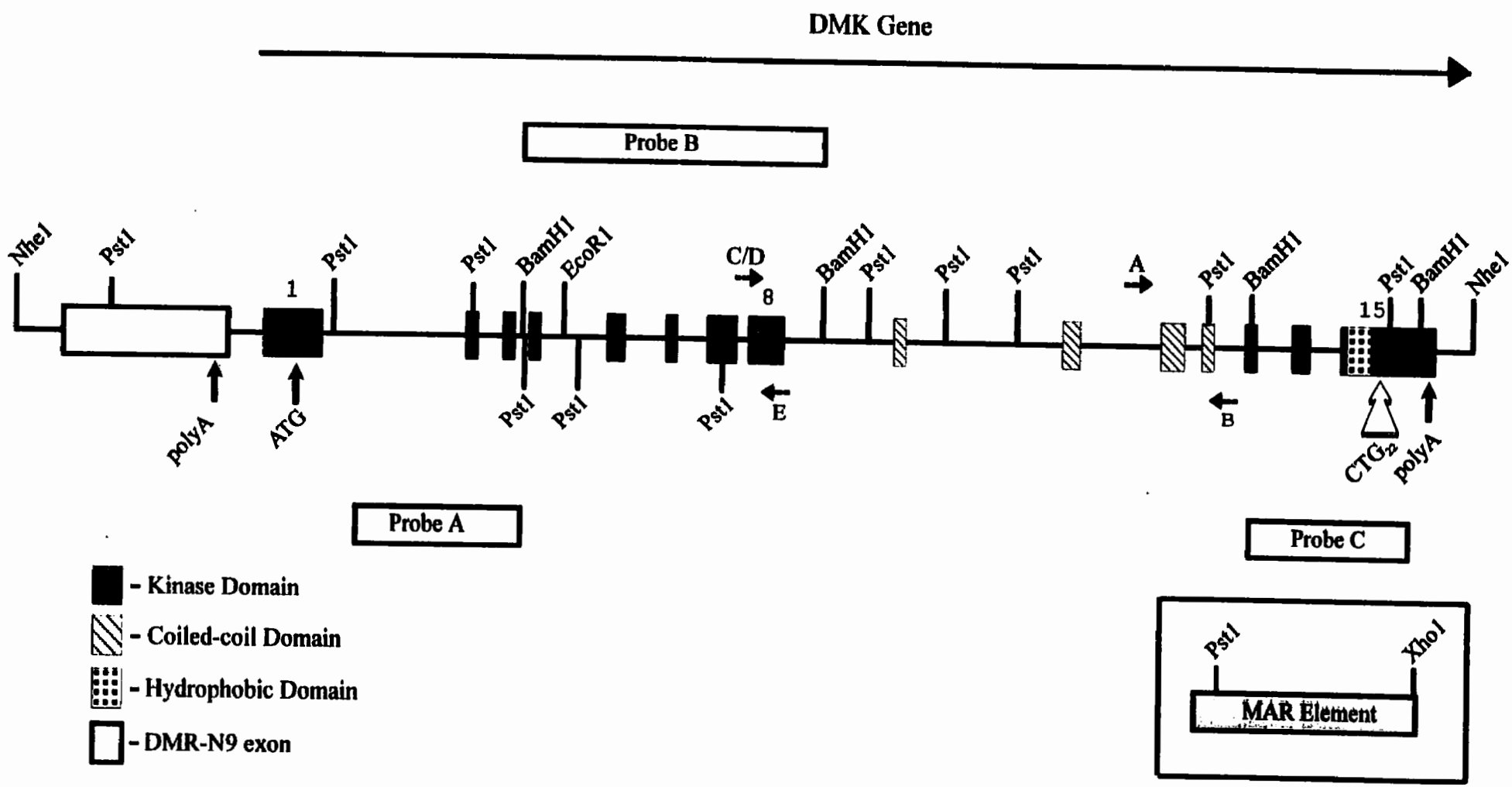
To ensure purity of the cell lines and that equivalent numbers of myoblasts were present for each sample, cells were stained with desmin. For fusion assays, 4 x 10^5 cells were plated in 35 mm dishes in triplicate. After 24 hours, the cells were washed once in PBS and incubated for 72 hours in fusion media (α-MEM medium supplemented with 2% horse serum) (Gibco/BRL). The cells were then washed in PBS and fixed with 100% methanol for 10 minutes. Cells were stained with Giemsa for 2 min following myosin heavy chain (MHC) staining using monoclonal antibody MF20 (courtesy of Dr. P. Merrifield, University of Western Ontario) and peroxidase-conjugated anti-mouse antibody. Cells were washed with PBS once again, and nuclei were counted. The fusion index was calculated as the percentage of the total nuclei in the field which were found in myotubes. For each sample, three separate experiments were performed in this manner.

4.3 RESULTS

4.3.1 Generation of Transgenic Mice:

To study the effect of DMK over-expression in a tissue-specific manner, a genomic fragment containing 5' and 3' flanking sequence of the human DMK gene along with all intronic sequences was used to generate transgenic mice (Figure 4-1).

Figure 4-1: Schematic diagram of the human DMK gene used to create transgenic mice. Shaded boxes indicate human DMK exons. Horizontal arrows indicate the location and direction of the oligonucleotide primers that were used to identify transgenic mice (primers A and B) and determine copy number (C, D and E). Restriction sites, localization of probes, the CTG repeat, translation initiation and polyadenylation sites are indicated. A schematic diagram of a MAR sequence is also shown.


DMK Gene
Probe B
Nhe1
Pst1
Pst1
Pst1
BamH1
EcoR1
C/D
BamH1
Pst1
Pst1
Pst1
A
Pst1
BamH1
Pst1
BamH1
Nhe1
1
8
15
polyA
ATG
Pst1
Pst1
Pst1
E
B
CTG2
polyA
Probe A
Probe C
- Kinase Domain
- Coiled-coil Domain
- Hydrophobic Domain
- DMR-N9 exon
Pst1
Xho1
MAR Element

Transgenic mouse lines were identified by PCR. Co-integration of transgene and MAR sequence without re-arrangement, was verified using Southern blot analysis (Figure 4-2A and B). FISH analysis was used to determine the distribution of integrated transgene in the mouse genome. The presence of only one signal per chromosomal spread confirmed the occurrence of single sites of integration at different loci in all three founder lines (Figure 4-3). Lines were out-bred to establish stable transmission. Densitometric comparison of the transgene to an endogenous, single copy mouse fragment, generated using semi-quantitative PCR (as described in Chapter 2.7), indicated transgene copy numbers ranging from 10 to 50 (Table 4-1).

4.3.2 Transgene Expression:

Expression of the transgene in each line was assessed by Northern blot analysis (Figure 4-4, Table 4-1). Over-expression of human DMK transcript was particularly enhanced in brain, muscle, tongue and eye, tissues typically affected in DM (Harper, 1989), at levels greater than endogenous mouse DMK of wild-type littermates (Figure 4-4, Table 4-1). In transgenic heart, only moderate expression of human DMK transcript was observed (where line A>B>C) (Figure 4-4, Table 4-1). Lines A, B and C, respectively, showed decreasing levels of DMK transcript in all tissues examined here, consistent with decreasing copy number (Table 4-1). No expression of MAR sequences was detected, as expected. The lines with higher levels of expression (A and B) were used for further analysis.

A human DMK-specific polyclonal antibody (Whiting *et al.*, 1995) was used to

Figure 4-2: Identification of DMK transgenic mice using Southern analysis. Total DNA was prepared from mouse tails and equivalent amounts (5 μg) were digested with. (A) *Bam*HI and probed with a 2.2 kb *Bam*HI fragment (probe B) or, (B) *Pst* I and probed with a 1.4 kb *Bam*HI fragment (probe C). The differences in intensity reflects variable transgene copy numbers among the lines. The lower band in (B) represents the co-integration of a MAR element which possesses a Pst I site close to its 5' end.

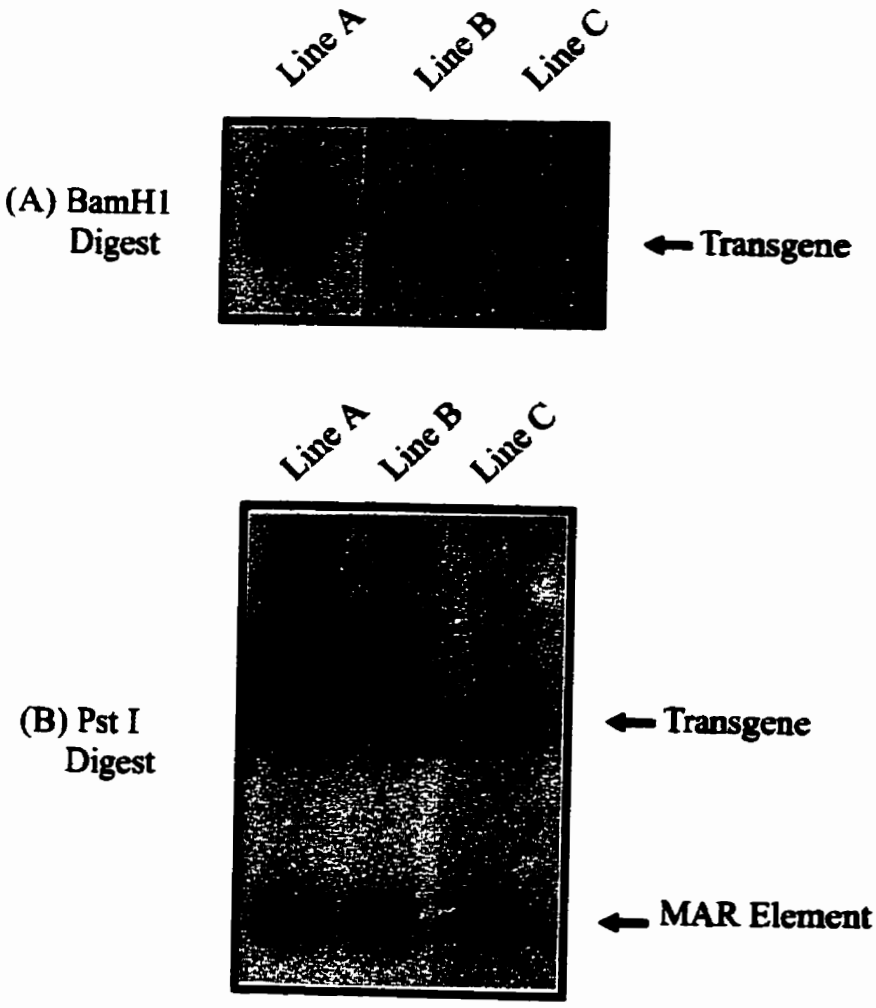

Line A
Line B
Line C
(A) BamH1 Digest
Transgene
Line A
Line B
Line C
(B) Pst I Digest
Transgene
MAR Element

Figure 4-3: FISH analysis of interphase/metaphase spreads derived from transgenic lines A, B and C. Arrows indicate singlets in chromosomal spreads confirming single sites of integration of the human DMK transgene in these founder lines.

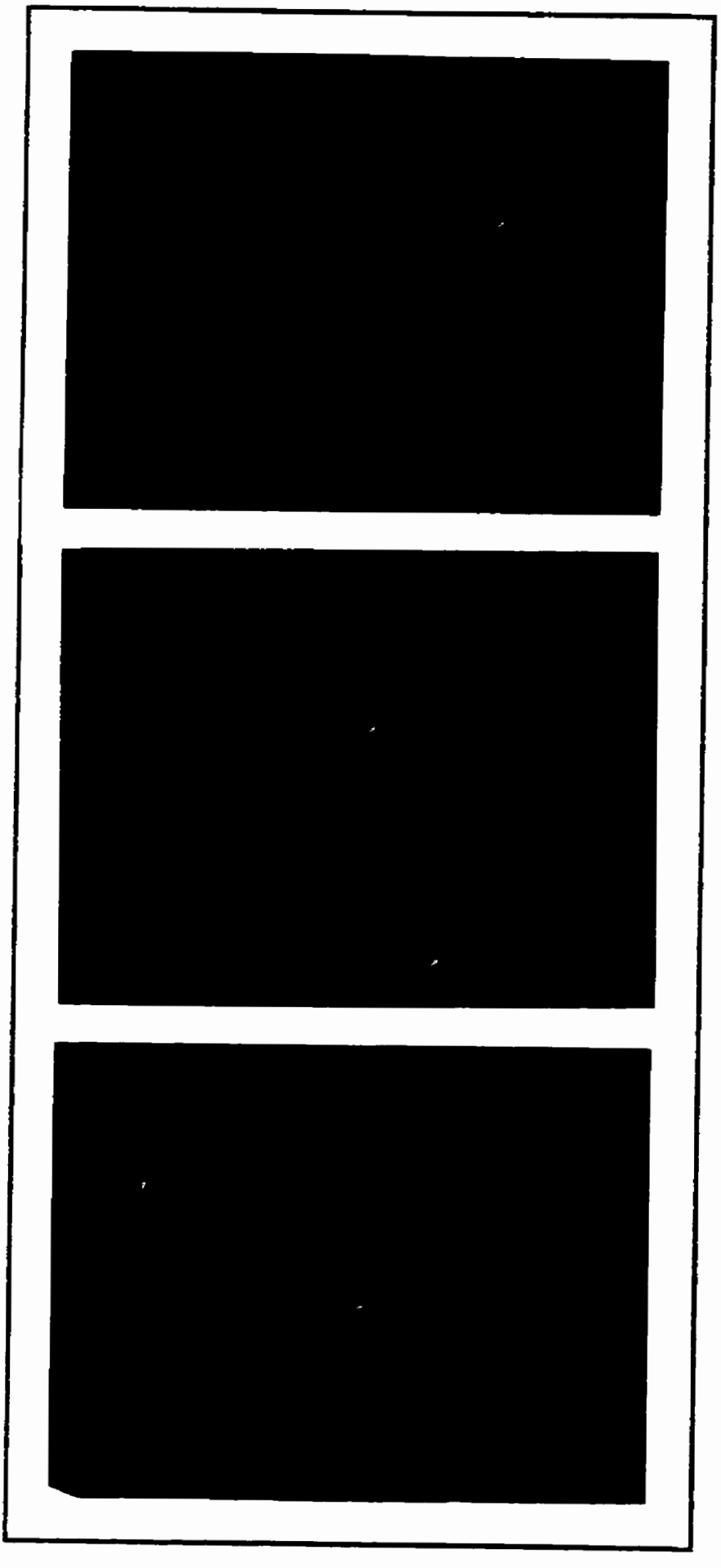
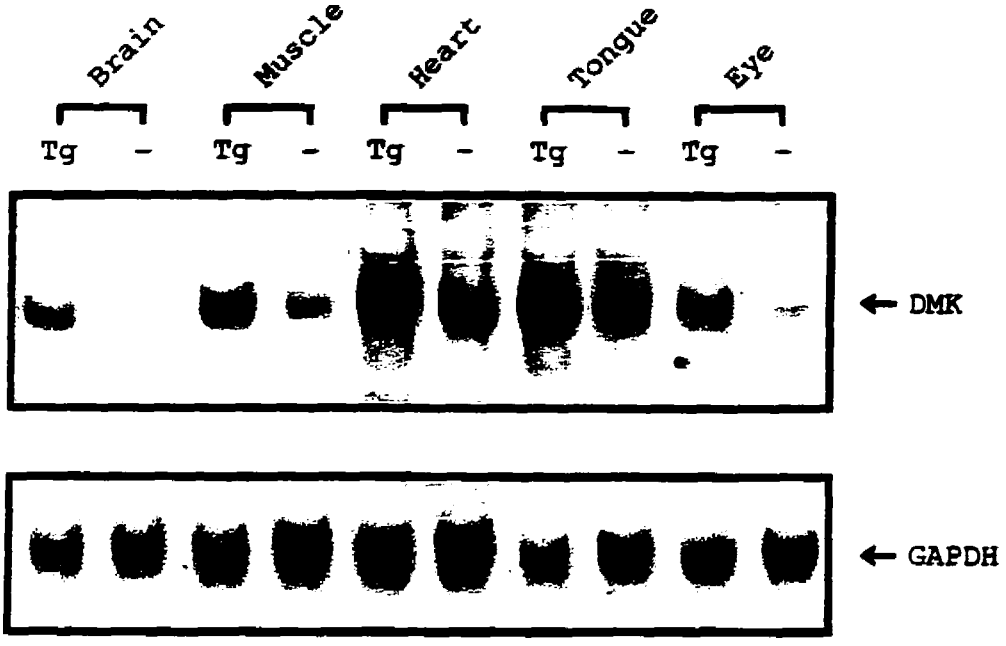

Line A
Line B
Line C

Table 4-1: Transgene Copy Number and Tissue Expression Levels

Line	Transgene Copy No	Transgene Expression Levels *									
		RNA					Protein				
		Brain	Muscle	Heart	Tongue	Eye	Brain	Muscle	Heart	Tongue	Ey
A	50	20	6	2	8	5	12	4	1.5	5	3
B	35	12	4	1.5	5	3.5	7	1.5	1	3	1.
C	10	2	1	1	1	1	1.5	1	1	1	1

* Numbers represent fold increases in transgene levels over mouse endogeneous levels determined by densitometric scans. RNA levels were normalized to GAPDH levels. Protein levels were normalized to a 60 kDa cross-reactive species (56 kDa species for heart). Increases in transgene protein levels were determined by combining the area of the DMK isoforms together and comparing this to identical areas in endogeneous lanes.

Figure 4-4: Expression of human DMK transcripts from transgenic mice. Total RNA was prepared from transgenic mouse (Tg) line A and wild-type littermates (-) and analyzed by Northern blot. Sequential hybridization was performed with human DMK and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probes. Over-expression of human DMK transcript was observed in transgenic brain, skeletal muscle, tongue and eye at levels 1.5 to 20 fold greater than endogenous mouse DMK. A similar level of over-expression was not detected in transgenic heart samples.


Brain
Muscle
Heart
Tongue
Eye
Tg
-
Tg
-
Tg
-
Tg
-
Tg
-
DMK
GAPDH

detect expression of human DMK protein in transgenic mice by immunoblot analysis (Figure 4-5). A number of immunoreactive bands were detected, however, similar to human frontal cortex control, two prominent species, close to the predicted size of DMK (74 kDa and 82 kDa) were observed to be over-expressed in transgenic tissue extracts when compared to wild-type controls (Figure 4-5). This was most striking in brain samples where neither isoform was detected in wild-type brain extract (Figure 4-5). Over-expression of human DMK protein was detected in tissues consistent with the Northern analysis (Figure 4-5, Table 4-1). Furthermore, in transgenic skeletal muscle, the 74 kDa species was over-expressed, whereas in brain, tongue and eye, both isoforms were expressed at elevated levels (Figure 4-5). Significant over-expression was not observed in the hearts of these transgenic mice consistent with RNA levels (Figure 4-5). Similar isoforms representing endogenous mouse DMK protein were recognized in all wild-type littermate tissues examined here, except brain (Figure 4-5). However, in muscle extracts, it appears that the endogenous mouse DMK species is smaller than its 74 kDa human counterpart, indicating that there is a size difference of this isoform between human and mouse DMK (Figure 4-5).

4.3.3 Muscle Pathology:

Cryostat sections of skeletal muscle were examined for evidence of any distinguishing histological features. Using haematoxylin and eosin staining, subsarcolemmal structures, known as sarcoplasmic masses, harboring disorganized myofilaments, free ribosomes and aggregations of tubules (Harper, 1989; Karpati, 1979)

Figure 4-5: Expression of human DMK protein from transgenic mice. Protein extracts from tissues of transgenic mouse (Tg) line A and wild-type littermates (-) were prepared and analyzed by western blot. Anti-DMK antibody, a polyclonal immunoglobulin derived from a fusion protein, was used at a final concentration of 0.1 μg/ml. The blot was developed using an ECL kit as recommended. DMK isoforms correspond to a 74 kDa and 82 kDa species as shown. Both isoforms were over-expressed in transgenic brain, tongue and eye extracts whereas the 74 kDa species was predominant in transgenic muscle extracts. Mouse DMK protein isoforms were detected in extracts of all wild-type tissues shown here, except brain.

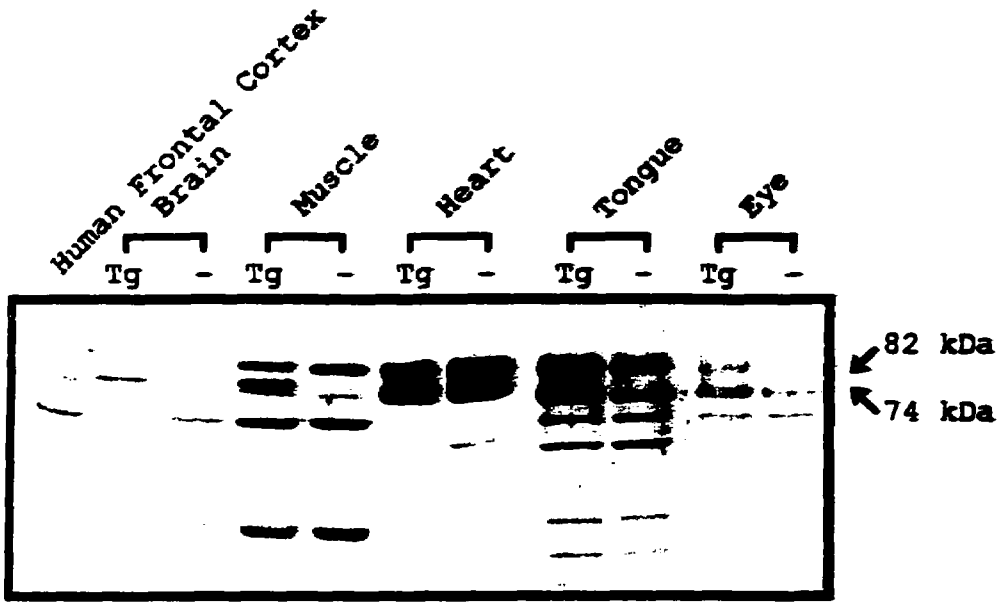
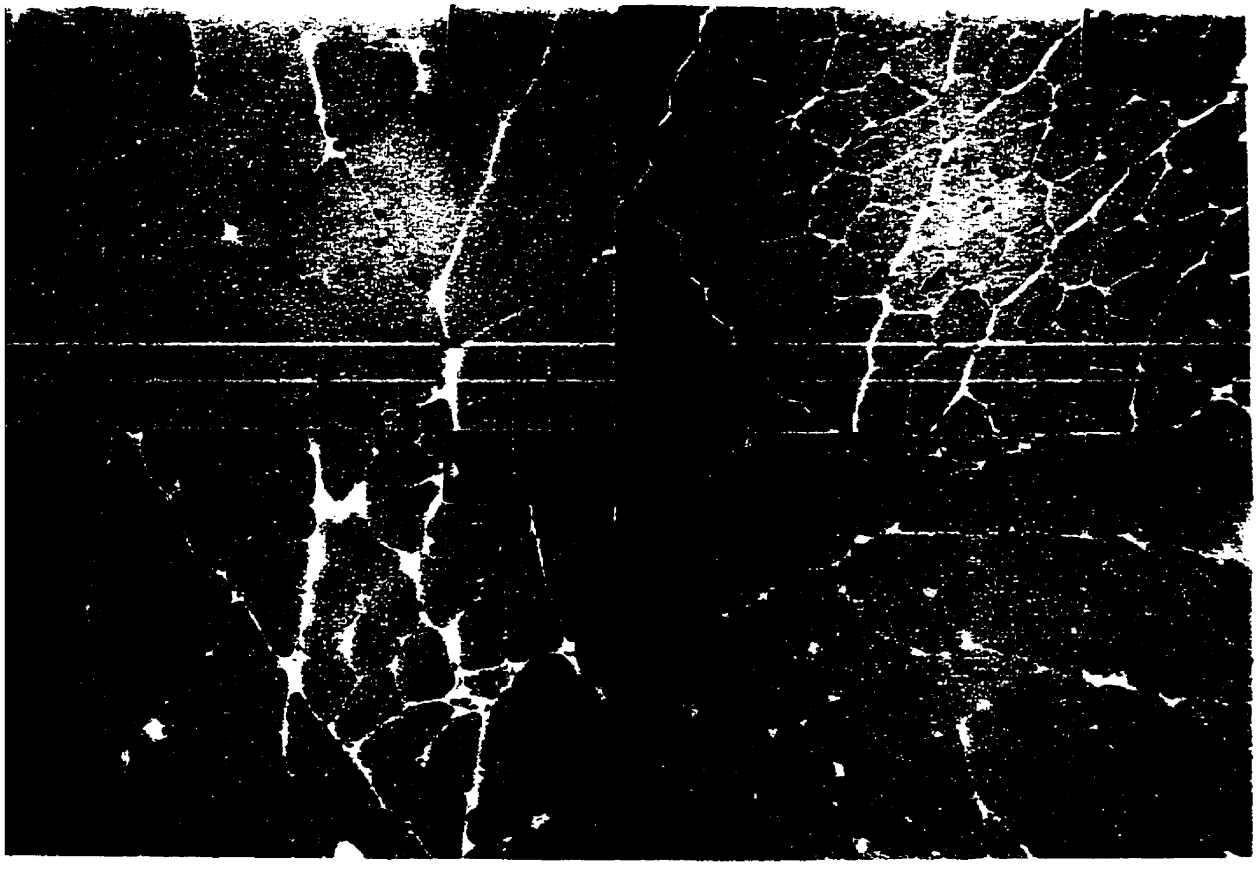

Human Frontal Cortex Brain
Muscle
Heart
Tongue
Eye
Tg
-
Tg
-
Tg
-
Tg
-
Tg
-
82 kDa
74 kDa

Figure 4-6: Histological examination of transgenic and wild-type cross-sectioned skeletal muscle. Representative haematoxylin and eosin stained gastrocnemius muscle are shown for, (A) transgenic mouse line A. Calibration bar = 25 μm, (B) transgenic mouse line A, (C) transgenic mouse line B and, (D) transgenic mouse line A wild-type littermate. (B, C, D) Calibration bar = 50 μm. The arrowhead indicates sarcoplasmic mass and arrows indicate centrally nucleated muscle fibers. Sarcoplasmic masses and an increase in centronucleated fibers were detected in transgenic mice.



were observed in 5% of fibers examined (Figure 4-6A). No sarcoplasmic masses were observed in wild-type sections. In addition, a dramatic increase in central nuclei was observed (that appeared variable in shape, size and intensity) in muscle fibers of transgenic mice (Figure 4-6B and 4-6C) compared to the wild-type littermates (Figure 4-6D). The prevalence of centronucleated muscle fibers was determined to be in the range of 25-35% for transgenic mice, in contrast to 2-5% for wild-type littermates (Figure 4-7, Student's 2-tailed t-test, $p<0.005$). The degree of centronucleation was greater in litters expressing higher levels of human DMK (Figure 4-7, Table 4-1, Student's 2-tailed t-test, $p<0.01$).

Using muscle fiber type-specific monoclonal antibodies, the area of type I, type IIa and type IIb fibers was determined in transgenic and wild-type littermates. The fiber area of type IIa and IIb were similar and type II fiber area was not significantly different between transgenic muscle samples and wild-type matched controls (Figure 4-8). A significant reduction in the size (atrophy) of type I fibers was observed in transgenic skeletal muscle as compared to wild-type littermates (Figure 4-8, Student's 2-tailed t-test, $p<0.005$). The degree of fiber atrophy was greater in litters expressing higher levels of human DMK (Figure 4-8, Table 4-1, Student's 2-tailed t-test, $p<0.025$).

4.3.4 Primary Myoblast Fusion Index:

In order to determine if there is a defect in muscle maturation in these DMK transgenic mice, I established primary myoblast cell lines from these mice and wild-type littermates. The fusion index of primary myoblasts was determined by assessing

Figure 4-7: Prevalence of centronucleated fibers in transgenic animals (Tg) and wild-type littermates (-). Muscle samples were obtained from 3 litters in transgenic lines A and B. Values are means ± S.D. derived from 10 randomly selected areas containing 100 fibers each. Transgenic mice showed a significant increase in centronucleated fibers as compared to wild-type littermates (Student's 2 tailed t-test, $p<0.005$).

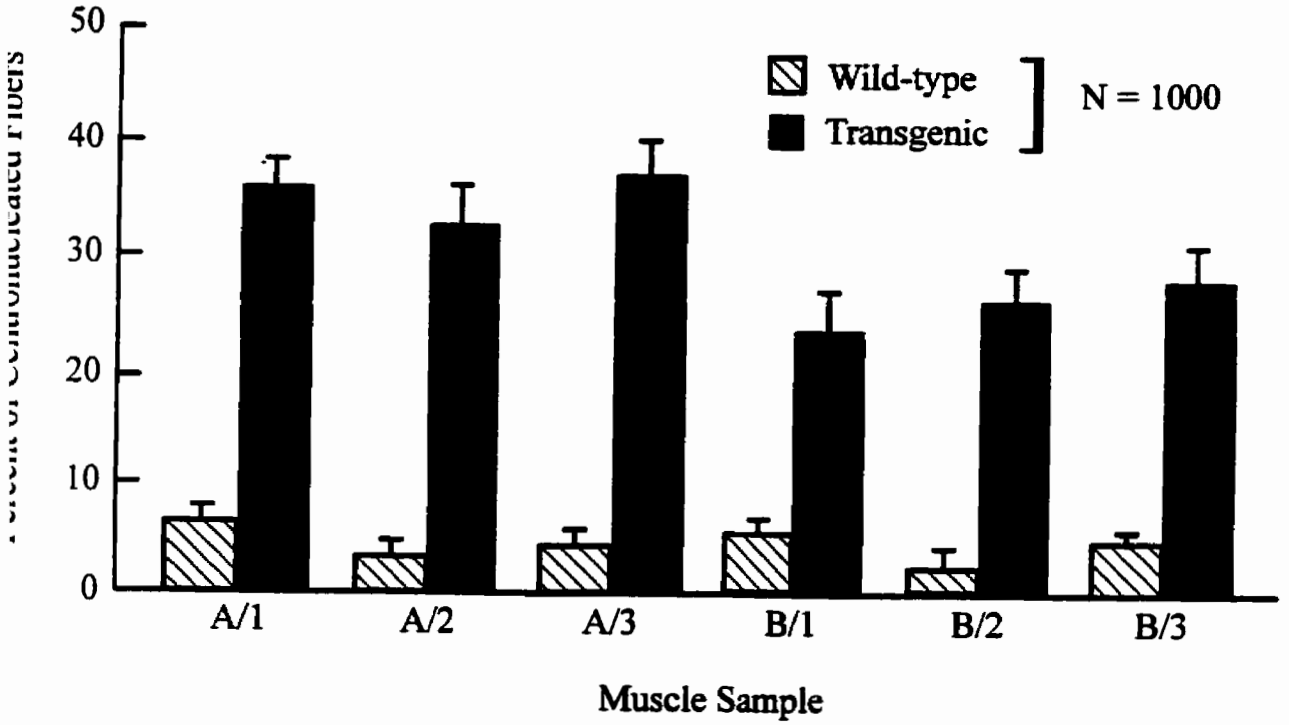

50
40
30
20
10
0
Wild-type
Transgenic
N = 1000
A/1
A/2
A/3
B/1
B/2
B/3
Muscle Sample

Figure 4-8: Mean muscle fiber area in transgenic mice (Tg) and wild-type littermates (-). Mean fiber area ± S.D. of type I and type II fibers in transgenic mice (Tg) and wild-type littermates (-) found in 1000 randomly chosen fibers from muscle samples obtained from 3 litters in transgenic lines A and B. Significant atrophy of type I fibers was observed in transgenic skeletal muscle as compared to wild-type littermates (Student's 2 tailed t-test, $p<0.005$).

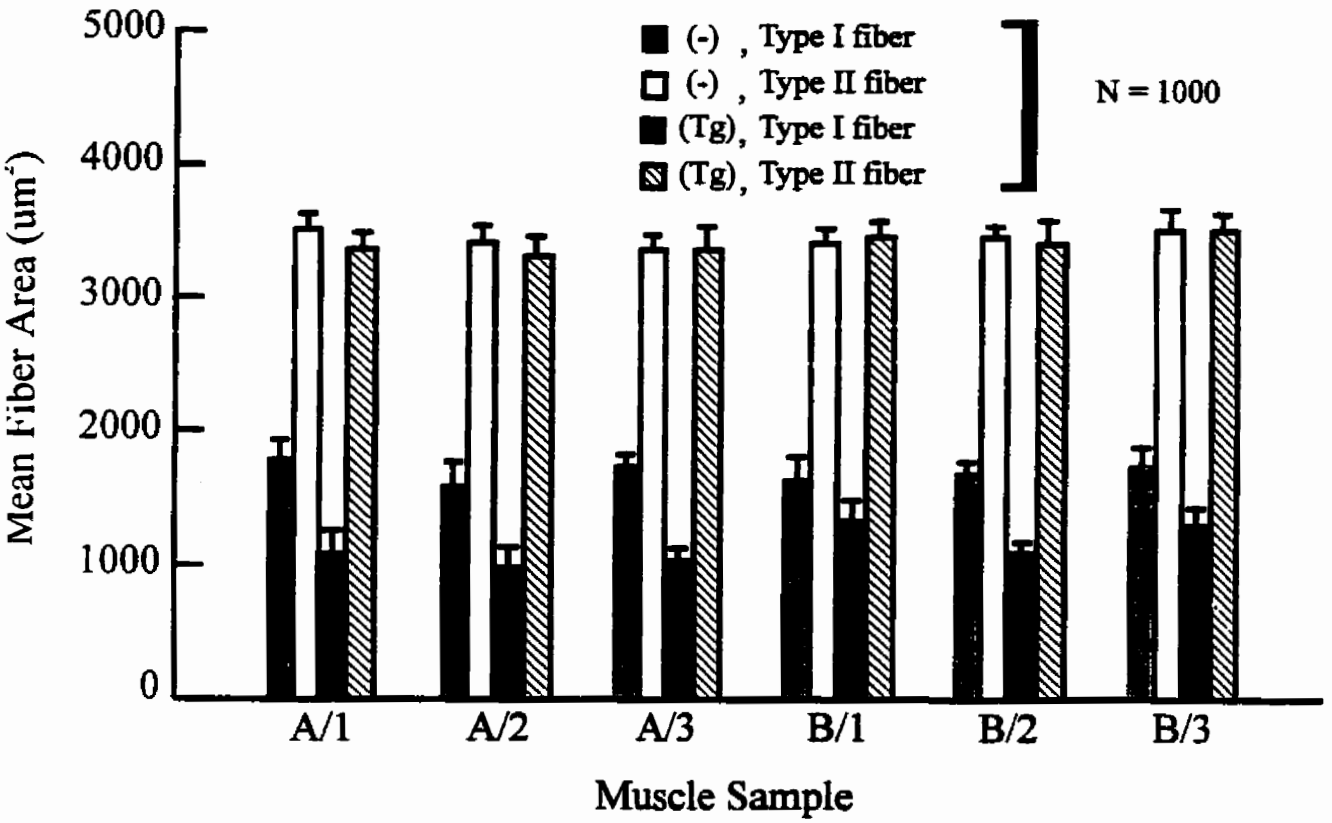
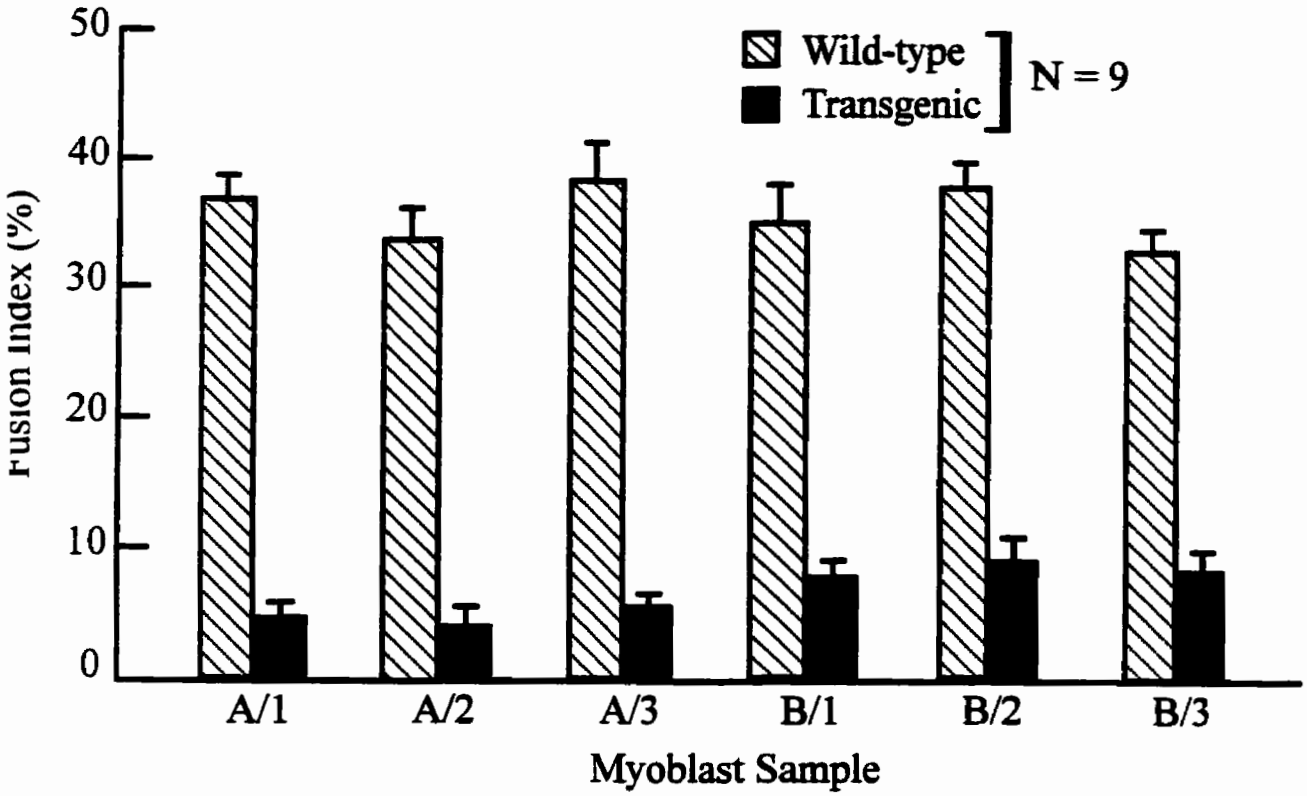

5000
4000
3000
2000
1000
0
Mean Fiber Area (um²)
(-) , Type I fiber
(-) , Type II fiber
(Tg), Type I fiber
(Tg), Type II fiber
N = 1000
A/1
A/2
A/3
B/1
B/2
B/3
Muscle Sample

Figure 4-9: Percent fusion of primary myoblasts from transgenic and wild-type littermates following induction of differentiation. Primary myoblasts were established from 3 litters in transgenic lines A and B. Values are mean percentages ± S.D. derived from 3 independent experiments performed in triplicate. Myoblasts established from transgenic mice exhibited a reduction in fusion potential as compared to wild-type control myoblasts (Student's 2 tailed t-test, $p<0.005$).


50
40
30
20
10
0
Fusion Index (%)
Wild-type
Transgenic
N = 9
A/1
A/2
A/3
B/1
B/2
B/3
Myoblast Sample

myoblast differentiation potential (Figure 4-9). Myoblasts established from transgenic animals exhibited a 4-8 fold reduction in fusion potential as compared to wild-type matched control myoblasts (Figure 4-9). The fusion index was lower in myoblasts established from litters (line A) expressing higher levels of human DMK (Figure 4-9, Table 4-1, Student's 2 tailed t-test, $p<0.01$).

4.4 DISCUSSION:

Evidence is presented in this chapter that over-expression of the human DMK gene in transgenic mice results in a myopathy with features similar to those observed in DM. It is unlikely that the pathology observed was due to the disruption of a gene at the site of integration since this phenotype was associated with independent transgenic lines, each with a different integration site. The pattern of over-expression observed in these mice indicates *cis*-acting regulatory elements are present in this transgene. Characterization of these regulatory elements has shown that a muscle specific enhancer, located in intron 1, is responsible for increased expression of DMK in skeletal muscle (Storbeck *et al.*, 1997). Similar to RNA expression levels, two human DMK protein isoforms (a 74 kDa and a 82 kDa species) were detected at elevated levels in transgenic tissues with respect to wild-type controls (Figure 4-5). Consistent with DMK isoforms reported in rat tissues (Whiting *et al.*, 1995), the endogenous mouse DMK protein in muscle extracts appears smaller than its 74 kDa human counterpart (Figure 4-5). Furthermore, neither species is detected in wild-type brain extract but is clearly evident in

transgenic brain extract verifying the 74 kDa and 82 kDa species (Maeda *et al.*, 1995: Waring *et al.*, 1996) as authentic human DMK isoforms. The skeletal muscle pathology in these DMK transgenic mice is characterized by an increase in centronucleation. the presence of sarcoplasmic masses and reduction in size (atrophy) of type I fibers. Although each of these observed characteristics is found individually in other myopathies, the presence of two or more of these changes is considered diagnostic of DM muscle biopsies from affected patients (Brooke, 1986; Roses *et al.*, 1979).

An increase in the number of central nuclei is typically found in DM muscle (Carpenter and Karpati, 1986). Consistent with the observations presented here, nuclei associated with DM muscle have been reported to be variable in shape, size and intensity of staining (Korenyi-Both, 1983; Sarnat and Silbert, 1976). Although increased centronucleation is associated with regeneration in other myopathies like Duchenne muscular dystrophy, the relative lack of fibrosis associated with DM suggests that repeated necrosis and regeneration is not a factor with this disease (Carpenter and Karpati, 1984). Similarly, no evidence of fibrosis was detected in these DMK transgenic mice.

Previous histological studies have shown that there is a marked delay of myoblast terminal differentiation in skeletal muscle tissue of severely affected DM patients (Dubowitz, 1985; Iannaccone *et al.*, 1986). This delay in differentiation has been proposed to be a cause of the increase in centronucleation in DM muscle as nuclei of embryonic muscle cells, which are internalized, fail to re-locate to the sarcolemma by

birth (Carpenter and Karpati, 1984; Dubowitz, 1985; Iannaccone *et al.*, 1986). In addition, although type I fiber atrophy is apparent in DM patients, the eventual loss of this fiber type is not seen as the disease progresses (Carpenter and Karpati, 1984). The atrophy instead, may be due to a delay or defect in maturation of these muscle fibers (Sarnat and Silbert, 1976). To gain further insight into the status of muscle maturation in the DMK transgenic mice, primary myoblast cell cultures were established from these mice and wild-type littermates. Fusion assays demonstrate that over-expression of human DMK in these mice significantly reduces myoblast differentiation potential (Figure 4-9). In addition, the fusion potential appears to be inversely correlated to the level of human DMK expression.

Although atrophy of type I fibers and centronucleation are diagnostic hallmarks of DM, the role of these features in the pathophysiology of this disease is unresolved (Harper, 1989). Type I fiber atrophy is occasionally associated with hypertrophy of type II fibers (Dubowitz, 1985). It has been postulated that the loss of type I fiber size is compensated for by an increase of type II fiber size (hypertrophy) as a means to maintain muscle bulk (Karpati, 1979; Tohgi *et al.*, 1994). Furthermore, type I fiber atrophy and myotonia are early disease findings (Dubowitz, 1985). It is the increase in type II fiber size which is thought to be responsible for muscle weakness and contractibility defects (Karpati, 1979). A significant reduction in the size of type I fibers in our DMK transgenic mice was observed but the relative fiber size of type II fibers of transgenic mice and their wild-type littermates were similar, indicating that the type II fibers were

not enlarged due to the over-expression of DMK (Figure 4-8). It is possible that hypertrophy of type II fibers is age-dependent and may present itself as these transgenic mice age. However, as a compensation of type II fiber size was not observed in these mice at six months of age, six month old DMK transgenics should be useful for determining the role of type I atrophy itself in inducing myotonia by EMG studies.

Sarcoplasmic masses are subsarcolemmal area structures that occur in a number of neuromuscular diseases, including DM (Karpati, 1979). Although their role in the pathogenesis of DM is also unknown, they are thought to arise as a result of unregulated synthesis of muscle components, harbouring disoriented myofilaments and other cytoplasmic organelles (Harper, 1989; Karpati, 1979). Such abnormalities on the edge of the muscle fiber have been postulated to affect the cytoskeleton, perhaps resulting in centronucleation (Karpati, 1979). Of particular interest is the recent report that the DMK protein is re-localized to sarcoplasmic masses found in type I fibers from a muscle sample of a severely affected DM patient (Dunne *et al.*, 1996). Investigation of whether this is true for the sarcoplasmic masses found in these transgenic mice is warranted.

Another DM transgenic model has been recently reported by Jansen *et al* (1996). These investigators do not observe a similar skeletal muscle phenotype in their model, although the same genomic fragment was used to generate both sets of transgenic mice. They report an irregular appearance of the heart, resembling cardiac myopathy, in those mice with the highest levels of human DMK expression. Although the RNA expression profile in their mice is not known, the difference in phenotype between these two models

is perhaps due to both the effect of MAR sequences in enhancing position-independent, copy number-dependent expression (McKnight *et al.*, 1992; Phi-Van *et al.*, 1990; Stief *et al.*, 1989) and the higher absolute copy number in my lines (Table 3-1). In addition, it has been reported that strain-dependent genetic factors can modify phenotype in transgenic mice and may contribute to the differences between these mouse models (Hsaio *et al.*, 1996; Rozmahal *et al.*, 1996). Nevertheless, the observation of cardiac myopathy in the Jansen model and the DM-like features I have observed strongly suggests that over-expression plays a role in DM pathology. Whether these effects are due to increased levels of human DMK transcript and/or protein remains to be determined.

In contrast to the results obtained with DMK transgenic mice, muscle from DMK deficient mice exhibit changes which are only mildly myopathic and not consistent with alterations observed in DM affected muscle (Hamshere and Brooke, 1996; Harper, 1989; Harris *et al.*, 1996; Karpati, 1979; Korenyi-Both, 1983; Reddy *et al.*, 1996). The lack of a DM-like phenotype in any tissue suggests that haplo-insufficiency of DMK mRNA is not responsible for disease. Furthermore, it has been shown that DMK protein is still present in DM affected muscle (Dunne *et al.*, 1996; van der Ven *et al.*, 1993; Chapter 3). Previous work has found DMK protein localized post-synaptically at neuromuscular junctions in skeletal muscle indicating its role in synaptic function and conductance (van der Ven *et al.*, 1993; Whiting *et al.*, 1995). However, the observation of minor ultrastructure changes, fibrosis and degeneration in the skeletal muscle of DMK deficient

mice may be indicative of another role of DMK protein in skeletal muscle structural integrity (Reddy *et al.*, 1996).

A recent report presents evidence that expansion of the CTG repeat results in the accumulation of DMK transcripts in the nucleus (Taneja *et al.*, 1995) perhaps by disrupting its processing or transport (Krahe *et al.*, 1995; Wang *et al.*, 1995). The accumulation of DMK transcripts in the nucleus may confer on them a *trans*-acting gain of function. A recently cloned CUG binding protein (Timchenko *et al.*, 1996) may have a role in this mechanism by stabilizing the transcript or by being sequestered by the CTG repeat, preventing it from its normal cellular function. A dependence of accumulation of DMK transcripts on the size of the CTG repeat would explain the observed correlation between the degree of repeat expansion and disease severity. Possibly, the high levels of DMK transcription in these DMK transgenic mice mimics this accumulation of transcript in the nucleus and results in the DM-like phenotype observed. Identification of this CUG binding protein in mouse would provide further evidence of the role of over-expression of DMK transcript in disease pathogenesis, particularly in muscle.

In conclusion, the model presented here demonstrates a skeletal muscle phenotype similar to that seen in DM patients. The fusion assay experiment indicates that over-expression of human DMK in the muscle of these transgenic mice interferes with muscle maturation which may be causative of the histological phenotype which I have observed. Further studies of these mice may allow elucidation of the molecular basis of this observed phenotype in DM skeletal muscle. These observations suggest that over-

expression of DMK transcript and/or protein are key mediators of disease pathogenesis.

CHAPTER 5: RETINAL DEGENERATION IN ADULT TRANSGENIC MICE OVER-EXPRESSING THE HUMAN MYOTONIC DYSTROPHY (DM) KINASE GENE

5.1 INTRODUCTION:

In the absence of muscle involvement in adults, DM is frequently diagnosed on the basis of unique lens changes comprised of multicoloured, iridescent dust-like opacities that eventually evolve into cataracts (Harper, 1989). Light and electron microscopic studies have shown that these characteristic opacities result from whorls of plasma membrane present in vacuoles located in the subcapsular lens cortex (Dark *et al.*, 1977; Eshaghian *et al.*, 1978). However, cataracts are not the only eye change associated with DM. Numerous progressive retinal changes have also been observed in DM with migration of retinal pigment epithelium (RPE) into the photoreceptor layer being the most common change encountered (Ginsberg *et al.*, 1978). Macular and paramacular lesions have also been detected in at least 20% of DM cases although resulting serious visual impairment has rarely been reported (Ginsberg *et al.*, 1978). Finally, degeneration of photoreceptor cells similar to retinitis pigmentosa (RP) has been described in a number of cases of DM (Harper, 1989; Green, 1985).

In an effort to establish a model of DM in the mouse, transgenic animals that over-express human DMK transcript and protein in skeletal muscle, brain, tongue and eye were generated (Chapter 4). Studies of these mice revealed characteristic histological features of DM including centronucleation and type I fiber atrophy (Chapter 4) and learning and memory deficits (Appendix I) consistent with over-expression of DMK in skeletal muscle and brain, respectively. In this study, the eyes of DMK transgenic mice

were examined for the unique lens changes associated with DM. The retinal alterations described here provide further evidence of the pathogenic effect of DMK over-expression in transgenic mice.

5.2 MATERIALS AND METHODS

5.2.1 Transgenic Mice:

transgenic mice were generated as previously described (Chapter 2.5-2.12). DMK transcript and protein expression were assessed as described in Chapter 4.

5.2.2 Enucleation:

Transgenic mice and wild-type matched controls from lines A and B were anesthesized and subsequently euthanized by an overdose of somnitol (MTC pharmaceuticals). Eyes were dilated and examined for cataracts. Both eyes were removed from each mouse with an effort to keep the optic nerve intact. One eye was frozen in isopentane cooled in liquid nitrogen and stored at -80 °C until further use. The other eye was stored in formalin for 48 hours and then embedded in paraffin. Cryostat sections of eyes from 3, 4, 5 and 12 month old transgenic mice and controls were examined following haematoxylin and eosin staining according to the protocol in Chapter 2.9.

5.3 RESULTS:

No evidence of cataracts was detected in any sample examined. Eyes from transgenic mice from both lines, up to 4 months old, were indistinguishable from wild-type littermate controls. All of these samples possessed 8 to 10 rows of photoreceptor

cell nuclei and well formed stacks of disks in the outer segment of the retina (Figure 5-1A). A single site of disruption of the photoreceptor cell layer by retinal pigment epithelium (RPE) migration was observed in 5 month old transgenic mice derived from line A (Figure 5-1B). By 12 months, migration of RPE around blood vessels in these transgenic mice was widespread (Figure 5-2A). Furthermore, transgenic retinas derived from line A were devoid of a photoreceptor cell layer by 12 months (Figure 5-2B). The outer nuclear layer was reduced to a single row of sparse photoreceptor nuclei. Whether the residual photoreceptors were rods or cones was difficult to distinguish. The inner retinal layers (inner nuclear and ganglion cell layers) appeared slightly disturbed. Conversely, eyes isolated from transgenic line B showed a single focus of retinal alteration at 12 months (Figure 5-2C). No abnormality was detected in any of the wild-type matched controls examined here.

5.4 Discussion:

The significance of these observations in the retinas of DMK transgenic mice are three-fold. First, these findings support the role of over-expression of DMK transcript and/or protein in the pathogenesis of DM. Lens abnormalities are predominant in older affected individuals and not a common feature in congenital DM patients (Harper, 1989). As previously shown (Chapter 3), adult DM patients appear to have more DMK protein than normal matched controls while congenital DM cases appear to have slightly less protein, perhaps due to the inability of transcripts harbouring large CTG repeats to be transported out of the nucleus (Taneja *et al.*, 1995). Likewise, photoreceptor cells are fairly sensitive to protein levels (Olsson *et al.*, 1992), thereby indicating that over-

Figure 5-1: Histological appearance of line A DMK transgenic (A) and wild-type littermate (B) 5 month old retinas. Enucleated eyes were fixed in paraffin, sectioned and stained with haematoxylin and eosin. Unlike the wild-type control, the transgenic retina is becoming disorganized at a single site in the photoreceptor layer (OS, IS) as a result of retinal pigment epithelium migration. RPE, retinal pigment epithelium; OS, outer segment; IS, inner segment; ONL, outer nuclear layer; INL, inner nuclear layer, GC, ganglion cell layer. The bracket indicates the photoreceptor cell layer. 400x magnification.

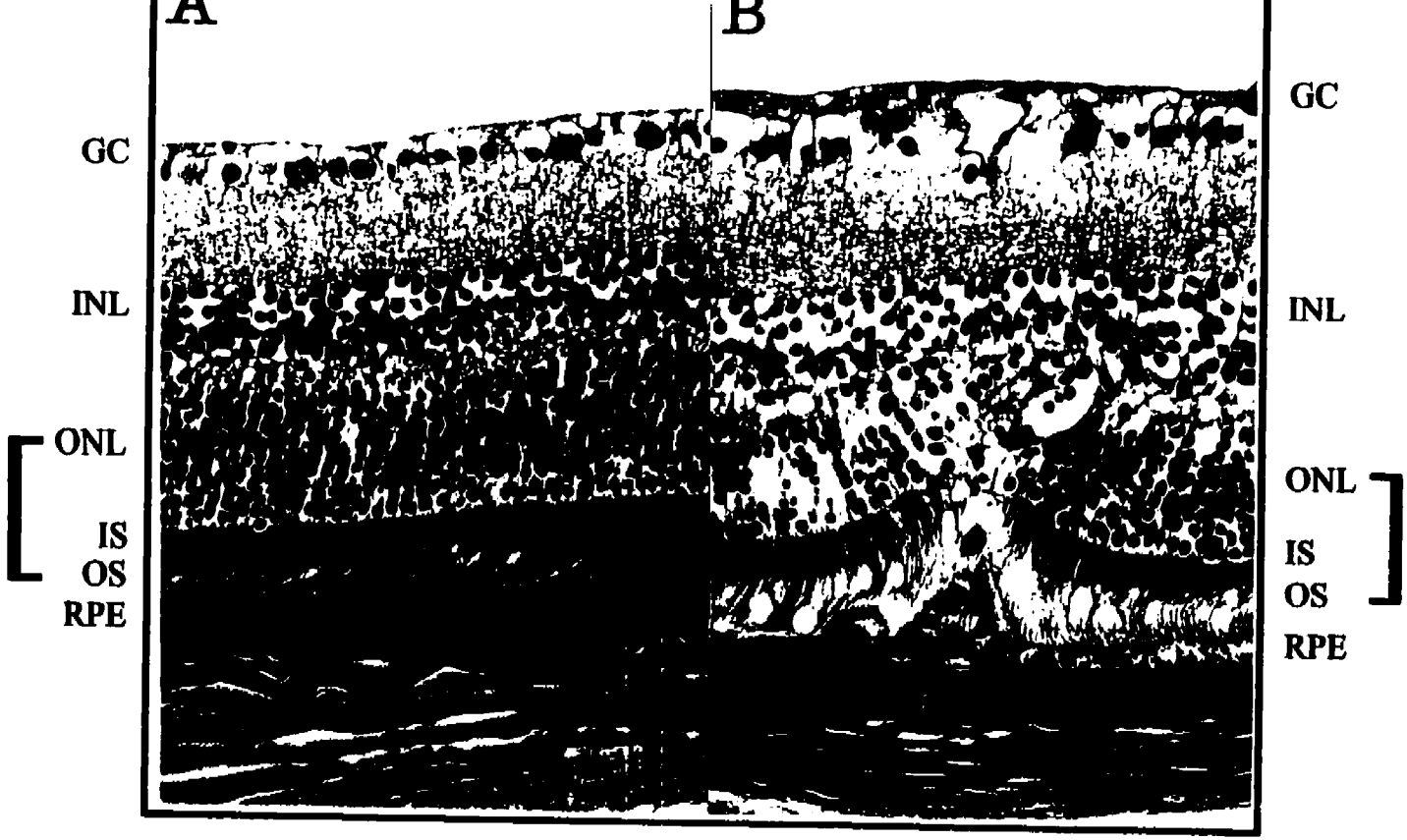

A
B
GC
INL
ONL
IS
OS
RPE
GC
INL
ONL
IS
OS
RPE

Figure 5-2: Histological appearance of 12 month old retinas from line A (A, B) and line B (C) DMK transgenic mice. Representative haematoxylin and eosin stained eye sections indicating major retinal changes in line A mice including, (A) widespread retinal pigment epithelium (RPE) migration and, (B) loss of most of the photoreceptor cell layer (OS, IS) and shortened and disorganized outer and inner nuclear layer (ONL/INL). 400x magnification. A single focus of retinal disruption was observed in line B transgenic mice (C). 200x magnification.

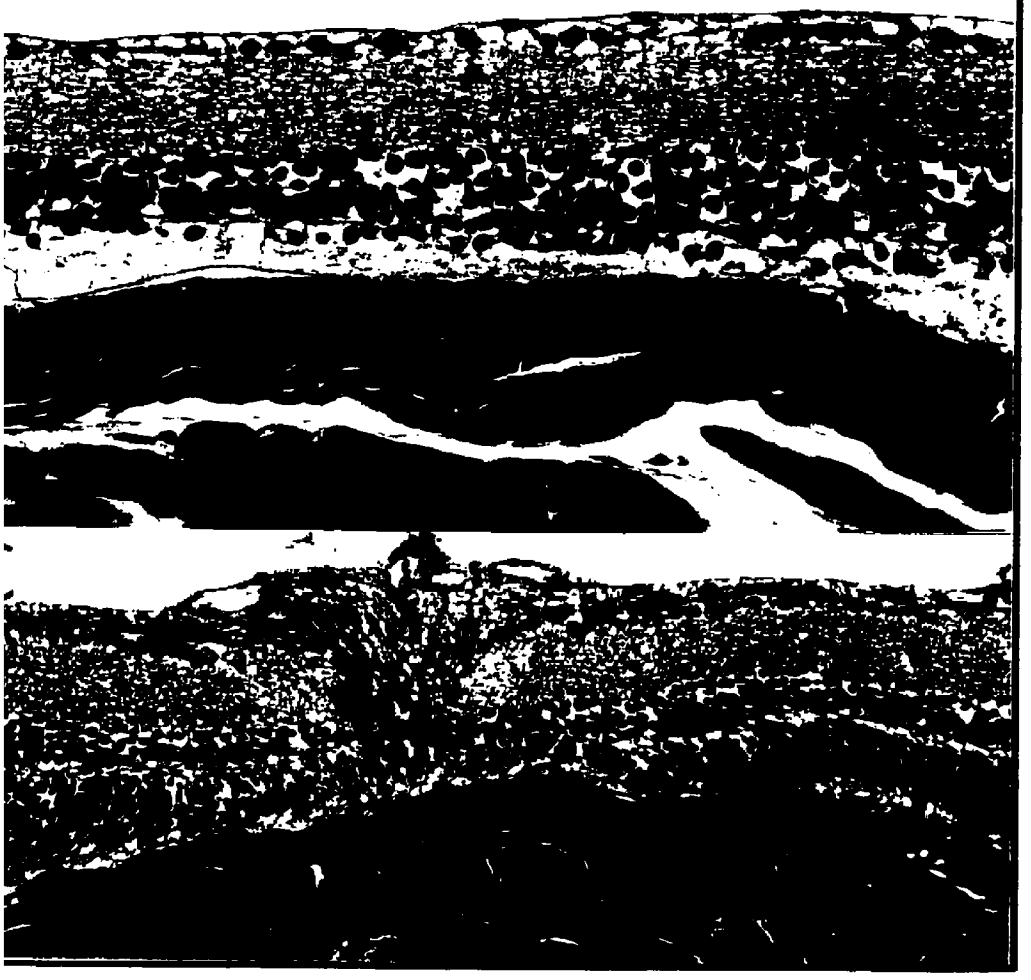
A

GC

ONL/INL

RPE

B



GC

ONL/INL

RPE

GC

INL

ONL

IS

OS

RPE

expression of DMK protein, driven by the endogeneous DMK promoter in the retina, may be the culprit leading to the retinal degeneration observed here and associated with DM. DMK transcripts have been detected in the retina of mice 14 days post-natal (Jansen et al., 1996) but clearly, localization of DMK protein to this region of the eye is required. Furthermore, in DM, retinal changes are slowly degenerative, occurring over the lifetime of a DM patient (Ginsberg *et al.*, 1978). In these DMK transgenic mice, the first signs of retinal degeneration appear in the fifth month post-natal, substantially slower than other RP mouse models (Chang *et al.*, 1993b).

It is possible that the striking phenotype observed in line A is due to the inactivation of an endogeneous gene as a result of integration of the transgene. However, the milder phenotype of RPE migration in transgenic mice from line B compared to line A mice reflects the varying expression levels between the two lines. Comparison of line A and B expression levels in eye suggests that DMK transcript and protein levels above 5 and 3 fold, respectively, are necessary to elicit a phenotype in a 12 month period and may provide a basis for the role of DMK protein levels as a diagnostic indicator for retinal changes in DM patients. It is very likely that a similar phenotype to line A will become evident in line B as these mice age. Moreover, increasing expression levels in line B by breeding to homozygosity may lead to the complete degeneration of the photoreceptor cell layer as seen in line A.

Secondly, these findings represent a potential new model of RP, a term applied to retinal degenerative diseases in humans (Olson *et al.*, 1992). To date, naturally occurring or transgenic mouse models exist for three photoreceptor-specific genes in humans that

are associated with RP. The retinal degeneration (*rd*) mouse contains viral DNA insertion in β-cGMP phosphodiesterase which prevents its production. In this model, degeneration of the retina is rapid and complete by day 17 post-natal (Chang *et al.*, 1993; Tsang *et al.*, 1996). The retinal degeneration slow (*rds*) mouse carries an insertion in the peripherin gene thereby interfering with its production. Unlike *rd*, degeneration in *rds* mice is slower, beginning at 3 weeks, 50% of nuclei are lost by week 12 and completely by 12 months (Chang *et al.*, 1993). Finally, transgenic mice expressing mutant rhodopsin begin retinal degeneration by week 3, lose 50% of the nuclei in the outer nuclear layer of the retina by week 7 and have virtually no nuclei left by 12 months (Olson *et al.*, 1992).

Unlike these models, photoreceptor degeneration in mice over-expressing DMK appears to be much slower and progressive, occurring between 5 and 12 months (Figure 6-1 and 6-2). However, electron microscopic studies will be required to determine if rods are the first cells degenerating thereby confirming the RP phenotype. Coincidentally, a sixth locus for autosomal dominant RP (adRP) has been mapped to 19q13.4, a linkage interval which is relatively close to the DM mutation (Al-Maghtheh *et al.*, 1994). Considering that ocular abnormalities are associated with adult onset DM, the possible role of DMK in these families with adRP gives way to speculation and may warrant a study of the extent of CTG expansion or levels of DMK protein in these adRP families.

Lastly, these findings are significant in that photoreceptor cell death generally occurs by apoptosis or programmed cell death, which has not been associated with DM previously. Apoptosis is a process used to control the growth of developing cells (Chang *et al.*, 1993; Portera-Cailliau *et al.*, 1994). Morphologically, cell death associated with

RP is characterized by condensation of the nucleus and the cytoplasm of the dying rod cell which would appear pyknotic (Nickells, 1996). As the nucleus condenses, degradation of the unprotected stretches of DNA that link nucleosomes together occurs resulting in a distinctive laddering of DNA fragments upon isolation and resolution of total DNA (Nickells, 1996). Instead of lysis, the cell begins to break up into smaller membrane-bound forms, or apoptotic bodies, which contain individual organelles and remnants of the nucleus (Adler, 1996; Nickells, 1996). These bodies are rapidly phagocytosed by surrounding cells including, coincidentally, retinal pigment epithelial (RPE) cells (Adler, 1996; Nickells, 1996; Sung *et al.*, 1994).

Thus, the early appearance of RPE migration into the photoreceptor cell layer in these DMK transgenics may mark the beginning of retinal degeneration by way of apoptosis. Identification of hallmarks of apoptosis will be required for evidence of cell death. These apoptotic indicators include the appearance of pyknotic nuclei and internucleosomal DNA fragmentation as detected by the appearance of characteristic DNA ladders by agarose gel electrophoresis and the *in situ* labelling of apoptotic cell nuclei with a terminal deoxynucleotidyl transferase-mediated incorporation of biotinylated nucleotides into the 3' ends of DNA fragments (TUNEL). Finally, if retinal degeneration is occurring by apoptosis in these mice, breeding the DMK transgenics with mice over-expressing inhibitors of apoptosis such as bcl-2 (Boise et al., 1993) or the recently reported IAP family (Liston *et al.*, 1996) may rescue or modify the phenotype and lead to a better understanding of apoptotic pathways and their role in DM pathophysiology.

CHAPTER 6: THE EFFECT OF THE MYOTONIC DYSTROPHY (DM) MUTATION IN CELL CULTURE AND IN MICE

6.1 INTRODUCTION:

Expanded trinucleotide repeats (TNR) have been identified as the cause of an ever-increasing list of human genetic diseases including: Fragile X retardation syndrome, FRAXA (Fu *et al.*, 1991; Verkerk *et al.*, 1991) and FRAXE (Knight *et al.*, 1993), spinal and bulbar muscular atrophy (SBMA; LaSpada *et al.*, 1991), myotonic dystrophy (DM; Brooke *et al.*, 1992; Fu *et al.*, 1992; Mahadevan *et al.*, 1992), Huntington's disease (HD; The Huntington's Disease Collaborative Group, 1993), the spinocerebellar ataxias (SCA1-3; Orr *et al.*, 1993; Imbert *et al.*, 1996; Pulst *et al.*, 1996; Sanpei *et al.*, 1996), denatatorubral pallidolysian atrophy (DRPLA; Koide *et al.*, 1994; Nagafuchi *et al.*, 1994), Machado-Joseph disease (MJD; Ikeda *et al.*, 1996) and Fredereick's ataxia (FRDA; Campuzano *et al.*, 1996). In most of these diseases, there is a positive correlation between increases in repeat size with increased severity of disease and earlier age of onset.

The extent of expansion in DM affected individuals is large and this is presumably due to the fact that the CTG repeat is located in the 3'-untranslated region (UTR) of the DM gene. The size of the CTG repeat generally correlates with clinical severity and earlier age of onset such that allele sizes in the range of 80-150 repeats are associated with a mildly affected adult DM state, whereas allele sizes of 500-2000 repeats are associated with a severely affected congenital DM state (Harley *et al.*, 1993; Hunter *et al.*, 1992; Tsilfidis *et al.*, 1992). Furthermore, intergenerational expansion provides the molecular basis for the observed genetic anticipation associated with DM

and a number of triplet repeat diseases whereby severity of disease increases in successive generations. Although this relationship between CTG repeat size and disease severity was identified some time ago, the mechanism of repeat expansion and how it causes disease remains unclear.

In order to understand the role of TNR expansion in disease pathogenesis, many groups have been trying to create a transgenic model for repeat instability and disease manifestation. Transgenic mouse models have been generated harbouring cDNA constructs for SBMA (45 repeats; Bingham *et al.*, 1995), HD (44 repeats; Goldberg *et al.*, 1996) and SCA1 (82 repeats; Burright *et al.*, 1995). However, instability was not observed in any of these models, including SCA1 transgenic mice which did exhibit ataxia (Burright *et al.*, 1995). This lead to a belief that mice may not be suitable for modeling TNR diseases due to inherent differences in their replication and repair processes (Bingham *et al.*, 1995; Burright *et al.*, 1995). Recently however, three groups have demonstrated moderate somatic and intergenerational instability in the form of a gain or loss of up to 6 repeats in transgenic mouse models harbouring approximately 113 CAG repeats in a partial HD cDNA (Mangiarini *et al.*, 1997), 55 CTG repeats within a genomic fragment containing the DM gene and two neighbouring genes (Gourdon *et al.*, 1997) and a non-coding 162 CTG repeat array (Monckton *et al.*, 1997). However, only the HD mouse elicited a phenotype and no genetic anticipation was observed.

To facilitate the study of the role of an expanded CTG repeat in DM pathogenesis in transgenic mice, I have generated DMK cDNA constructs with 5 (DMK5) and 100 (DMK100) CTG repeats. These constructs were used to transfect cell-

lines to test their integrity and their effects on DMK transcript stability and protein expression. Seven lines of transgenic mice, two harbouring DMK5 and five expressing DMK100, were analyzed for transgene expression, TNR instability and muscle pathology. This mouse model provides further convincing evidence that TNR instability, similar to the degree of which is observed in DM patients is achievable in mice. Moreover, there is evidence for DM-like pathology and an increase in phenotypic severity with increasing TNR length (genetic anticipation) in these transgenic mice.

6.2 MATERIALS AND METHODS

6.2.1 Cloning the DM Mutation:

Using primers 404 (5'-ACGCGCCCTATGCCTCTTTC-3') and 407 (5'-CAGAGCAGGGCGTCATGCACA-3'), genomic sequence from exon 13 to the 3' end of the DMK gene including 5, 45, 60 and 100 CTG repeats were amplified from a nonaffected individual and various DM patients, respectively. These genomic sequences were cloned into vector pCRII (Invitrogen) named pCTG and then subcloned into a pBluescript II SK+ vector (Stratagene) harbouring a cDNA encoding DMK reconstructed from various partial cDNA clones (cDNA1 and cDNA2) and upstream genomic sequences (pATG). The final transgene constructs were cloned into a expression vector regulated by the CMV promoter and enhancer and terminated by rabbit β-globin polyadenylation signal (Figure 6-1). In a second class of constructs, genomic sequences harbouring the 3'UTR from DMK with various CTG repeats were also subcloned into expression vector pcDNA3 (Invitrogen), downstream of a CMV regulated reporter gene, chloramphenicol acetyltransferase (CAT) (Figure 6-5). Deletion

analysis of these CAT-CTG repeat clones included the substitution of the CTG repeat and flanking DMK sequences with the multiple cloning site sequences from pUC8 (Figure 6-6).

6.2.2 Stable Transfections:

For stable transfections, mouse myoblasts (C2C12), obtained from ATCC, were plated at 5 x 10^5 cells per 100 mm dish 24 hours prior to transfection. Cells were incubated with precipitate, prepared by standard calcium precipitation (Sambrook *et al.*, 1989) for 8 hours and then replenished with fresh growth medium. Neomycin (G418: Gibco/BRL) was added at a final concentration of 400 μg/ml 24 hours later. After 2 weeks, resistant cells were isolated using cloning rings, replated and maintained in fresh medium with G418. Aliquots were resuspended in medium with 10% DMSO and stored in liquid nitrogen.

6.2.3 Transient Transfections:

For transient transfections, mouse myoblasts (C2C12), fibroblasts (C3H10T1/2), rhabydomyosarcoma cells (TE32) and HeLa cells were plated at 3 x 10^5 cells per 60 mm dish 24 hours prior to transfection. The next day, plated cells were re-fed with fresh growth medium 2 hours prior to the addition of the precipitate prepared with the test construct and control plasmid encoding β-galactosidase, as described in section 6.2.2. Cells were transfected for 6 hours and then replenished with fresh growth medium. After 24 hours, cells were harvested in 0.25 M Tris-HCl, pH 8.0 and collected by microfugation at 4 °C for 10 minutes at maximum speed. For protein extracts, cell

pellets were resuspended in an equal volume of 3x loading dye (187.5 mM Tris-HCl pH 6.8, 6% SDS, 30% glycerol. 0.03% bromophenol blue and 1.25 M β-mercaptoethanol) and boiled for 10 minutes before loading equal aliquots on discontinuous SDS polyacrylamide gels. For CAT assays, cell pellets were lyzed following 3 freeze-thaw cycles and heated at 60 °C for 10 minutes. Samples were spun for 10 minutes at maximum speed before transferring supernatants to fresh tubes which were stored at -80 °C. CAT activity was measured from frozen extracts in a liquid scintillation based assay (^{14}C) as described by the manufacturer (Promega).

6.2.4 Estimation of mRNA Half-Life Using Actinomycin D:

Stably transfected C2C12 cells were plated at 3 x 10^5 cell per 60 mm dish 24 hours prior to the addition of actinomycin D(5 μg/ml). Cycloheximide (10 μg/ml), when used, was added 2 hours prior to the addition of actinomycin D. Cells were harvested at intervals of 0, 2, 4, 6, 8, 10 and 24 hours after the addition of the transcriptional inhibitor, actinomycin D. RNA was isolated from cells as described in Chapter 2.2.1.

6.2.5 Generating Transgenic Mice:

Transgenes consisting of a minigene DMK cDNA with 100 and 5 CTG repeats, regulated by a CMV promoter and enhancer, were isolated following digestion with *Sal* I and *Not* I. Fragments were purified according to Chapter 2.5. Transgenic mice, copy number estimation, protein preparation, immunoblot analysis, sectioning tissues and histological examination were done as described in Chapters 2.6 to 2.12.

6.2.6 CTG Repeat Instability:

Genomic DNA was prepared from mouse tail as described in Chapter 2.1.1. PCR analysis was performed using upstream primer 409, 5-GAAGGGTCCTTGTAGCCGGGAAT-3' end-labeled with [γ-^{32}P]ATP and cold downstream primer 410, 5'-AGAAATGGTCTGTGATCCC-3'. Genomic DNA (0.1-0.5 μg) was amplified using a commercial DNA amplification kit (Perkin-Elmer/Cetus) in a total volume of 25 μl with 50 ng of each primer at an initial denaturation of 96 °C for 3 minutes followed by 30 cycles of 96 °C for 1 minute, 62 °C for 1 minute and 72 °C for 1 minute. PCR products (6 μl) were mixed with 4 μl of formamide loading buffer and heated for 3 minutes at 96 °C. An aliquot of 5 μl of heated product was separated on a 6% denaturing acrylamide gel along with a control sequencing ladder prepared using ^{33}P according to sequencing kit instructions (US Biochemicals). Gels were exposed to X-OMAT/AR x-ray film (Kodak) for 1-3 hours at 80 °C.

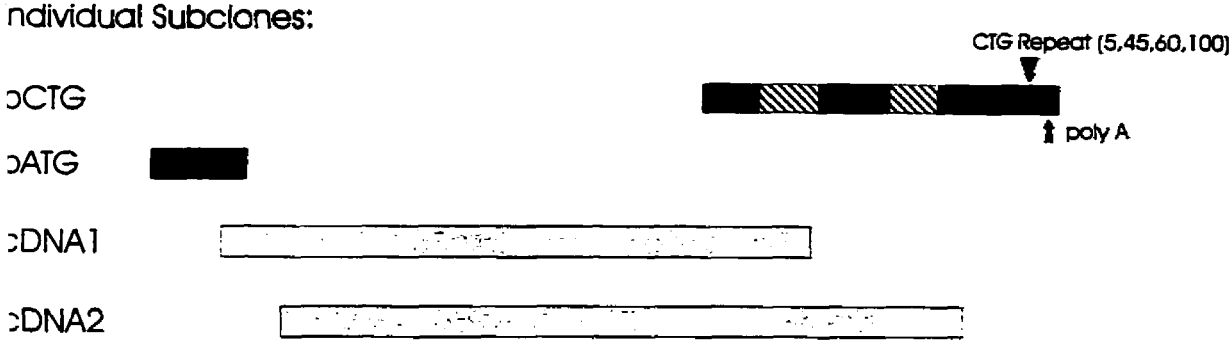
6.3 RESULTS

6.3.1 Transgene Expression in Cell Culture:

To study the effect of the DM mutation, various sizes of CTG repeat were introduced into a human DMK minigene construct regulated by the CMV promoter and enhancer (Figure 6-1). These clones were reconstructed from cDNAs that contained various portions of the gene along with some genomic sequences to provide stability to the repeats for cloning purposes. The final transgene constructs were expressed in C2C12 cells to confirm the integrity of the repeat clones (Figure 6-2). In addition to

Figure 6-1: Construction of human DMK transgene carrying 5 (DMK5) or 100 (DMK100) CTG repeats as described in materials and methods. Shaded boxes indicate DMK sequences. Hatched boxes indicate human DMK introns. pCR1 and pCR2 represent genomic sequences amplified by PCR and subcloned into pCRII vector. cDNA1 and cDNA2 indicate partial DMK cDNAs isolated from a human skeletal muscle library. Horizontal arrows indicate the location and direction of oligonucleotide primers that were used to identify transgenic mice (C and E) and determine copy number (C, D and E). Restriction sites, location of probes, the CTG repeat, exons, CMV promoter and enhancer, translation initiation and polyadenylation sites are indicated. A schematic diagram of a MAR sequence is also shown.

ndividual Subclones:



inal Construct:

MK Transgene:

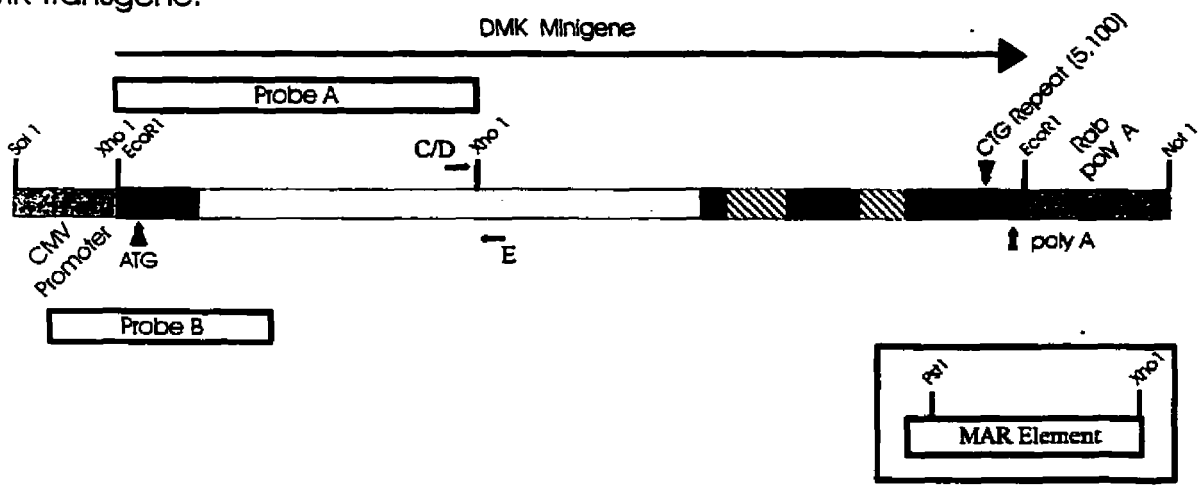
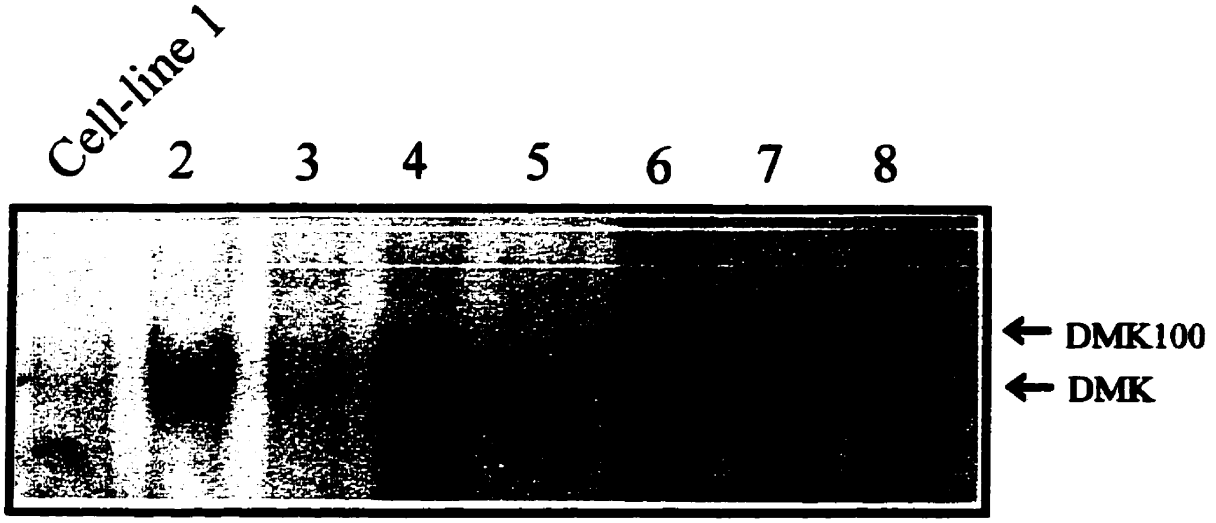


Figure 6-2: Expression of DMK transgene transcripts in stably transfected mouse myoblasts. Total RNA was prepared from a few clones for each construct and analyzed by Northern blot. Expression levels were variable but larger transcripts were expressed from transgene constructs carrying 100 CTG repeats. Cell-line 1, C2C12; 2, DMK5; 3, DMK5; 4, DMK5; 5, DMK60; 6, DMK60; 7, DMK100; 8, DMK100.


Cell-line 1
2
3
4
5
6
7
8
DMK100
DMK

endogenous mouse DMK transcript, DMK repeat constructs were expressed to varying degrees in stably transfected C2C12 cell-lines. As predicted, larger DMK transgene-specific transcripts were identified in cell-lines transfected with DMK100 (Figure 6-2).

A human DMK polyclonal antibody (Whiting *et al.*, 1995) was used to detect expression of the human DMK protein, derived from repeat constructs (Figure 6-3). A number of immunoreactive bands were detected, however, similar to a human frontal cortex extract control, the larger DMK species (82 kDa) was observed to be over-expressed in transfected cell-lines. Previous characterization shows that the larger species is one of only two prominent DMK species detected using this antiserum (Chapter 3 and 4). The smaller species have been shown to be cross-reactive as they are detected in DMK deficient mice (Jansen *et al.*, 1996).

6.3.2 Transcript Stability:

Transcript derived from DMK5 was no longer detectable after 4 hours (Figure 6-4A) in the presence of the transcriptional inhibitor, actinomycin D and appeared to have a half-life of no more than 1.2 hours (Figure 6-4B). However, transcripts expressing 100 CTG repeats were detectable up to 8-10 hours following actinomycin D treatment (Figure 6-4A) with a half-life of 3.5 hours (Figure 6-4B). Endogenous mouse DMK, which has an identical transcript size to DMK5, was found to have a similar half-life to DMK5 message as well (Figure 6-4B). The addition of cycloheximide followed by actinomycin D treatment had no further affect on transcript half-life.

Figure 6-3: Expression of human DMK protein in mouse myoblasts stably transfected with DMK transgenes. Protein extracts were prepared and analyzed by Western blot as described in General Material and Methods (Chapter 2.8-2.9). The DMK transgene corresponds to the 82 kDa species expressed from a human frontal cortex control.

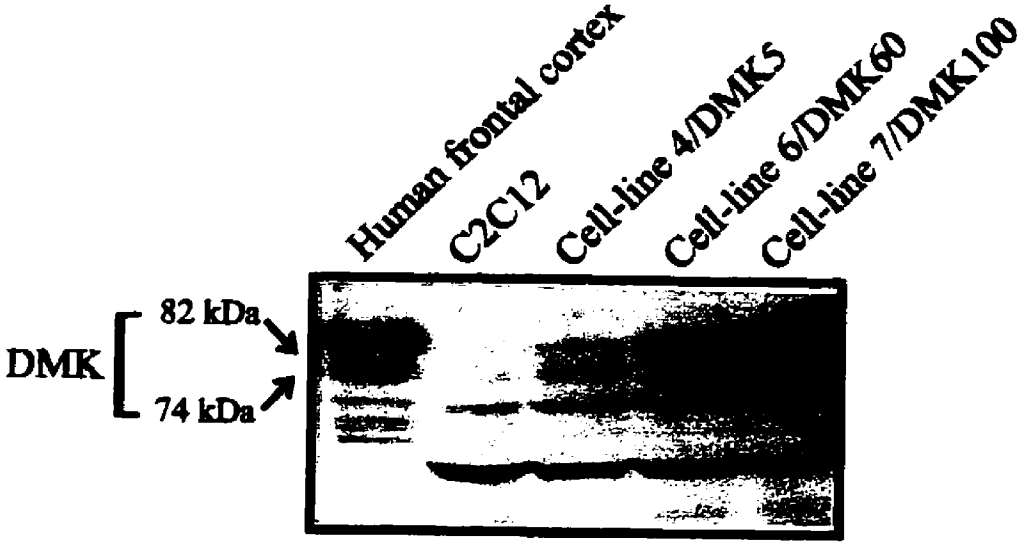
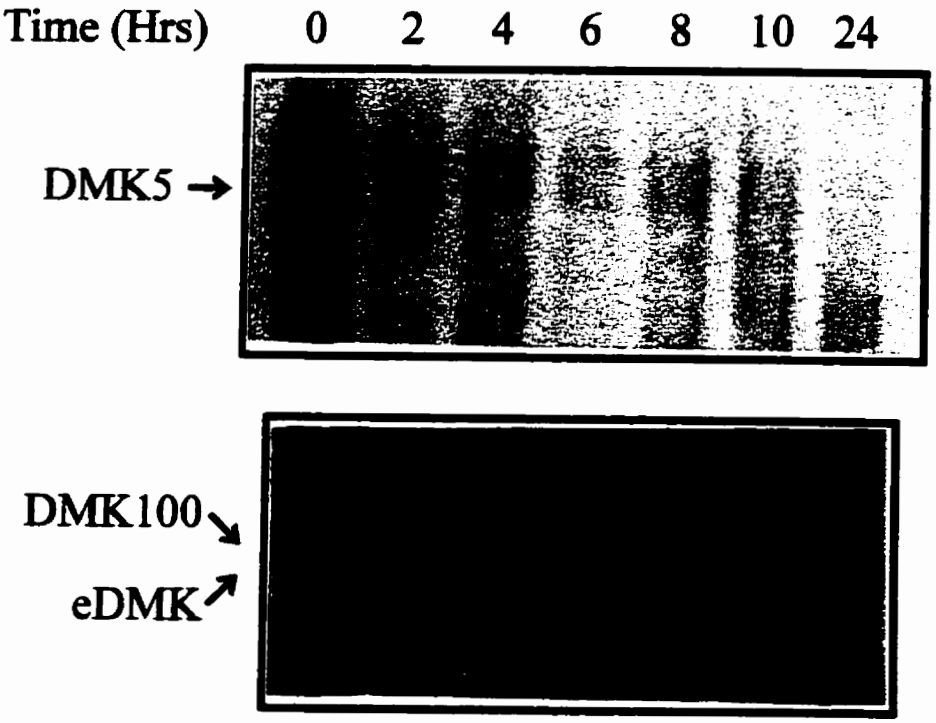
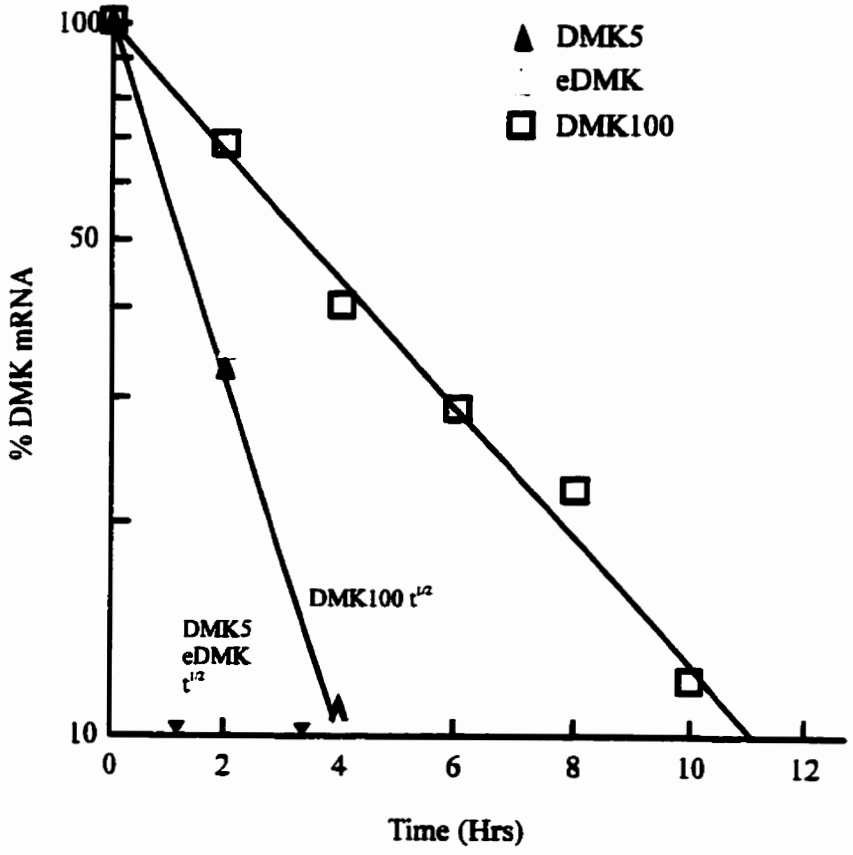

Human frontal cortex
C2C12
Cell-line 4/DMK5
Cell-line 6/DMK60
Cell-line 7/DMK100
DMK
82 kDa
74 kDa

Figure 6-4: Stability of DMK5 and DMK100 transcripts following inhibition of transcription using actinomycin D. (A) Levels of transcript were assessed by Northern blot of total RNA. DMK transcripts carrying 100 CTG repeats appeared to be more stable than DMK5 transcripts. e, endogenous mouse DMK. (B) The amount of DMK mRNA was measured from autoradiograms representing different exposures and three independent experiments by laser densitometry. The log of the amount of transcript remaining was plotted as a function of time (hrs), from which the half-life ($t^{1/2}$) was extrapolated. The half-life of DMK100 transcripts was consistently found to be 3 fold greater than DMK5 transcripts. e, endogeneous mouse DMK.

A


Time (Hrs)
0
2
4
6
8
10
24
DMK5
DMK100
eDMK

B


100
50
10
% DMK mRNA
DMK5
eDMK
DMK100
DMK100 t1/2
DMK5
eDMK
t1/2
0
2
4
6
8
10
12
Time (Hrs)

6.3.3 The Effect of CTG Repeat Size on CAT Activity:

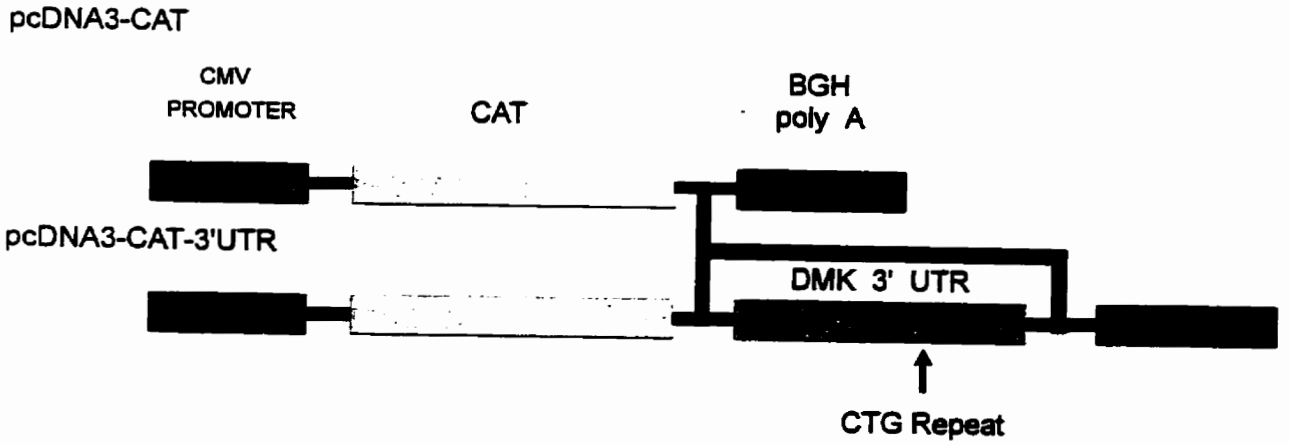
Chloramphenicol acetyltransferase (CAT) activity was used to monitor the effect of CTG repeat size on protein expression (Figure 6-5A). Direct dependence of CAT activity on size of the CTG repeat was observed in all the cell-lines tested here (Figure 6-5B). Deletion analysis of the DMK 3'UTR indicated that the most important element affecting CAT activity was the presence of the CTG repeat itself (Figure 6-6A). Substitution of the CTG repeat with an unrelated sequence did not restore CAT activity at levels obtained with the CTG repeat present (Figure 6-6H). However, the repeat alone was not sufficient to maintain substantial CAT activity as higher levels were observed in the presence of sequences upstream of the CTG repeat as compared to downstream sequences (Figure 6-6B and 6-6C).

6.3.4 Generation of Transgenic Mice:

Seven transgenic lines, two containing the 5 repeat construct (DMK5) and five harbouring the 100 repeat construct (DMK100), regulated by the CMV promoter and enhancer (Figure 6-1), were identified by PCR. Co-integration of the transgene with micro-injected MAR sequences without rearrangement was verified by Southern analysis (Figure 6-7A and 6-7B). Lines were out-bred to establish stable transmission. Densitometric comparison of the transgene to an endogenous, single copy mouse fragment, generated using semi-quantitative PCR (Chapter 2.7), indicated transgene copy numbers to be 2 and 20 for lines 1 and 2, respectively, with DMK5 and 2, 26, 15, 10 and 20 for lines 3 to 7, respectively, for DMK100 (Table 6-1).

Figure 6-5: The effect of CTG repeat size on chloramphenicol acetyltransferase (CAT) activity. (A) A schematic of CAT hybrid constructs. CMV promoter, CAT gene, DMK 3'UTR with various CTG repeat sizes and the polyadenylation site of the bovine growth hormone (BGH) is indicated. (B) CAT activity was determined in a number of cell-lines, following normalization to co-transfected β-galactosidase. Activity was found to increase with increase CTG repeat size.

(A)


pcDNA3-CAT
CMV PROMOTER
CAT
BGH poly A
pcDNA3-CAT-3'UTR
DMK 3' UTR
CTG Repeat

(B)

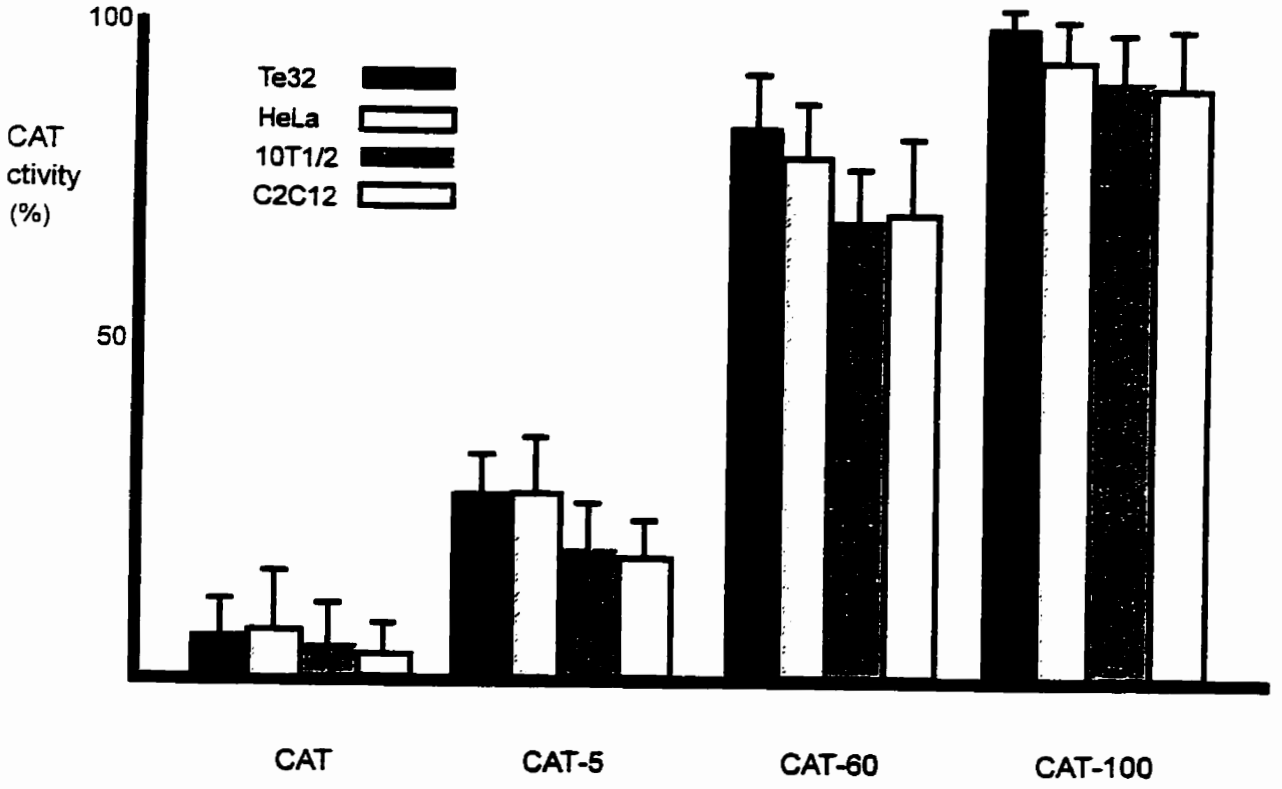

100
50
CAT ctivity (%)
Te32
HeLa
10T1/2
C2C12
CAT
CAT-5
CAT-60
CAT-100

Figure 6-6: The effect of deletion analysis of the DMK 3'UTR on CAT activity. A schematic diagram of CAT hybrid deletion constructs is shown in conjunction with the effect of each on CAT activity. CMV promoter, CAT gene, DMK 3'UTR with CTG repeats and polyadenylation site is indicated. The highest CAT activity was detected in a deletion construct containing the CTG repeat and 3'UTR upstream sequences.

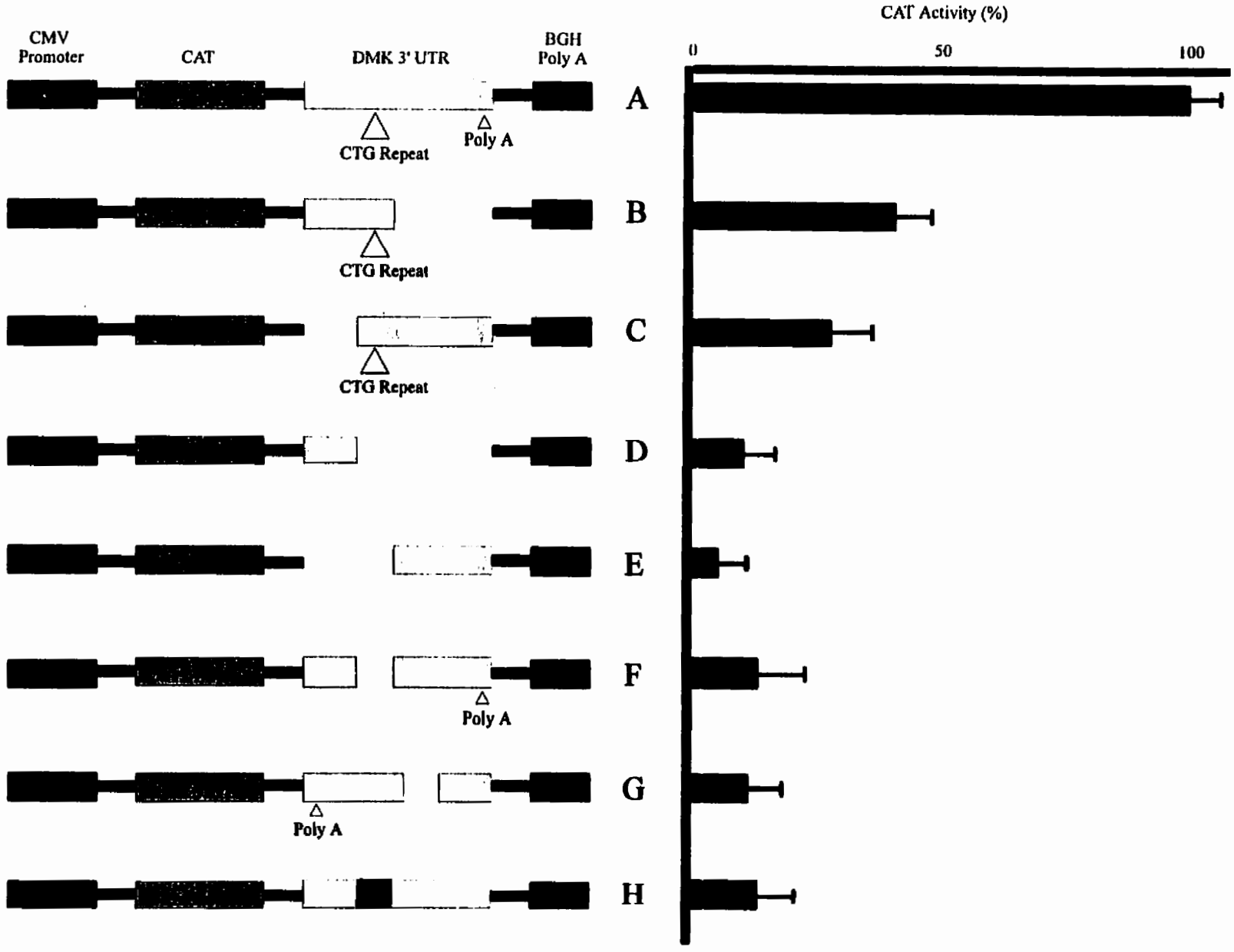
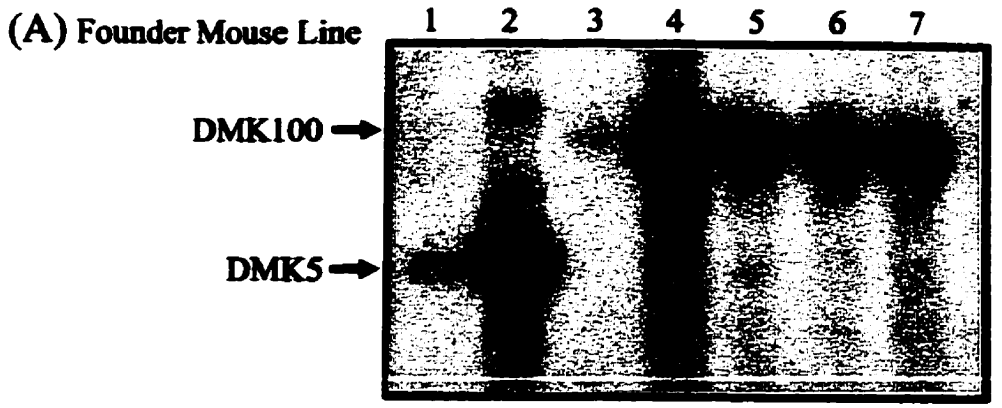

CMV Promoter
CAT
DMK 3' UTR
BGH Poly A
CTG Repeat
Poly A
A
B
C
D
E
F
G
H
CAT Activity (%)
0
50
100

Figure 6-7: Identification of DMK repeat transgenic mice using Southern analysis. Total DNA was prepared from mouse tails, equal amounts (5 μg) were digested with, (A) EcoR1 and probed with a 1.2 kb Xho1 fragment (probe A) or. (B) Xho1 and probed with a 980 bp Nde1/BspH1 fragment (probe B). The differences in intensity reflects variable transgene copy numbers among these lines. The lower band in (B) represents the presence of MAR sequences which possess a Xho1 site close to it 3'end in all founder lines.


(A) Founder Mouse Line
1 2 3 4 5 6 7
DMK100
DMK5

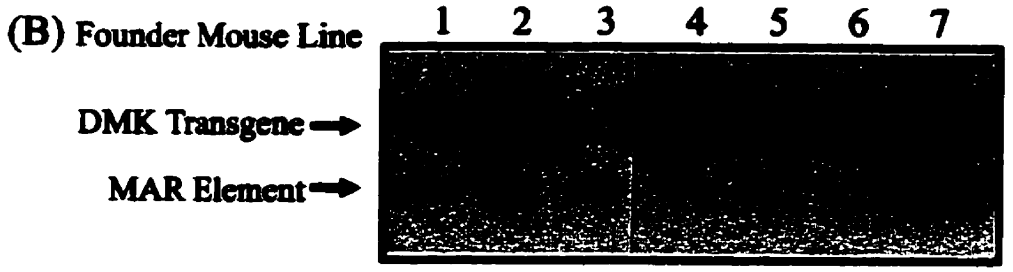

(B) Founder Mouse Line
1 2 3 4 5 6 7
DMK Transgene
MAR Element

Table 6-1: Transgene Copy Number and Tissue Expression Levels

Line	CTG No	Transgene Copy No	Transgene Expression Levels*				
			Protein				
			Brain	Muscle	Heart	Tongue	Eye
1	5	2	2	n.c.	1.3	n.c.	n.c.
2	5	20	2.5	1.1	2	n.c.	n.c.
3	100	2	1.3	1.3	1.2	n.c.	n.c.
4	100	26	n.c.	2.5	2	2	2
5	100	15	n.c.	1.8	1.5	n.c.	n.c.
6	100	10	n.c.	1.5	2	n.c.	2
7	100	20	1.8	2	n.c.	1.5	1.7

* Numbers represent fold increases in transgene levels over mouse endogeneous levels determined densitometric scans. Protein levels were normalized to a 60 kDa cross-reactive species (56 kDa spec in heart). n.c. indicates no change in expression levels between transgenic mice and wild-type litter controls.

6.3.5 Transgene Expression:

Due to the large number of lines and tissues requiring survey, transgene expression was assessed by immunoblot analysis using a human DMK-specific polyclonal antibody as explained in Chapter 2.8-2.9. Transgene expression was found to be variable per tissue per line likely due to the presence of the CMV promoter and enhancer (summarized in Table 6-1). Human DMK protein was expressed in muscle of lines 1 and 3 to 7. In heart, lines 1-6 showed elevated levels of human DMK protein. Lines 1, 2, 3 and 7 expressed human DMK in brain whereas transgene expression in tongue was detected in lines 4 and 7. Finally, lines 4, 6 and 7 expressed human DMK protein in eye. In all cases, only the human-specific 82 kDa DMK species was detected as shown in a representative immunoblot of brain extracts derived from transgenic lines (Figure 6-8). Cross-reactive bands were detected in all lines but were not over-expressed in transgenic lines.

6.3.6 CTG Repeat Instability in Mice:

Intergenerational CTG expansion was detected in all mouse lines expressing the DMK100 transgene (Figure 6-9). The founder (F) generations, from all lines, harbour the original 100 CTG repeat present in the transgene. However, in lines 4-6, some evidence of expansion of the CTG repeat was detected in the founder suggesting some somatic instability (Figure 6-9). The degree of expansion was variable between mouse lines ranging from an increase of 6 repeat units (18 nucleotides) in line 3 up to 18 repeat units (54 nucleotides) in line 6 (Figure 6-9). The largest degree of expansion of 48

Figure 6-8: Expression of human DMK protein from CTG repeat (DMK5 and DMK100) transgenic mice. Protein extracts from brain of transgenic mice (Tg) from mouse line 1 (Tg1) to line 7 (Tg7) and wild-type littermates (-) of DMK5 (-/5) and DMK100 (-/100). Expression of the 82 kDa human DMK species was detected as shown.

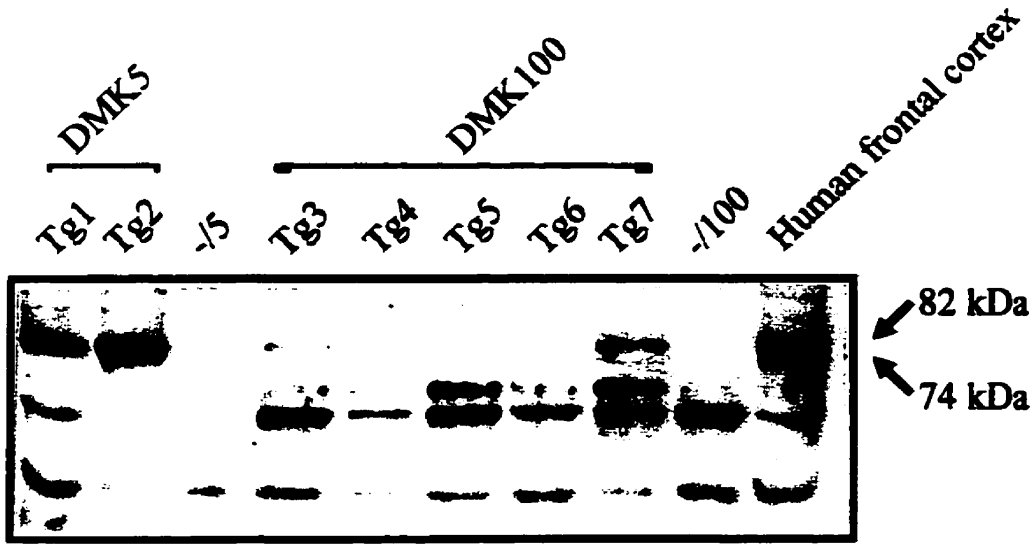
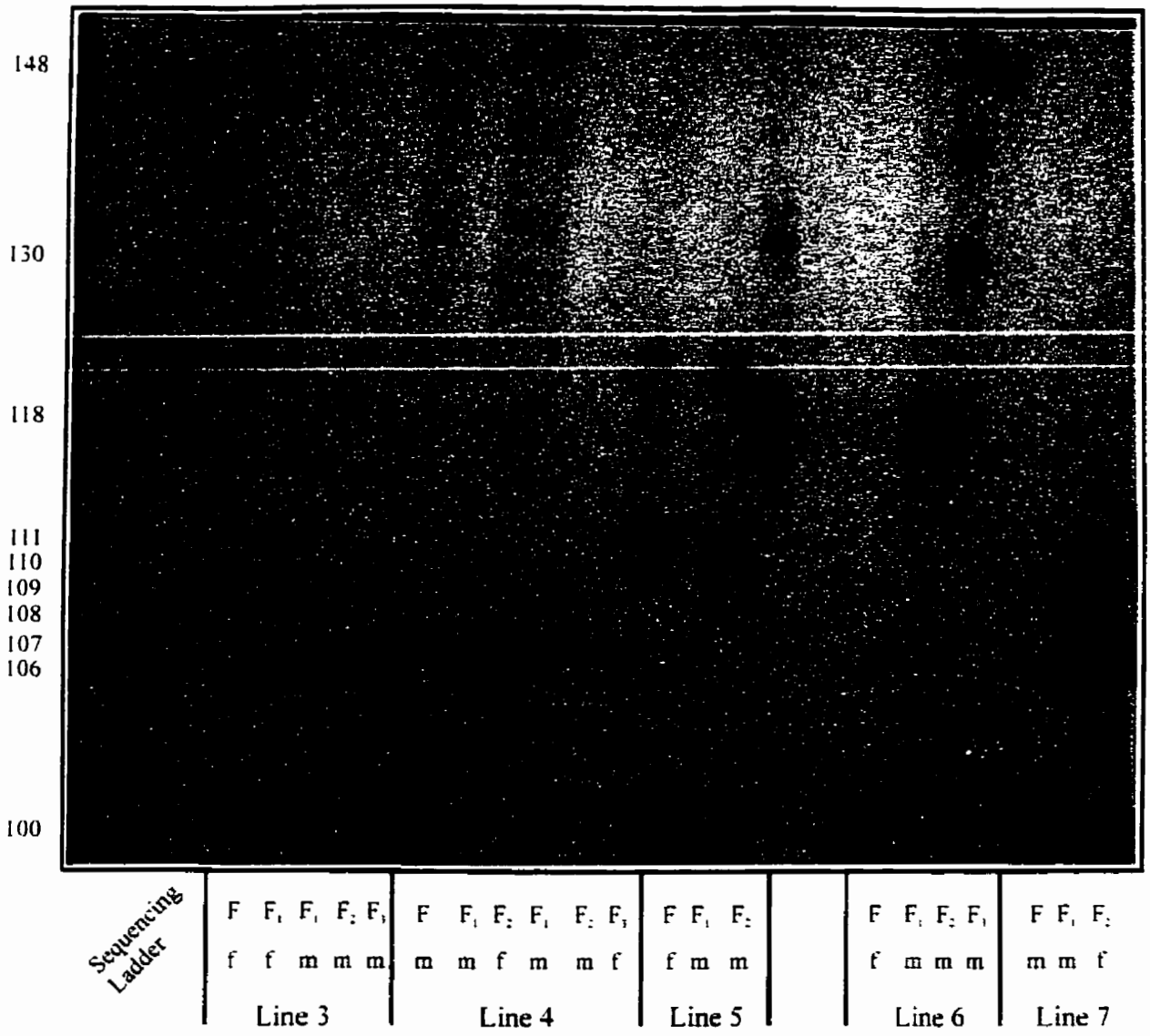

DMK5
DMK100
Tg1
Tg2
-/5
Tg3
Tg4
Tg5
Tg6
Tg7
-/100
Human frontal cortex
82 kDa
74 kDa

Figure 6-9: Documentation of intergenerational instability of the CTG repeat in transgenic mice. Genomic DNA from transgenic mice carrying DMK100 was amplified using hot PCR and resolved on a 6% sequencing gel. The length of CTG repeat (number of repeats) was determined from an adjacent sequencing ladder and is indicated. CTG expansion was detected in all mouse lines expressing the DMK100 transgene, independent of the sex of the transmitting transgenic mouse. F, founder mouse; F_1, F_2 and F_3, 1st, 2nd and 3rd generation offspring;, f, female; m, male.


148
130
118
111
110
109
108
107
106
100
Sequencing Ladder
F F1 F1 F2 F3
f f m m m
Line 3
F F1 F2 F1 F2 F3
m m f m m f
Line 4
F F1 F2
f m m
Line 5
F F1 F2 F3
f m m m
Line 6
F F1 F2
m m f
Line 7

repeat units occurred in the F3 generation of line 6. However, complete loss of the 100 CTG repeat present in the transgene was not observed. Finally, expansion of the CTG repeat appeared to be independent of the sex of the transmitting transgenic mouse.

6.3.7 Muscle Pathology of Transgenic Mice:

Using haematoxylin and eosin staining, no sarcoplasmic masses were observed in any sections examined (Figure 6-10). Line 1 transgenic mice, which did not have detectable levels of human DMK protein in muscle, but was co-microinjected with MARs, exhibited the normal range of centronucleated fibers (Figure 6-10A). No substantial change in centronucleation was detected intergenerationally in line 3 mice (Figure 6-10B) in contrast to line 6 mice where centronucleation became more predominant in the F3 generation compared to the F1 generation (Figure 6-10C and 6-10D; Table 6-2). Overall, the percentage of centronucleated fibers was determined to be in the range of 8-23% for transgenic mice from lines 3-7 in contrast to 2-7% for wild-type littermates (Figure 6-11; Table 6-2).

6.4 DISCUSSION:

According to the *in vitro* studies presented here, an expanded CTG repeat can increase transcript stability. Furthermore, transcript stability is independent of protein synthesis, shown by the lack of effect of the protein synthesis inhibitor cycloheximide. This indicates that transcript stability may be mediated by a *cis*-acting element such as a stable hairpin structure, formed by an expanded CTG repeat. These secondary structures have been previously demonstrated for all trinucleotide repeats (Gacy et al., 1995).

Figure 6-10: Histological examination of transgenic cross-sectioned skeletal muscle. Representative haematoxylin and eosin stained gastrocnemius muscle are shown for, (A) transgenic mouse line 1 that does not express human DMK protein in skeletal muscle, (B) the F_3 generation of transgenic mouse line 3, (C) the F_1 generation of transgenic mouse line 6 and, (D) the F_3 generation of transgenic mouse line 6. An increase in centronucleation was detected in line 3 and 6 mice, which express human DMK in skeletal muscle. It was observed to increase intergenerationally in line 6 mice. Arrows indicate centronucleated muscle fibers. Calibration bar = 50 μm.

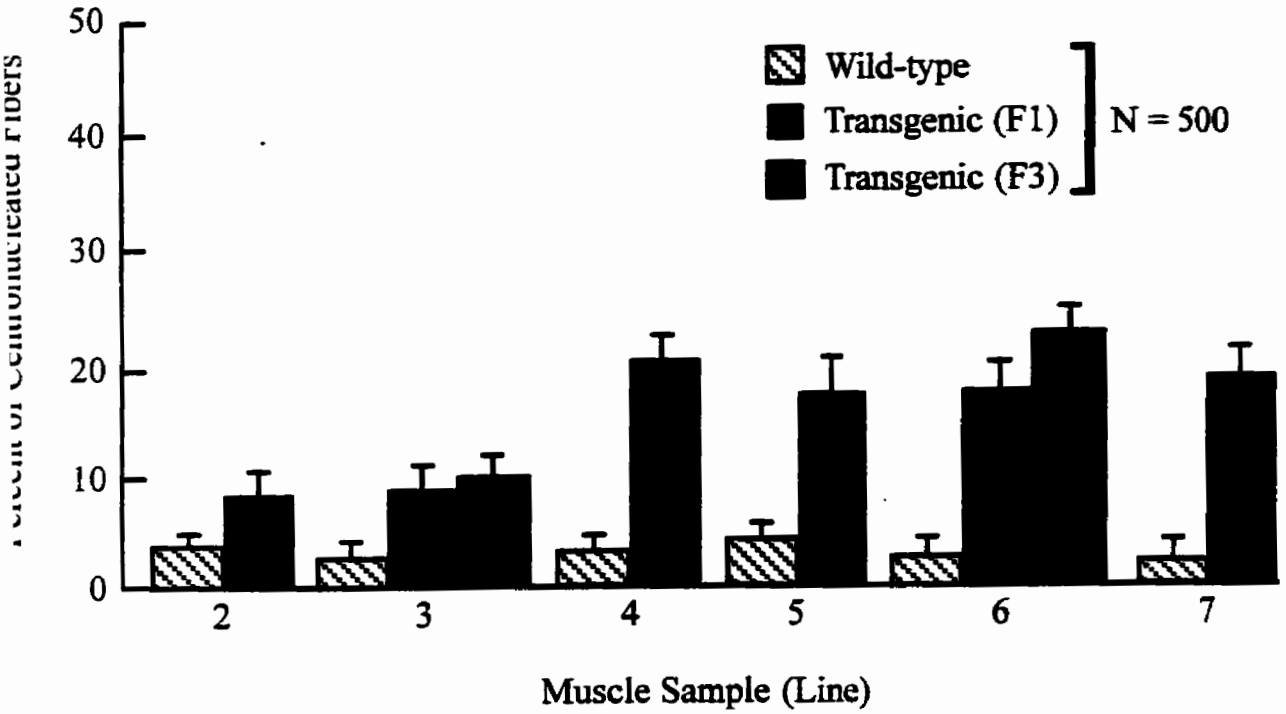

Percent of Centronucleated Fibers
50
40
30
20
10
0
Wild-type
Transgenic (F1)
Transgenic (F3)
N = 500
2
3
4
5
6
7
Muscle Sample (Line)

Figure 6-11: Histogram depicting the prevalence of centronucleated fibers in transgenic mice and wild-type littermates. Muscle samples were obtained from transgenic lines 2-7. Values are means ∓ S.D. derived from 5 randomly selected areas containing 100 fibers each. Transgenic mice from lines 4-7 showed a substantial increase in centronucleated fibers as compared to wild-type littermates ($p<0.005$). Centronucleation increased intergenerationally in line 6 mice.

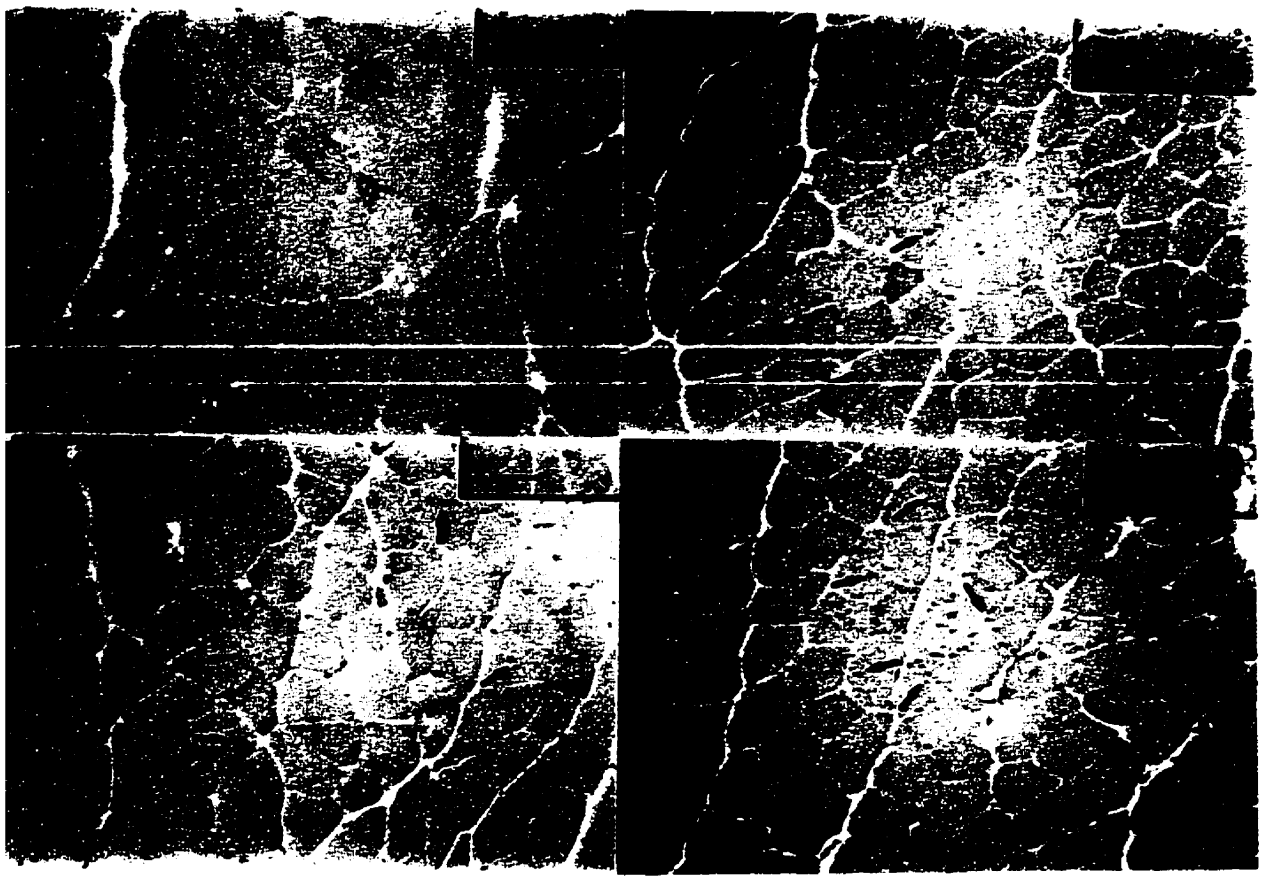


Table 6-2: Summary of CTG Instability, DMK Protein Levels in Skeletal Muscle Prevalence of Centronucleation in Transgenic Mice

Line	CTG No	Transgene Copy No	Change in CTG No			Muscle DMK Levels *			% Central Nuclei *		
			F_1	F_2	F_3	F_1	F_2	F_3	F_1	F_2	F_3
1	5	2				n.c.					
2	5	20				1.1		1.2	8		
3	100	2	+6			1.3		1.2	9		10
4	100	26	+6		+11	2.5		2.5	22		
5	100	15	+11	+18		1.8		1.9	18		
6	100	10	+18	+30	+48	1.5		2	18		24
7	100	20	+6	+11		2		2	20		

* Numbers represent fold increases in DMK protein levels in transgenic mice over mouse endogeneous levels as described previously. Percent of centronucleation does not include standard deviation but are indicated in Figu 6-11.

Alternatively, transcript stability may be achieved by the binding of a cycloheximide insensitive factor to the expanded CTG repeat. Transient expression experiments showed that increasing amounts of the reporter gene product, chloramphenicol acetyltransferase (CAT), are produced proportional to the increasing size of the CTG repeat. Moreover, protein level is directly dependent on the presence of the CTG repeat itself. One could postulate that up to a certain CTG repeat size, increased transcript stability leads to elevated DMK protein levels such as detected in adult onset patients (Chapter 3).

In transgenic mice, instability of the CTG repeat is observed which is reminiscent of CTG repeat transmission patterns observed in DM families (Brunner, 1992; Harley *et al.*, 1993; Lavedan *et al.*, 1993; Mulley *et al.*, 1993; Shelbourne *et al.*, 1992; Tsilfidis *et al.*, 1992). Preliminary data for genetic anticipation is detected and is most evident in transgenic mouse line 6, where an increase in CTG expansion was accompanied by a corresponding increase in centronucleation in successive generations. Additionally, there appears to be a positive correlation between CTG repeat size and phenotypic severity. This is most striking by comparison of the percent of centrally nucleated fibers in lines 3 and 6 (10% and 23%, respectively) which undergo the smallest and largest increase, respectively, in CTG size by the F3 generation. Presumably, a more severe phenotype akin to the human disease should become evident in these mice as the CTG repeat continues to expand.

The use of MAR sequences to enhance copy number-dependent expression of transgenes, independent of their integration point in the genome has been shown

previously (Chapter 4) and likewise appears to promote a similar effect in these lines (Table 6-1 and 6-2). An exception to this observation was detected in transgenic mouse line 6 where a high level of DMK protein was detected in F3 mice even though these mice harbour a relatively small number of transgenes. Interestingly, mice from this line underwent the greatest degree of CTG expansion from 100 CTG repeats to 148 CTG repeats by the F3 generation. The transgenic mice from this line also showed the largest increase in centronucleation. This correlates well with the cell culture results in which increased protein level is due to increased stability of DMK transcripts because of the increases in CTG repeat size. A similar effect may be occurring in transgenic mouse line where increases in CTG repeat size leads to an elevation in protein levels. It would prove worthwhile to monitor DMK transcript and protein levels in each generation, particularly in line 6, to confirm this effect of CTG repeat size on expression levels. It should be noted that besides increased stability, there also may be accumulation of transcript due to impairment in export or processing of RNA transcripts as a result of a large CTG repeat such as those consistently found in congenital DM patients. One might therefore expect increasing protein levels to a point, then decreasing levels as the repeat size expands past a certain threshold.

The role of over-expression DMK protein in the manifestation of DM is unknown. Over-expression of DMK protein in adult DM patients has been previously reported (Dunne *et al.*, 1996; Chapter 3), although this level of increased expression is not dramatic. However, as documented in this thesis (Chapter 4 and Chapter 6), 1.5 fold over-expression of DMK protein was detected in transgenic mice exhibiting some DM-

like histopathology. It is therefore possible that greater DM kinase levels in a cell may account for at least some of the symptoms of DM. In this respect, it is interesting that DMK shares homology to rho-binding kinase, ROK (Leung *et al.*, 1996). ROK phosphorylates the myosin-binding subunit of myosin phosphatase upon interaction with GTP-bound rho, resulting in the down regulation of the phosphatase's ability to dephosphorylate myosin light chain. This results in increasing contraction of smooth muscle and the formation of stress fibers in non-muscle cells. It will be important to determine whether or not the normal function of DMK is in a similar pathway.

DMK transcripts harbouring large CTG repeats have previously been shown to become trapped within the nuclei of cells from DM patients (Taneja *et al.*, 1995). This phenomenon may account for the reduction in DMK protein in congenital DM patients who characteristically carry a large TNR (Chapter 3). Furthermore, a ribonucleoprotein constituent which binds CUG repeats and which is ubiquitously expressed has been isolated (Timchenko *et al.*, 1996). The CUG binding protein may contribute to disease by increasing transcript stability by preventing RNA export or by becoming sequestered, preventing it from performing its normal function within the cell. Generating a transgenic mouse that does not express DMK protein but only DMK message should help to decipher the role of DMK transcript over-expression. However, it may be that the multisystemic and phenotypically variable nature of DM is the result of both elevated DMK transcript and protein levels.

With regard to TNR instability, the observations obtained with these transgenic mice suggest that the *cis*-acting elements that effect expansion must be present on the

transgene. In conjunction with work by Monckton *et al.* (1997), it is clear that the CTG repeats themselves are capable of promoting instability. Despite the fact that the transgene used in the Monckton model has more CTG repeats, the degree of instability was far greater in our model, suggesting that other factors outside the 3'UTR or even the genetic background of the mice contribute to TNR expansion. Moreover, the genomic context in which the TNR is situated may not be as important as initially surmised as expansion was detected in all mouse lines examined here as well as in those already reported. Contrary to the other TNR transgenic models, no deletions or sex-specific transmission differences were detected in these mice although a more comprehensive study needs to be conducted in order to thoroughly examine this issue. In the human disease, the CTG repeats tend to be more unstable, in terms of increasing repeat array size, during maternal transmission (Harley *et al.*, 1993; Lavedan *et al.*, 1993; Mulley *et al.*, 1993; Tsilfidis *et al.*, 1992). This correlation may become more evident as the repeat expands or may represent inherent differences between humans and mice.

The pattern of repeat instability that was detected in these mice, some repeats expanding intergenerationally with others remaining stable, suggests that some mouse lines harbour transgenes integrated at multiple sites. As a consequence, flanking chromatin may have an effect on the degree of instability of each transgene. However, if this is true, the multiple loci can be bred out until only one locus in each line is obtained. The other possibility is that only a portion of the transgenes in multicopy lines is expanding. It has been suggested that transcription is a necessary component for the mechanism of expansion of TNRs (Richards and Sutherland, 1992). The one mouse line

generated by Mangiarini *et al.* (1997) that did not show any signs of instability also did not express the transgene. It is quite possible that in the model presented here, expression of the transgene can influence CTG instability, particularly in light of the fact that DMK5 and DMK100 transgenes were regulated by a CMV promoter which has been known to become inactivated *in vivo*.

Although the model described here leaves many unanswered questions, it presents a first important step to understanding disease pathogenesis. Achieving TNR instability in mice was previously thought to be impossible. But now, given that repeat instability has been clearly documented in the transgenic mouse, much work can now be undertaken. This includes examining somatic instability and studying possible mechanisms of genetic anticipation. The role of DMK transcript and changes in protein levels as a consequence of CTG repeat size *in vivo* needs to be analyzed, particularly with respect to DM pathophysiology. No doubt this will be a useful model especially for the study of the mechanisms of TNR instability and disease with the eventual goal of initiating therapeutic strategies.

CHAPTER 7: CONCLUDING REMARKS

The data presented in this thesis reflect the assessment of the effects of human DMK over-expression in transgenic mice. It is obvious from current literature that the mechanism whereby CTG expansion results in DM is unclear, particularly in light of its unprecedented location in the 3'UTR of a gene that encodes a serine-threonine kinase with unknown function. The controversy surrounding the effect of TNR expansion on DMK expression has been complicated by the limited availability of suitable patient tissues and normal controls. Nevertheless, from a study presented this thesis (Chapter 3), modest over-expression of DMK protein was observed in adult DM patients providing further evidence for an over-expression model of pathogenesis. The lack of DMK protein over-expression in congenital DM skeletal muscle samples is likely a consequence of the previously reported reduction in processed (Krahe *et al.*, 1995) or poly A+ DMK transcripts (Wang *et al.*, 1995) as a result of increased accumulation of mutant transcript in the nucleus (Taneja *et al.*, 1995).

This disparity in DMK protein levels in congenital and adult DM tissue may, in fact, reflect alternative roles for transcript and protein over-expression in the pathogenic process. Clinically, these two forms of DM are quite distinct. Whereas congenital DM patients present predominantly with muscular hypotonia, adult DM patients exhibit mostly myotonia, muscle weakness and wasting (Harper, 1989). However, both forms of DM are associated with similar histological changes in skeletal muscle (Harper, 1989; Korenyi-Both, 1983). The transgenic mice generated in this thesis work, over-express the human DMK transcript and protein. Significantly, many of the hallmark histological

features of DM including sarcoplasmic masses, increased centronucleation and type I fiber atrophy are seen in the skeletal muscle of these DMK transgenics (Chapter 4). Furthermore, increased centronucleation was also seen in mice expressing an expanded CTG repeat in the context of a DMK minigene construct (Chapter 6). Interestingly, the transgenic mice with the greatest degree of CTG expansion had the highest level of expression and the highest incidence of centronucleation.

In vitro studies of the mutant DMK minigene indicated that DMK transcript stability and protein expression was directly dependent on CTG repeat size, even when examining as few as 100 repeats (Chapter 6). Previous histological studies of DM patient skeletal muscle tissue have led to the proposal that a number of the histological features associated with DM muscle, such as increased centronucleation and type I fiber atrophy, appear to be a result of immaturity as opposed to degenerative/regenerative processes (Carpenter and Karpati, 1984; Dubowitz, 1985; Iannaccone *et al.*, 1986). Experiments presented here show that primary myoblasts established from DMK transgenic mice have reduced fusion potential. Additionally, in mouse myoblasts, over-expression of the 3'UTR alone is sufficient to cause this reduction (Sabourin *et al.*, 1996). Thus, the sole molecular feature shared between the mouse models, the cell culture studies, adult DM patients and congenital DM patients, is the increased expression of the DMK transcript suggesting that this may be responsible for the DM-like muscle histopathology .

Similarly, the over-expression of DMK transcript in brain is likely the cause of DM-associated mental and cognitive defects which are profound in congenital DM

patients but not progressive with aging (Damian *et al.*, 1994; Malloy *et al.*, 1990; Portwood *et al.*, 1986). Parallel to mental dysfunction noted in these patients, the behavioural alterations observed in transgenic mice expressing human DMK transcript and protein included impaired short-term memory and learning disability, attributes associated with the hippocampus (Appendix I). Interestingly, the murine hippocampus is one of the first regions of the CNS to express DMK transcript post-natally (Jansen *et al.*, 1996). Perhaps this area of the brain is sensitive to the early over-expression of DMK mRNA, which has been shown to be elevated by as a much as 14-fold in the brain of congenital DM patient (Sabourin *et al.*, 1993). However, DMK protein has been localized to other regions of the brain as well and may contribute to the behavioural impairment observed in the disease state (Appendix I; Whiting et al., 1995).

Degenerative changes in the retina appear to occur exclusively in the adult onset form of the disease, which is associated with elevated DMK protein levels (Chapter 3; Dunne *et al.*, 1996). Transgenic mice over-expressing human DMK transcript and protein do develop migration of retinal pigment epithelium (RPE) and a retinitis pigmentosa (RP)- like photoreceptor cell degeneration (Chapter 5) similar to retinal manifestations previously documented in DM patients. The lack of retinal degeneration and lack of elevated DMK protein levels in congenital DM patients suggests that over-expression of DMK protein and not perturbations of DMK transcript levels is likely the cause of retinal defects in DM patients. This is further supported by the fact that photoreceptor cells are sensitive to changes in protein levels (Olsson *et al.*, 1992). However, this mechanism of pathogenesis by DMK protein is extrapolated from the

levels of transcript and protein determined in muscle samples obtained from congenital and adult DM patients. Although a previous survey of congenital tissues detected elevated levels of transcript in all of them, quantification of DMK transcript and protein levels in adult and congenital DM retina with the proper matched controls would be required to substantiate this hypothesis.

The observation of a DM-like phenotype in the tissues of these transgenic animals supports a role for over-expression of DMK in this disease. Furthermore, the reproduction of the clinical aspects of DM in these transgenic mice supplies further evidence that over-expression of DMK transcript and/or protein in various tissues causes the classical form of the disease. In congenital DM, significant accumulation of DMK message levels in the nucleus, perhaps as a result of altered transcript processing or export, leads to disease. Haploinsufficiency at the protein level may occur in congenital DM patients but likely does not directly contribute to disease given the lack of a DM-like phenotype in DMK deficient mice (Jansen *et al.*, 1996; Reddy *et al.*, 1996).

The failure to observe a similar phenotype in a recently reported DMK transgenic mouse is not easily explained. However, the model analyzed here does exhibit higher RNA and protein levels, possibly due to the role of MAR sequences as enhancers promoting copy-number dependent, position-independent expression. Moreover, the strains of mice used to generate transgenic mice were different in both models, something which can greatly influence the onset of a phenotype as has been recently shown for cystic fibrosis (Rozmahel *et al.*, 1996) and Alzheimer disease mouse models (Hsaio *et al.*, 1996). Nonetheless, the model described here will be useful for

deciphering the molecular mechanisms of disease, particularly in skeletal muscle, eye and brain.

Mere support for the DMK over-expression model is provided by its reproduction in mice expressing a human DMK minigene construct with expanded repeats (Chapter 6). As a consequence of using a CMV promoter and enhancer to regulate expression, not all lines over-express DMK in tissues typically affected by DM. However, those that do over-express DMK in skeletal muscle show substantial centronucleation of muscle fibers. A more complete assessment of skeletal muscle as was done for the previous model is required to confirm reiteration of characteristic DM myopathy in the CTG repeat transgenic mice.

The expansion of the CTG repeat observed in these mice suggest that they are a valuable *in vivo* resource for the study of mechanisms of instability of TNRs. As there is a correlation between repeat size and severity of disease in DM, CTG repeat size must have a role in determining transcript levels and ultimately protein levels. From the cell culture work presented in Chapter 6, transcript stability and protein levels were observed to be directly dependent on CTG repeat size, up to 100 CTG repeats. However, there must be a threshold repeat size at which transcript processing or export is hampered resulting in the observed reduction in processed mutant transcript (Krahe *et al.*, 1995; Wang *et al.*, 1995) leading to reduced DMK levels as found in congenital DM patients (Chapter 3). Using these CTG transgenic mice, it will thus be possible to conduct a study of the effect of expanded CTG repeat size on transcript stability, processing and export, something which has not been possible until now due to the unavailability of

patient and control tissues. Finally, preliminary evidence, presented here, suggests that there is some form of genetic anticipation occurring in these repeat mice which has not been noted previously in any other TNR mouse model (Gourdon *et al.*, 1997; Margiarini *et al.*, 1997; Monckton *et al.*, 1997). At the observed rate of CTG expansion, the phenotype of these repeat mice should increase in severity, as found in the human disease. Alternatively, a moderation in the phenotype as a consequence of intergenerational contraction would be most interesting in validating this as an authentic TNR mouse model for DM.

In the presence of the necessary *cis*-acting elements, targeting a large CTG repeat to the mouse DMK gene would allow examination of the effect of the amplified repeat on the possible perturbation of the expression levels of endogenous DMK transcript and protein. In addition, the impact of the repeat on neighbouring genes and on the chromatin structure could be studied with the availability of littermate matched controls. Another useful model would be the generation of transgenic mice over-expressing human DMK minigene constructs with expanded CTG repeats regulated by the endogenous DMK promoter. It would be interesting to see if a multisystemic phenotype would arise in this latter model. Engineering mice to have only a single copy by micro-injecting a dilute transgene solution would help to eliminate the potential effects of transgene copy number and CTG repeat size on transgene expression levels. Nevertheless, based on the findings presented in this thesis, DM pathology and CTG instability can be reproduced in mice as a consequence of DMK over-expression and introduction of an expanded CTG repeat, respectively. Although a new challenge arises

to combine both of these aspects into one model, based on the analysis of the present results, the majority of mechanisms proposed for DM pathogenesis could be tested in these mice. As a consequence, they provide a framework for future investigations, facilitated by the availability of these DMK transgenic mouse models generated during the course of this Ph.D. study.

APPENDIX I: OVER-EXPRESSION OF THE HUMAN MYOTONIC DYSTROPHY (DM) KINASE GENE LEADS TO IMPAIRED EXPLORATORY BEHAVIOUR IN TRANSGENIC MICE

The contents of this chapter have been submitted as:

Gerlai, R.*, Narang, M.A.*, Whiting, E., Roder, J., and Korneluk, R.G. (1997) Behavioural Brain Research.
(*co-authors)

I.1 INTRODUCTION:

Although predominately regarded as a neuromuscular disease, myotonic dystrophy (DM) is also associated with mental and cognitive impairment including reduced intelligence, poor concentration and increased apathy (Harper, 1989; Huber *et al.*, 1989; Portwood *et al.*, 1984; Turnpenny *et al.*, 1993). Studies indicate that language and verbal memory remain unaffected although nonverbal abilities are significantly impaired indicating involvement of the right hemisphere of the brain (Malloy *et al.*, 1990). Cognitive defects have been shown to involve short-term memory, spatial perception and orientation (Malloy *et al.*, 1990; Portwood *et al.*, 1986). Furthermore, investigators have noted physical changes including altered skull thickness, cerebral atrophy, white matter lesions, marked microencephaly and increased ventricular size (Avrahami *et al.*, 1987; Chang *et al.*, 1993; Damian *et al.*, 1994; Portwood *et al.*, 1994). In addition, a decrease in cerebral glucose utilization has been reported suggesting abnormal brain function (Fiorelli *et al.*, 1992). Finally, although these alterations are variable, they do correlate with disease state whereby the extent of mental and cognitive

defects, which is not degenerative with age, is greater in congenital DM, a more severe form of the disease (Damian *et al.*, 1994; Malloy *et al.*, 1990; Portwood *et al.*, 1986).

In Chapter 4, initial characterization was presented of transgenic mice expressing the human DMK gene in tissues typically affected by DM such as muscle, eye and brain. Substantial over-expression of DMK transcript (up to 20 fold) and protein (up to 12 fold) was detected in these tissues. Furthermore, a skeletal muscle pathology that bears resemblance to DM was observed in these transgenic mice including increased centronucleation and type I fiber atrophy. In this chapter, behaviour of these transgenic mice was examined as a means of indentifying altered brain function as a result of over-expression of DMK in brain. In addition, the localization of human DMK protein to discrete synaptic sites in the transgenic brain is described and its effects on transgenic mouse behaviour is discussed.

I.2 METHODS AND MATERIALS

I.2.1 Transgenic Mice:

Transgenic mice have been described in Chapter 4 and 5. In the neuroanatomical and behavioural analysis all three lines (A, B and C) were used. In the final data analyses, their data were pooled as no significant line differences, or copy number effects, were seen.

I.2.2 Behavioural Testing (performed by Dr. Robert Gerlai, Neuroscience Dept., Genentech, San Francisco, USA):

A total of 26 mice (12 control and 14 transgenic mice) were tested in two behavioural paradigms, open field and T-maze. The data of different transgenic lines and sexes are pooled in the final data analyses as no significant line or sex effects were found. The mice were tested between 10:00 and 16:00 hours individually first in the open field (Gerlai *et al.*, 1993), and a week later in the T-maze spontaneous alternation test (Gerlai *et al.*, 1994). The sequence of experimental subjects was randomized over genotype, transgenic line and sex. The observer did not know whether the individual mice were transgenic or control at the time of the test.

The open field apparatus, commonly used to study novelty induced exploratory activities (Crusio *et al.*, 1989) was a plastic box (46 x 25 x 15 cm) whose bottom was covered with a sheet of paper with a 5 cm^2 grid pattern. The open field contained a novel object, a plastic bottle (8 cm diameter, 20 cm high). The mice were placed in the center of the field individually and their behaviour was videorecorded for 5 min. The recordings were later replayed and quantified using an event recorder computer program (Gerlai and Hogan, 1992). The frequency and duration of spontaneous motor patterns appearing upon exposure to the test situation were measured. The advantage of this recording method is that the observer can measure a large number of events with high precision. In addition to this practical advantage, this method is based on one of the fundamental tenets of ethology, which assumes that the apparently continuous stream of behaviour can be

broken down into mutually exclusive, distinct and successive motor patterns that represent specific units of behaviour (Huntingford, 1984). The behavioural measures recorded with the program were expressed as either cumulative frequency (f=number of times per test period) or relative duration (d=duration as a percentage of the test period). The following behavioural units were quantified and analyzed: LEANING (f) leaning against the wall with one or both forepaws while standing on the hindlegs; LONG BODY (d) extending the forepaws and the frontal part of the body, while anchoring the hindlegs resulting in an elongated body shape; DEFECATION (f) depositing fecal pellets; GROOMING (d) stereotypical face cleaning and fur licking movements; LOCOMOTION SCORE: number of squares crossed on the floor grid (a cross was counted when the mouse entered a new square with both of its forepaws); OBJECT EXPLORATION (f) leaning against or sniffing at the plastic bottle; PASSIVITY (d) remaining motionless; REARING (f) standing upright on the hindlegs.

A week after the open field test the mice were tested in another apparatus, the T-maze (length of start and goal stems=75 cm, width=10 cm, height=20 cm) (Gerlai *et al.*, 1994). The walls, made of transparent acrylic, were glued to a black acrylic square bottom piece. At the beginning of the start arm a removable black acrylic door separated a 10-cm long start box from the rest of the arm. Entry to the goal arms could be prevented by another black door that could be placed at the entrance to either the left or right goal arm. The apparatus was placed in a room rich in visual stimuli. The T-maze

was thoroughly cleaned with a disinfectant deodorizing spray after each individual mouse but not between trials of the same mouse.

The test session consisted of one forced trial and 14 subsequent choice trials. No reinforcement was applied. The alternation test was based on spontaneous activity, i.e. the exploratory tendency of the animals. Mice were individually placed in the startbox and after 5 seconds of confinement, by lifting the guillotine door, they were allowed to explore the start arm and one of the goal arms. Entry to the other goal arm was blocked. The first blocked arm was chosen according to a computer generated semi-random schedule. This was the first, forced, trial. The mice explored the area available to them and eventually reentered the start arm and moved down to the start box. When they entered the start box they were confined there for 5 seconds by putting the start box door in place. During confinement the door blocking one of the goal arms was removed. The start box door was then released and the second trial was started. However, this time the mice could choose between the two goal arms (first free choice). After the mouse had chosen and entered one goal arm half way down, the other arm was blocked by the door. The mice readily left the explored goal arm and moved down to the start box again. They were confined there for 5 seconds and the testing cycle continued with another free choice test as described above. Fourteen such free choice trials were carried out during the continuous recording session and the consecondsutive choices were recorded. The overall alternation rate during the 14 free choice trials (0%=no alternation, 100%=alternation at each trial, 50%=random choice) was calculated and analyzed. The

above described testing method has been suggested to provide the most precise measure of spontaneous alternation as it does not introduce unwanted confounding factors including negative reinforcement associated with handling (Gerlai, 1997; Gerlai *et al.*, 1994). This procedure has been shown to detect even mild hippocampal dysfunction (Gerlai, 1997).

I.2.3 Statistical analysis:

The effects of genotype or transgenic line origins was investigated by variance analyses (ANOVA). The results were also confirmed by non-parametric Mann-Whitney or Kruskal-Wallis tests. A potential correlation between transgene copy number and behaviour was studied by Spearman rank correlation.

I.2.4 Immunofluorescence:

Transgenic and age-matched control wild-type littermates were anesthetized and perfused transcardially with phosphate buffered saline, pH 7.2 (PBS). Brain was removed, cut along the coronal plane and frozen in isopentane cooled by liquid nitrogen. Cryostat sections (16 μm) were cut at -20 °C and thaw mounted onto superfrost glass slides (Fisher). Slide-mounted sections were incubated with rabbit anti-DMK or anti-DMK preabsorbed with 1 mg/ml GST-DMK immunogen and 1 mg/ml gluthathione-agarose in PBS containing 0.3% Triton X-100. Sections were rinsed with PBS following incubation with primary antiserum and incubated with secondary antiserum, sheep anti-

rabbit immunoglobulin CY3 (Sigma). Sections were photographed with Kodak Ektachrome 400 at 40x magnification using a Zeiss Axiophot microscope.

I.3 RESULTS

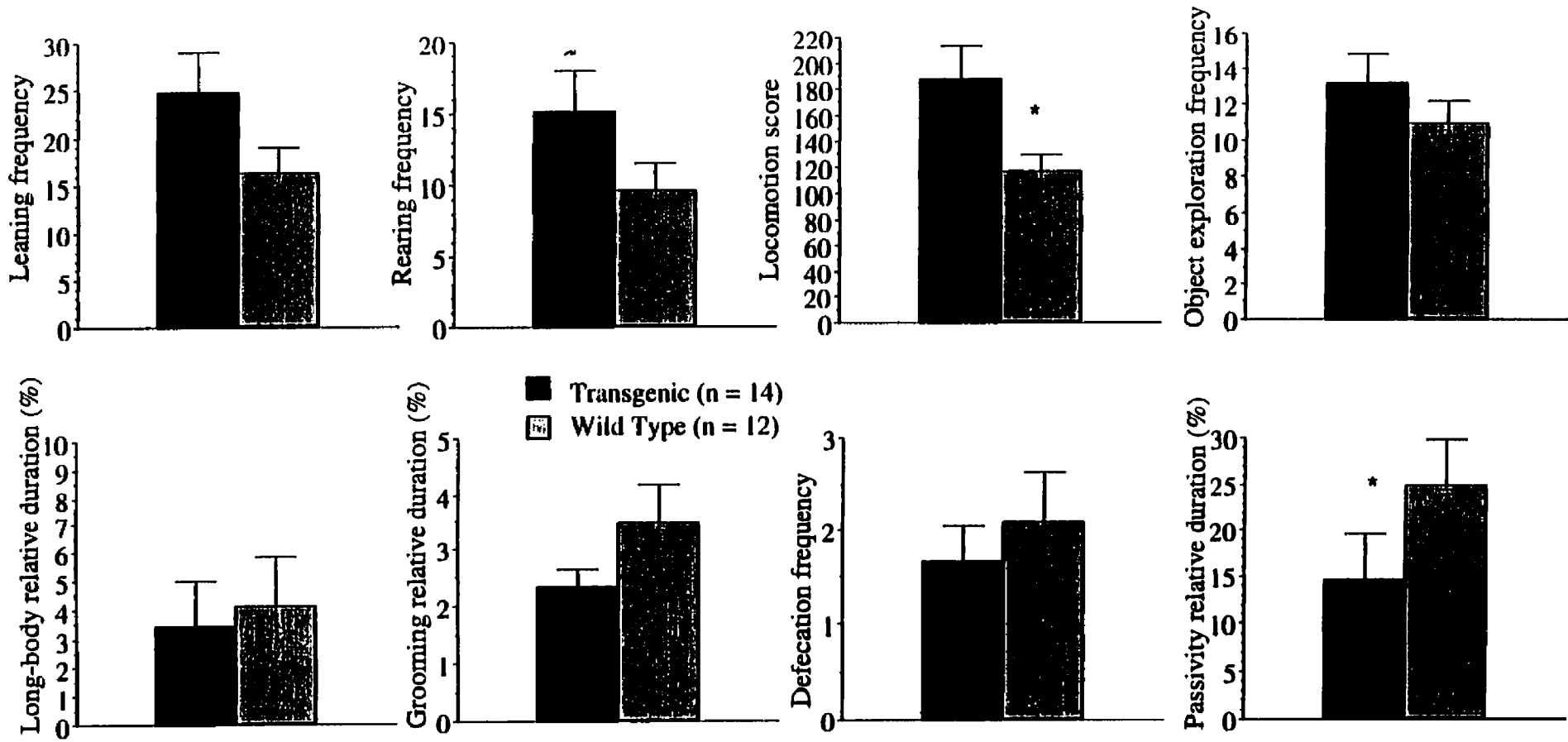
I.3.1 Transgene Expression:

Over-expression of human DMK transcript and protein was particularly pronounced in brain, muscle, tongue and eye at levels substantially greater than endogenous mouse DMK of wild-type littermates (Chapter 4). In brain, 2 to 20 fold increase in human DMK transcript was detected depending on transgenic line (line A=20, line B=12, line C=2 fold increase). Over-expression of human DMK protein was detected in tissues consistent with Northern blot analysis (brain: line A=12, line B=7, line C=1.5 fold increase). Expression levels were consistent with transgene copy numbers where line A>B>C.

I.3.2 Behavioural Analyses:

The general appearance of the transgenic mice suggested that no gross behavioural abnormalities were evident. Transgenic mice were active, well groomed and exhibited no increased mortality rate. However, in the open field they exhibited increased locomotion score (Mann Whitney, $p=0.04$) and remained passive for shorter duration of time (Figure I-1; Mann Whitney, $p=0.035$). It is also notable that they

Figure I-1: Behavioural elements measured in the open field. Mean ± Standard Error. Sample sizes (n) are indicated. Note that transgenic mice exhibited (A) a significantly elevated locomotion score and, (B) decreased duration of passivity (as indicated by asterisks). Also note that transgenic mice showed no signs of decreased muscle strength or obvious motor disturbance.


Leaning frequency
Rearing frequency
Locomotion score
Object exploration frequency
Long-body relative duration (%)
Grooming relative duration (%)
Defecation frequency
Passivity relative duration (%)
Transgenic (n = 14)
Wild Type (n = 12)
*
*

exhibited a markedly increased variance in their activity (Bartlett's test for variance in homogeneity $p=0.020$). The significantly altered activity level, however, did not affect other behaviours. Transgenic mice exhibited a normal level of leaning, long-body, defecation, grooming, object exploration and rearing ($p>0.110$).

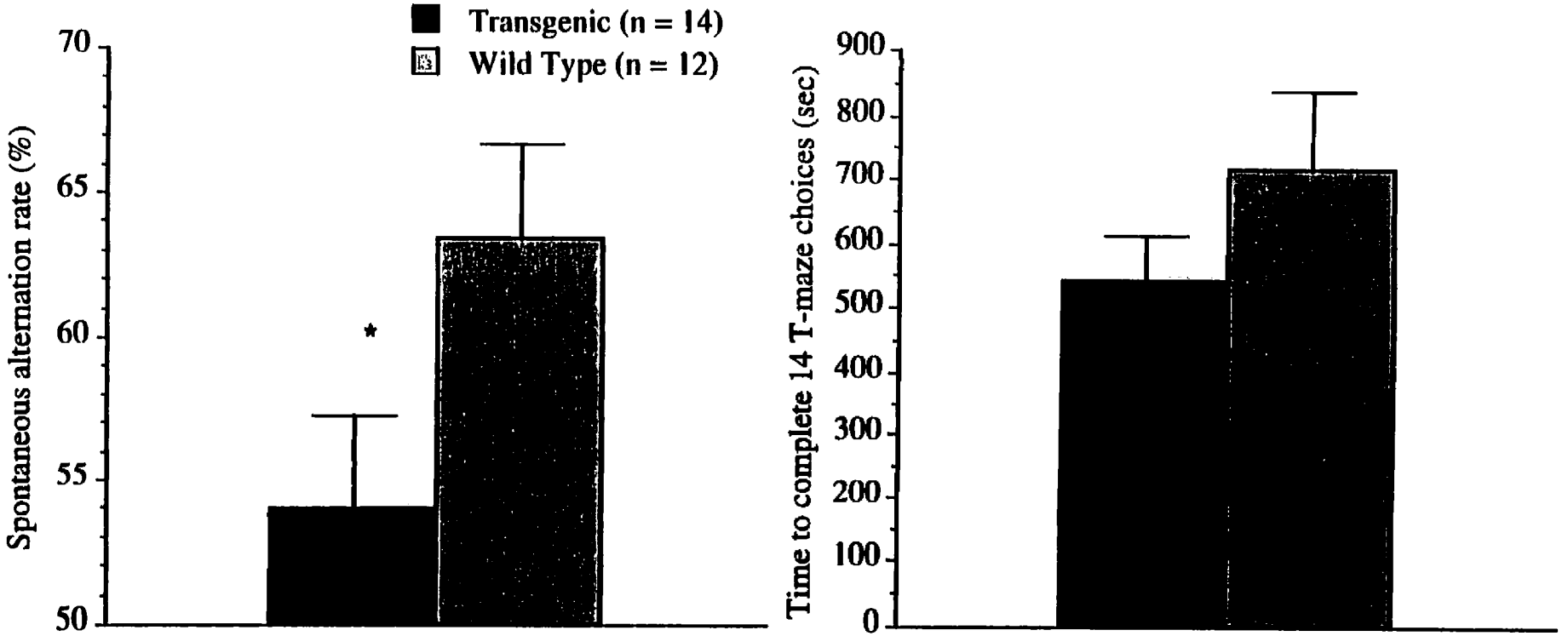
In the T-maze, transgenic mice showed a significantly decreased rate of alternation (Mann Whitney, $p=0.044$) compared to control (Figure I-2). Furthermore, the alternation rate of the transgenic mice (53.98%) was not significantly different from 50% random choice level ($p>0.20$) whereas wild type control mice alternated at 63.42%, a rate significantly above random choice ($p<0.01$). Interestingly, the alternation rate difference was not associated with changes in time required to complete the 14 free choices. The difference between transgenic and control mice was not significant (Mann Whitney, $p=0.198$).

It is also notable that no significant correlation was found between any behaviour and the copy number of the transgene the transgenic mice carried ($r<|\pm 0.352|$, $p<0.05$). Furthermore, no differences were found between transgenic lines in any behaviour (Kruskal-Wallis test, $p<0.05$).

I.3.3 Localization:

Analysis of transgenic and wild-type littermate brain by immunofluourescence revealed that DMK protein localizes to various synaptic sites. DMK immunoreactivity was observed in the ependyma and choroid plexus of the cerebral ventricle of transgenic

Figure I-2: Spontaneous alternation in the T-maze. Mean ± Standard Error. Sample sizes (n) are indicated. Note that transgenic mice exhibited a significantly decreased alternation rate (indicated by an asterisk) despite the fact that they completed the task within an amount of time not significantly different from that of the wild type control mice.

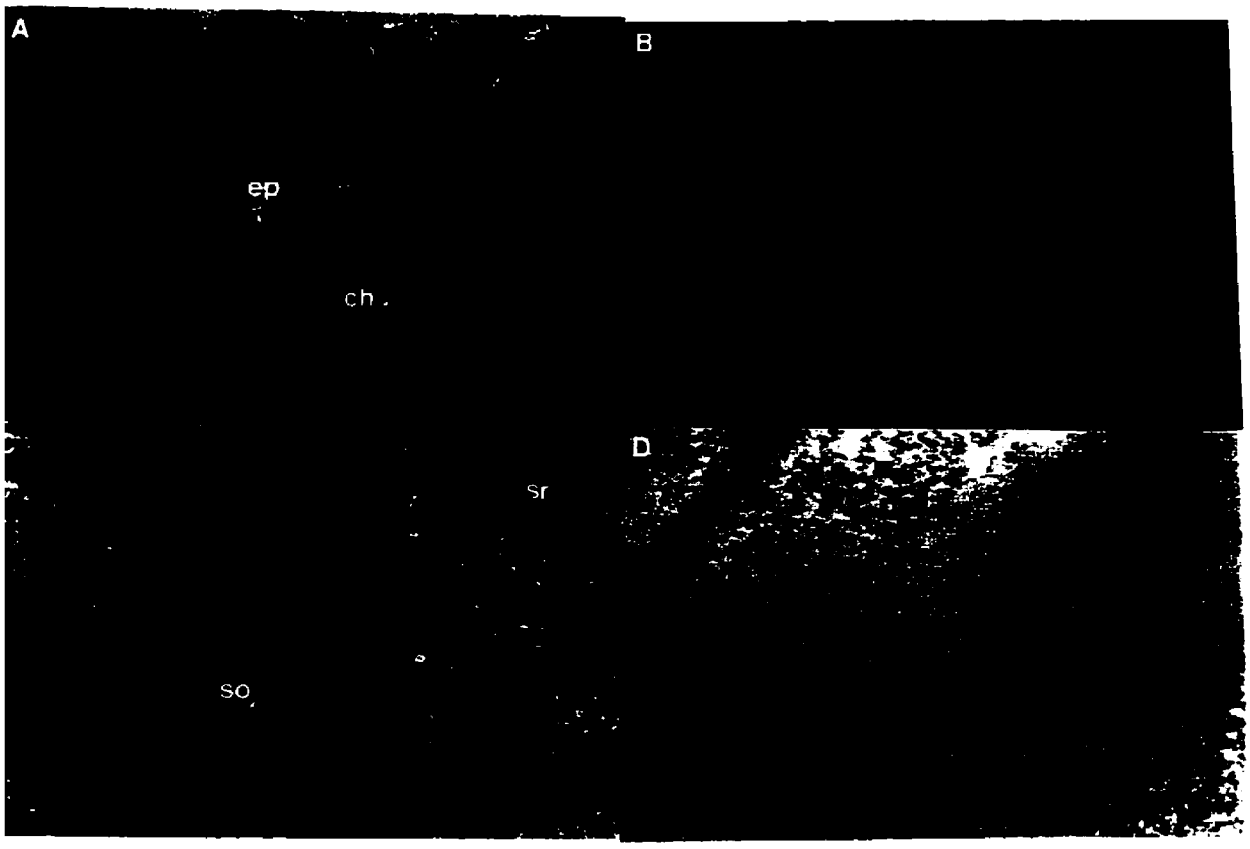

Transgenic (n = 14)
Wild Type (n = 12)
Spontaneous alternation rate (%)
70
65
60
55
50
*
Time to complete 14 T-maze choices (sec)
900
800
700
600
500
400
300
200
100
0

brain (Figure I-3A). Similarly, in the hippocampus, dense punctate staining was detected in the stratum radiatum and stratum oriens (Figure I-3C). Immunofluorescence was weak and difficult to discern in wild-type brain sections (Figure I-3B and I-3D). Staining was reduced to background levels in sections incubated with preabsorbed anti-DMK (data not shown). Furthermore, authenticity of human DMK localization in transgenic brain is provided by Western blot analysis of wild-type brain extracts which show no detectable DMK protein.

I.4 DISCUSSION:

Discrete localization of human DMK within different regions of the brain of these transgenic mice suggests that this protein may have specific functions in the central nervous system. Moreover, the pattern of localization is in agreement with studies previously reported in wild-type rat (Whiting *et al.*, 1995) suggesting the behavioural changes in these mice are due to increased levels of DMK and not due to the presence of DMK in areas not normally associated with its expression (Figure I-3). Behavioural tests such as the open field and T-maze indicate a mild behavioural deficit in these DMK transgenic mice. This deficit is not likely due to the disruption of an endogeneous mouse gene as behavioural alterations were observed in all transgenic lines that were generated by independent, random, single site insertions of the DMK transgene. Furthermore, no correlation between alterations in behaviour and transgene copy number was observed indicating that expression levels above 1.5 fold of DMK protein are sufficient

Figure I-3: Immunolocalization of human DMK protein in transgenic brain. Cryostat sections of transgenic cerebral ventricle (A) and hippocampus (C) and respective wild-type littermate controls (B and D) were incubated with anti-DMK. ep, ependyma; ch, choroid plexus; sr, statum radiatum; so, statum oriens; cp, cerebral peduncle. Panel D appears different due to over-exposure. Discrete localization of DMK protein within specific regions of the brain of these transgenic mice suggests that this protein has a role in signal transduction. 40x magnification.


A
B
ep
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for behavioural change to be detected. This latter finding appears to be in accordance with the dominant nature of the human DMK mutation (Harper, 1989).

Although the DMK transgene was found to be expressed not only in the brain but also in the muscle, eye and heart (Chapter 4), the general good health and normal appearance of the transgenic mice suggested that they did not suffer from gross motor abnormalities. This was confirmed by the open field test (Figure I-1), a test commonly used to study novelty induced exploratory activities, that showed unaltered level of leaning and rearing in the transgenic mice, behaviours that typically require good motor coordination, balance, and muscle strength. Furthermore, the transgenic mice showed no alterations in defecation or long body, behavioural measures of emotionality or fear. The only significant abnormality found in them was their elevated activity level.

Hyperactivity may have a diverse cause but has been seen in animals that are unable to process novel stimuli (Gerlai *et al.*, 1993), a function associated with the hippocampus. The exact cause or biological mechanisms underlying hyperactivity render the animal unable to learn or process novel stimuli implying that these DMK transgenic mice are less efficient in learning their environment shown by increased motor activity due to an increased need to recheck the field. This may lead to impaired performance in spatial learning tasks as well.

One task that has been shown to depend upon learning of extra maze spatial (visual) stimuli in mice is the T-maze spontaneous alternation task (Gerlai *et al.*, 1994; Gerlai, 1997). The significant impairment seen in these transgenic mice in this task also

suggests that their exploratory behaviour, or some biological mechanisms associated with exploration is abnormal (Figure I-2). Hyperactivity alone may not explain the impaired alternation seen in the transgenic mice since these transgenics finished the required 14 choice trials within the same amount of time as their normal control counterparts did. It is therefore possible that the abnormalities seen in these mice may be associated with altered exploratory tendencies, or stimulus processing.

The neuroanatomical substrate of the observed behavioural abnormalities is unclear. Previous *in situ* studies detected DMK transcript in the hippocampus of mice 14 days post-natal (Jansen *et al.*, 1996). Although the localization of the human DMK protein is not restricted to the hippocampus, it is possible that this brain region is most vulnerable to altered molecular mechanisms associated with the DMK transgene over-expression. However, the contribution of other brain regions cannot be ruled out. Certainly, the presence of DMK protein at synaptic sites in various regions of the brain suggest that it has a role in signal transduction and synaptic transmission. However, it may be perilous to relate the human conditions to the behavioural abnormalities seen in a potential mouse model. One may not expect a clear correlation between all aspects of a complex syndrome, like DM, between mouse and human. Nevertheless, the observed altered exploratory behaviour in the transgenic mouse appears to parallel some aspects of the cognitive deficits seen in the human including impaired short-term memory, spatial perception, and orientation (Malloy *et al.*, 1990; Portwood *et al.*, 1986). These

alterations, may be due, at least in part, to abnormal hippocampal function as a result of over-expression of human DMK.

REFERENCES:

Adams, R.D. (1975) in *Diseases of Muscle: A Study in Pathology, Third Edition.* Harper and Row, New York. pp. 269-271.

Adler, R. (1996) Mechanisms of photoreceptor death in retinal degeneration. Arch. Ophthalmol. **114**: 79-83.

Al-Maghtheh, M., Inglehearn, C.F., Keen, T.J., Evans, K., Moore, A.T., Jay, M., Bird, A.C., and Bhattacharya, S.S. (1994) Identification of a sixth locus for autosomal dominant retinitis pigmentosa on chromosome 19. Hum. Mol. Genet. **3**: 351-354.

Andrew, S.E., Goldberg, Y.P., Kremer, B., Telenius, H., Theilmann, J., Adam, S., Starr, E., Squitieri, F., Lin, B., Kalchman, M.A., Graham, R.K. and Hayden, M.R. (1993) The relationship between trinucleotide (CAG) repeat length and clinical features of Huntington's disease. Nat. Genet. **4**: 398-403.

Anvret, M., Ahlberg, G., Grandell, U., Hedberg, B., Johnson, K. and Edström, L. (1993) Larger expansion of the CTG repeat in muscle compared to lymphocytes from patients with myotonic dystrophy. Hum. Mol. Genet. **2**: 1397-1400.

Ashley Jr., C.T., Wilkinson, K.D., Reines, D. and Warren, S.T. (1993) FMR1 protein: conserved RNP family domains and selective RNA binding. Science **262**: 563-566.

Ashizawa, T., Dubel, J.R. and Harati, Y. (1993) Somatic instability of CTG repeat in myotonic dystrophy. Neurology **43**: 2674-2678.

Ashizawa, T., Dunne, P.W., Ward, P.A., Seltzer, W.K. and Richards, C.S. (1994) Effects of the sex of myotonic dystrophy patients on the unstable triplet repeat in their affected offspring. Neurology **44**: 120-122.

Aslanidis, C., Jansen, G., Amemiya, C., Shutler, G., Mahadevan, M., Tsilifidis, C., Chen, C., Alleman, J., Wormskamp, N.G.M., Vooijs, M., Buxton, J., Johnson, K., Smeets, H.J.M., Lennon, G.G., Carrano, A.V., Korneluk, R.G., Wierenga, B. and de Jong, P.J. (1992) Cloning of the essential myotonic dystrophy region and mapping of the putative defect. Nature **355**: 548-551.

Astrom, K.E. and Adams, R.D. (1979) in *Handbook of Clinical Neurology: Diseases of Muscle, Part I, Volume 40.* Edited by Vinken, P.J. and Bruyn, G.W. North Holland Publishing Co., New York. pp. 254-246.

Avrahami, E., Katz, A. and Bornstein, N. (1987) Computed tomographic findings of brain and skull in myotonic dystrophy. J Neurol Neurosurg Psych **50**: 435-438.

Barcelo, J.M., Mahadevan, M.S., Tsilifids, C., MacKenzie, A.E. and Korneluk. R.G. (1993) Intergenerational stability of the myotonic dystrophy protomutation. Hum. Mol. Genet. **2**: 705-709.

Bell, M.V., Hirst, M.C., Nakahori, Y., MacKinnon, R.N., Roche, A., Flint, T.J., Jacobs, P.A., Tommerup, N., Tranebjaerg, L., Froster-Iskenius, U., Kerr. B., Turner. G., Lindenbaum, R.H., Winter, R., Pembrey, M., Thibodeau, S. and Davies, K.E. (1991) Physical mapping across the fragile X: hypermethylation and clinical expression of the fragile X syndrome. Cell **64**: 861-866.

Bhagwati, S., Ghatpande, A. and Leung, B. (1996) Normal levels of DM RNA and myotonin protein kinase in skeletal muscle from adult myotonic dystrophy (DM) patients. Biochim. Biophys. Acta. **1317**: 155-157.

Bingham, P.M., Scott, M.O., Wang, S., McPhaul, M.J., Wilson, E.M., Garbern, J.Y., Merry, D.E. and Fischbeck, K.H. (1995) Stability of an expanded trinucleotide repeat in the androgen receptor gene in transgenic mice. Nat. Genet. **9**: 191-196.

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

Boucher, C.A., King, S.K., Carey, N., Krahe, R., Winchester, C.L., Rahman, S., Creavin, T., Meghji, P., Bailey, M.E.S., Chartier, F.L., Brown, S.D., Siciliano, M.J. and Johnson, K.J. (1995) A novel homeodomain-encoding gene is associated with a large CpG island interrupted by the myotonic dystrophy unstable $(CTG)_n$ repeat. Hum. Mol. Genet. **4**: 1919-1925.

Braga III, I.S., Oda, K., Kikuchi, T., Tanaka, S., Shin, Y., Sento, M., Itakura, C. and Mizutani, M. (1995) A new inherited muscular disorder in Japanese quails (*Coturnix coturnix japonica*). Vet. Pathol. **32**: 351-360.

Braun, T., Buschhausen-Denker, G., Bober, E., Tannich, E. and Arnold, H.H. (1989) A novel human muscle factor related to but distinct from *MyoD1* induces myogenic conversion I 10T1/2 fibroblasts. EMBO. J. **8**: 701-709.

Braun, T., Rudnicki, M., Arnold, H.H. and Jaenish, R. (1992) Targeted inactivation of the muscle regulatory gene *Myf-5* results in abnormal rib development and perinatal death. Cell **71**: 369-382.

Brewster, B.S., Jeal, S. and Strong, P.N. (1993) Identification of a protein product of the myotonic dystrophy gene using peptide specific antibodies. Biochem. Biophys. Res. Comm. **194**: 1256-1260.

Brook, J.D., McCurrach, M.E., Harley, H.G., Buckler, A.J., Church, D., Aburatani, H., Hunter, K., Stanton, V.P., Thirion, J-P., Hudson, T., Sohn, R., Zemelman, B., Snell, R.G., Rundle, S.A., Crow, S., Davies, J., Shelbourne, P., Buxton, J., Jones, C., Juvonen, V., Johnson, K., Harper, P.S., Shaw, D.J. and Housman, D.E. (1992) Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a protein kinase family member. Cell **68**: 799-808.

Brooke, M.H. (1986) in *Clinician's View of Neuromuscular Diseases: Second Edition.* Williams and Wilkins, Baltimore. pp. 192-205.

Brunner, H.G., Brüggewirth, H.T., Nillesen, W., Jansen, G., Hamel, B.C.J., Hoppe, R.L.E., de Die, C.E.M., Höweler, C.J., van Oost, B.A., Wieringa, B., Ropers, H.H. and Smeets, H.J.M. (1993) Influence of sex of the transmitting parent as well as of parental allele size on the CTG expansion in myotonic dystrophy (DM). Am. J. Hum. Genet. **53**: 1016-1023.

Burns, T.W., Dale, H.E. and Langley, P.L. (1965) The lipid and electrolyte composition of plasma and the erythrocyte of the myotonic goat. Clin. Res. **13**: 235.

Burright, E.N., Clark, H.B., Servadio, A., Matilla, T., Fedderson, R.M., Yunis, W.S., Duvick, L.A., Zoghbi, H.Y. and Orr, H.T. (1995) SCA1 transgenic mice: a model for neurodegeneration caused by an expanded CAG trinucleotide repeat. Cell **82**: 937-948.

Bush, E.W., Taft, C.S., Meixell, G.E. and Perryman, M.B. (1996) Overexpression of myotonic dystrophy kinase in BC3H1 cells induces the skeletal muscle phenotype. J. Biol. Chem. **271**: 548-552.

Buxton, J., Shelbourne, P., Davies, J., Jones, C., van Tongeren, T., Aslanidis, C., de Jong, P., Jansen, G., Anvret, M., Riley, B., Williamson, R. and Johnson, K. (1992) Detection of an unstable fragment of DNA specific to individuals with myotonic dystrophy. Nature **355**: 547-548.

Campuzano, V., Montermini, L., Molto, M.D., Pianese, L., Cossee, M., Cavalcanti, F., Monros, E., Rodius, F., Duclos, F., Monticelli, A., Zara, F., Canizares, J., Koutnikova, H., Bidichandani, S.I., Gellera, C., Brice, A., Trouillas, P., De Michele, G., Filla, A., De Frutos, R., Palau, F., Patel, P.I., Di Donato, S., Mandel, J.L., Cocozza, S., Koenig, M and Pandolfo, M. (1996) Friedreich's ataxia: autosomal recessive disease caused by an intronic GAA triplet repeat expansion. Science **271**: 1423-1427.

Carango, P., Noble, J.E., Marks, H.G. and Funanage, V.L. (1993) Absence of myotonic dystrophy protein kinase (DMPK) mRNA as a result of a triplet repeat expansion in myotonic dystrophy. Genomics **18**: 340-348.

Carpenter, S. and Karpati, G. (1984) in *Pathology of Skeletal Muscle*. Churchill Livingstone, New York. pp. 110-251.

Chang, L., Anderson, T., Migneco, O.A., Boone, K., Mehringer, C.M., Villanueva-Meyer, J., Berman, N. and Mena, I. (1993a) Cerebral Abnormalities in myotonic dystrophy. Arch. Neurol. **50**: 917-923.

Chang, G-Q., Hao, Y. and Wong, F. (1993b) Apoptosis: final common pathway of photoreceptor death in *rd*, *rds* and rhodopsin mutant mice. Neuron **11**: 595-605.

Crescenzi, M., Fleming, T.P., Lassar, A.B., Weintraub, H. and Aaronson, S.A. (1990) MyoD induces growth arrest independent of differentiation in normal and transformed cells. Proc. Natl. Acad. Sci. USA **87**: 8442-8446.

Crusio, W.E., Schwegler, H. and Van Abeelen, J.H.F. (1989) Behavioral responses to novelty and structural variation of the hippocampus in mice. I. Quantitative-genetic analysis of behavior in the open-field. Behav. Brain. Res. **2**: 75-80.

Curtis, D., Lehman, R. and Zamore, P.D. (1995) Translational regulation in development. Cell **81**: 171-178.

Damian, M.S., Bachmann, G., Koch, M.C., Schilling, G., Stoppler, S. and Domdoff, W. (1994) Brain disease and molecular analysis of myotonic dystrophy. Neuroreport. **5**: 2549-2552.

Dark., A.J. and Streeten, B.W. (1977) Ultrastructure study of cataract in myotonica dystrophica. Am. J. Ophthalmol. **84**: 666-674.

Davis, R.L.. Weintraub, H. and Lassar, A.B. (1987) Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell **51**: 987-1000.

Dubowitz, V. (1985) in *Muscle Biopsy: A Practical Approach*. Bailliere-Tindal, Toronto. pp. 380-395.

Dunne, P.W., Walch, E.T. and Epstein HF (1994) Phosphorylation reactions of recombinant human myotonic dystrophy protein kinase and their inhibition. Biochemistry **33**: 10809-10814.

Dunne, P.W., Ma, L., Casey, D.L., Harati, Y. and Epstein, H.F. (1996) Localization of myotonic dystrophy protein kinase in skeletal muscle and its alteration with disease. Cell. Mot. Cytoskel. **33**: 52-63.

Eberhart, D.E., Malter, H.E., Feng, Y. and Warren, S.T. (1996) The fragile X mental retardation protein is a ribonucleoprotein containing both nuclear localization and nuclear export signals. Hum. Mol. Genet. **5**: 1083-1091.

Edmondson, D.G. and Olson, E.N. (1989) A gene with homology to the *myc* similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. Genes and Dev. **3**: 628-640.

Eshaghian, J., March, W.F., Goossens, W. and Rafferty, N.S. (1978) Ultrastructure of cataract in myotonic dystrophy. Invest. Ophthalmol. Vis. Sci. **17**: 289-293.

Etongué-Mayer, P., Faure, R., Bouchard, J-P., Thibault, M-C. and Puymirat, J. (1994) The myotonin-protein kinase phosphorylates tyrosine residues in normal human skeletal muscle. Biochem. Biophys. Res. Comm. **199**: 89-92.

Fiorelli, M., Duboc, D., Mazoyer, B.M., Blin, J., Eymard, B., Fardeau, M. and Samson, Y. (1992) Decreased cerebral glucose utilization in myotonic dystrophy. *Neurology* **42**: 91-94.

Fu, Y-H., Kuhl, D., Pizzuti, A., Pierretti, M., Sutcliffe, J.S., Richards, S., Verkerk, A.J.M.H., Holden, J.J.A., Fenwick Jr., R.G., Warren, S.T., Oostra, B.A., Nelson, D.L. and Caskey, C.T. (1991) Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the sherman paradox. Cell **67**: 1047-1058.

Fu, Y-H., Pizutti, A., Fenwick, R.G., King Jr., J., Rajnarayan, S., Dunne, P.W., Dubel, J., Nasser, G.A., Ashizawa, T., de Jong, P.J., Wieringa, B., Korneluk, R., Perryman, M.B., Epstein, H.F. and Caskey CT (1992) An unstable triplet repeat in a gene related to myotonic dystrophy. Science **255**: 1256-1258.

Fu, Y-H., Friedman, D.L., Richards, S., Pearlman, J.A., Gibbs, R.A., Pizzuti, A., Ashizawa, T., Perryman, M.B., Scarlato, G., Fenwick Jr, R.G. and Caskey, C.T. (1993) Decreased expression on myotonin-protein kinase messenger RNA and protein in adult form of myotonic dystrophy. Science **260**: 235-238.

Gacy, A.M., Goellner, G., Juranic, N., Macura, S. and McMurray, C.T. (1995) Trinucleotide repeats that expand in human disease form hairpin structures *in vitro*. Cell **81**: 533-540.

Gerlai, R. and Hogan, J.A. (1992): Learning to find the opponent: an ethological analysis of the behavior of paradise fish (*Macropodus opercularis*, Anabantidae) in intra- and inter-specific encounters. J. Comp. Psychol. **106**: 306-315.

Gerlai, R., Friend, W., Becker, L., O'Hanlon, R., Marks, A., Roder, J. (1993) Female transgenic mice carrying the human gene for S100ß are hyperactive. Behav. Brain Res. **55**: 51-59.

Gerlai, R., Marks, A. & Roder, J. (1994) T-maze spontaneous alternation rate is decreased in S100ß transgenic mice. Behav. Neurosci. **108**: 100-106.

Gerlai, R. (1997) A new continuous alternation task in T-maze detects hippocampal dysfunction in mice: A strain comparison and lesion study (Behav. Brain Res. submitted).

Ginsberg, J., Hamblet, J. and Menefee, M. (1978) Ocular abnormality in myotonic dystrophy. Ann. Ophthalmol. **8**: 1021-1028.

Goldberg, Y.P., Katchman, M.A., Metzler, M., Nasir, J., Zeisler, J., Graham, R., Koide, H.B., O'Kusky, J., Sharp, A.N., Ross, C.A., Jirik, F. and Hayden, M.R. (1996) Absence of disease phenotype and intergenerational stability of the CAG repeat in transgenic mice expressing the human Huntington disease transcript. Hum. Mol. Genet. **5**: 177-185.

Green, W.R. (1985) Systemic diseases with retinal involvement in W.H. Spencer (ed) *Ophthalmic Pathology*. W.B. Saunders Co., Philadelphia. pp. 1172-1173.

Gourdon, G., Radvanyi, F., Lia, A-S., Duros, C., Blanche, M., Abitbol, M., Junien, C. and Hofmann-Radvanyi, H. (1997) Moderate intergenerational and somatic instability of a 55-CTG repeat in transgenic mice. Nat. Genet. **15**: 190-192.

Hamshere, M.G. and Brook, J.D. (1996) Myotonic dystrophy, knockouts, warts and all. Trends Genet. **12**: 332-334.

Harley, H.G., Brook, J.D., Rundle, S.A., Crow, S., Reardon, W., Buckler, A.J., Harper, P.S., Housman, D.E. and Shaw, D.J. (1992) Expansion of an unstable DNA region and phenotypic variation in myotonic dystrophy. Nature **355**: 545-546.

Harley, H.G., Rundle, S.A., MacMillan, J.C., Myring, J., Brook, J.D., Crow, S., Reardon, W., Fenton, I., Shaw, D.J. and Harper, P.S. (1993) Size of the unstable CTG repeat sequence in realtion to phenotype and parental transmission in myotonic dystrophy. Am. J. Hum. Genet. **52**: 1164-1174.

Harper, P.S. (1989) in *Myotonic dystrophy (Second edition)*. W.B. Saunders Company, London.

Harris, S., Moncrieff, C. and Johnson, K. (1996) Myotonic dystrophy: will the real gene please step forward! Hum. Mol. Genet. **5**: 1417-1423.

Hasty, P., Bradley, A., Morris, J.H., Edmondson, D.G., Venuti, J.M., Olson, E.N. and Klein, W.H. (1993) Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. Nature **364**: 501-506.

Hoffmann-Radvanyi, H., Lavedan, C., Rabès, J-P., Savoy, D., Duros, C., Johnson, K. and Junien, C. (1993) Myotonic dystrophy: absence of CTG enlarged transcript in congenital forms, and low expression of the normal allele. Hum. Mol. Genet. **2**: 1263-1266.

Howeler, C.J., Busch, H.F.M., Geraedts, J.P.M., Niermeijer, M.F. and Staal, A. (1989) Anticipation in myotonic dystrophy: fact or fiction? Brain **112**: 779-797.

Hsaio, K., Chapman, P., Nilsen, S., Eckman, C., Harigaya, Y., Younkin, S., Yang, F. and Cole, G. (1996) Correlative memory deficits, Aβ elevation, and amyloid plaques in transgenic mice. Science **274**: 99-102.

Huber, S.J., Kissel, J.T. and Shuttleworth, E.C. (1989) Magnetic resonance imaging and clinical correlates of intellectual impairment in myotonic dystrophy. Arch. Neurol. **46**: 536-540.

Hunter, A., Tsilfidis, C., Mettler, G., Jacob, P., Mahadevan, M., Surh, L. and Korneluk, R.G. (1992) The correlation of age of onset with CTG trinucleotide repeat amplification in myotonic dystrophy. J. Med. Genet. **29**: 774-779.

Huntingford, F. (1984) in The study of Animal Behaviour. Chapman and Hall, London.

Iannaccone, S.T., Bove, K.E., Vogler, C., Azzarelli, B. and Muller, J. (1986) Muscle maturation delay in infantile myotonic dystrophy. Arch. Pathol. Lab. Med. **110**: 405-411.

Ikeda, H., Yamaguchi, M., Sugai, S., Aze, Y., Narumiya, S. and Kakizuka, A. (1996) Expanded polyglutamine in the Machado-Joseph disease protein induces cell death in vitro and in vivo. Nat. Genet. **13**: 196-202.

Imbert, G., Saudou, F., Yvert, G., Devys, D., Trottier, Y., Garnier, J-M., Weber, C., Mandel, J-L., Cancel, G., Abbas, N., Durr, A., Didierjean, O., Stevanin, G., Agid, Y. and Brice, A. (1996) Cloning of the gene for spinocerebellar ataxia 2 reveals a locus with high sensitivity to expanded CAG/glutamine repeats. Nat. Genet. **14**: 285-291.

Jackson, R.J. (1995) Cytoplasmic regulation of mRNA function: the importance of the 3'untranslated region. Cell **74**: 9-14.

Jansen, G., de Jong, P.J., Amemiya, C., Aslandis, C., Shaw, D.J., Harley, H.G., Brook, J.D., Fenwick, R., Korneluk, R.G., Tsilfidis, C., Shutler, G., Hermens, R., Wormskamp, N.G.M., Smeets, H.J. and Wieringa, B. (1992a) Physical and genetic characterization of the distal segment of the myotonic dystrophy area on 19q. Genomics **13**: 509-517.

Jansen, G., Mahadevan, M., Amemiya, C., Wormskamp, N., Segers, B., Hendriks, W., O'Hoy, K., Baird, S., Sabourin, L., Lennon, G., Jap, P.L., Iles, D., Coerwinkel, M., Hofker, M., Carrano, A.V., de Jong, P.J., Korneluk, R.G. and Wieringa, B. (1992b) Characterization of the myotonic dystrophy region predicts multiple isoform-encoding mRNAs. Nat. Genet. **1**: 261-268.

Jansen, G., Bartolomei, M., Kalscheuer, V., Merkx, G., Wormskamp, N., Mariman, E., Smeets, D., Ropers, H-H. and Wieringa, B. (1993) No imprinting involved in the expression of DM-kinase mRNAs in mouse and human tissues. Hum. Mol. Genet. **2**: 1221-1227.

Jansen, G., Bächner, D., Coerwinkel, M., Wormskamp, N., Hameister, H. and Wieringa, B. (1995) Structural organization and developmental expression pattern of the mouse WD-repeat gene DMR-N9 immediately upstream of the myotonic dystrophy locus. Hum. Mol. Genet. **4**: 843-852.

Jansen, G., Groenen, P.J.T.A., Bächner, D., Jap, P.H.K., Coerwinkel, M., Oerlemans, F., van den Broek, W., Gohlsch, B., Pette, D., Plomb, J.J., Molenaar, P.C., Nederhoff, M.G.J., van Echteld, C.J.A., Dekker, M., Berns, A., Hameister, H. and Wieringa B (1996) Abnormal myotonic dystrophy protein kinase levels produce only mild myopathy in mice. Nat. Genet. **13**: 316-324.

Justice, R.W., Zilian, O., Woods, D.F., Noll, M. and Bryant, P.J. (1995) The *Dropsophilia* tumor suppressor gene *warts* encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferatrion. Genes and Dev. **9**: 534-546.

Karpati, G. (1979) in *Handbook of Clinical Neurology. Diseases of Muscle, Part I, Volume 40.* Edited by Vinken, P.J. and Bruyn, G.W. North Holland Publishing, New York. pp. 1-61.

Kawaguchi, Y., Okamoto, T., Taniwaki, M., Aizawa, M., Inoue, M., Katayama, S., Kawakami, H., Nakamura, S., Nishimura, M., Akiguchi, I., Kimura, J., Narumiya, S. and Kakizuka, A. (1994) CAG expansions in a novel gene for Machado-Joseph disease at chromosome 14q32.1. Nat. Genet. **8**: 221-227.

Kinoshita, M., Takahashi, R., Hasegawa, T., Komori, T., Nagasawa, R., Hirose, K. and Tanabe, H. (1996) $(CTG)_n$ expansions in various tissues from a myotonic dystrophy patient. Muscle and Nerve **19**: 240-242.

Knight, S.J.L., Flannery, A.V., Hirst, M.C., Campbell, L., Christodoulou, Z., Phelps, S.R., Pointon, J., Middleton-Price, H.R., Barnicoat, A., Pembrey, M.E., Holland, J., Oostra, B.A., Bobrow, M. and Davies, K.E. (1993) Trinucleotide repeat amplification and hypermethylation of a CpG island in FRAXE mental retardation. Cell **74**: 127-134.

Koga, R., Nakao, Y., Kurano, Y., Tsukahara, T., Nakamura, A., Ishiura, S., Nonaka, I. and Arahata, K. (1994) Decreased myotonin-protein kinase in the skeletal and cardiac muscles in myotonic dystrophy. Biochem. Biophys. Res. Comm. **202**: 577-585.

Koide, R., Ikeuchi, T., Onodera, O., Tanaka, H., Igarashi, S., Endo, K., Takahashi, H., Kondo, R., Ishikawa, A., Hayashi, T., Saito, M., Tomoda, A., Miike, T., Naito, H., Ikuta, F. and Tsuji, S. (1994) Unstable expansion of CAG repeat in hereditary dentatorubral-pallidoluysian atrophy (DRPLA). Nat. Genet. **6**: 9-13.

Kolb, L.C. (1938) Congenital myotonia in goats. Bull. Johns Hopkins Hosp. **63**: 221-237.

Korenyi-Both, A.L. (1983) in *Muscle Pathology in Neuromuscular Disease*. Charles C. Thomas publishing, Illinois. pp. 255-265.

Krahe, R., Ashizawa, T., Abbruzzese, C., Roeder, E., Carango, P., Giacanelli, M., Funanage, V.L. and Siciliano, M.J. (1995) Effect of myotonic dystrophy trinucleotide repeat expansion on DMPK transcription and processing. Genomics **28**: 1-14.

La Spada, A.R., Wilson, E.M., Lubahn, D.B., Harding, A.E. and Fischbeck, K.H. (1991) Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. Nature **352**: 77-79.

Lassar, A.B., Buskin, J.N., Lockshon, D., Davis, R.L., Apone, S., Haushka, S.D. and Weintraub, H. (1989) MyoD is a sequence-specific DNA binding protein requiring a region of *myc* homology to bind to the muscle creatine kinase enhancer. Cell **58**: 823-831.

Lassar, A.B., Davis, R.L., Wright, W.E., Kadesch, T., Murre, C., Voronova, A., Baltimore, D. and Weintraub, H. (1991) Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins *in vivo*. Cell **66**: 305-315.

Lavedan, C., Hoffman-Radvanyi, H., Shelbourne, P., Rabes, J-P., Duros, C., Savoy, D., Dehaupas, I., Luce, S., Johnson, K. and Junien, C. (1993) Myotonic dystrophy: size- and sex-dependent dynamics of CTG meiotic instability, and somatic mosaicism. Am. J. Hum. Genet. **52**: 875-883.

Leung, T., Chen, X-Q., Manser, E. and Lim, L. (1996) The p160 RhoA-binding kinase ROKα is a member of a kinase family and is involved in the reorganization of the cytoskeleton. Mol. Cell. Biol. **16**: 5313-5327.

Liston, P., Roy, N., Tamai, K., Lefebvre, C., Baird, S., Cherton-Horvat, G., Farahani, R., McLean, M., Ikeda, J., MacKenzie, A. and Korneluk, R.G. (1996) Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. Nature **379**: 349-353.

Maeda, M., Taft, C.S., Bush, E.W., Holder, E., Bailey, W.M., Neville, H., Perryman, M.B. and Bies, R.D. (1995) Identification, tissue-specific expression, and subcellular localization of the 80- and 71-kDa forms of myotonic dystrophy kinase protein. J. Biol. Chem. **270**: 20246-20249.

Mahadevan, M., Tsilfidis, C., Sabourin, L., Shutler, G., Amemiya, C., Jansen, G., Neville, C., Narang, M., Barceló, J., O'Hoy, K., Leblond, S., Earle-MacDonald, J., de Jong, P.J., Wieringa, B. and Korneluk, R.G. (1992) Myotonic dystrophy mutation: an unstable CTG repeat in the 3' untranslated region of the gene. Science **255**: 1253-1255.

Mahadevan, M.S., Amemiya, C., Jansen, G., Sabourin, L., Baird, S., Neville, C.E., Wormskamp, N., Segers, B., Batzer, M., Lamerdin, J., de Jong, P., Wieringa, B. and Korneluk, R.G. (1993) Structure and genomic sequence of the myotonic dystrophy (DM kinase) gene. Hum Mol Genet **2**: 299-304.

Malloy, P., Mishra, S.K. and Adler, S.H. (1990) Neuropsychological deficits in myotonic muscular dystrophy. **53**: 1011-1013.

Mangiarini, L., Kathasiwam, K., Seller, M., Cozens, B., Harper, A., Hetherington, C., Lawton, M., Trottier, Y., Lehrach, H., Davies, S.W. and Bates, G.P. (1996) Exon 1 of the *HD* gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. Cell **87**: 493-506.

Mangiarini, L., Sathasivam, K., Mahal, A., Mott, R., Seller, M. and Bates, G.P. (1997) Instability of highly expanded CAG repeats in mice transgenic for the Huntington's disease mutation. Nat. Genet. **15**: 197-200.

Martorell, L., Martinez, J.M., Carey, N., Johnson, K. and Baiget, M. (1995) Comparison of CTG repeat length expansion and clinical progression of myotonic dystrophy over a five year period. J. Med. Genet. **32**: 593-596.

McKnight, R.A., Shamay, A., Sankaran, L., Wall, R.J. and Henninghausen, L. (1992) Matrix-attachment regions can impart position-independent regulation of a tissue-specific gene in transgenic mice. Proc. Natl. Acad. Sci. USA **89**: 6943-6947.

Miner, J.H. and Wold, B.J. (1990) Herculin, a fourth member of the MyoD family of myogenic regulator. Proc. Natl. Acad. Sci. USA **87**: 1089-1093.

Monckton, D.G., Coolbaugh, M.I., Ashizawa, K.T., Siciliano, M.J. and Caskey, C.T. (1997) Hypermutable myotonic dystrophy CTG repeats in transgenic mice. Nat. Genet. **15**: 193-196.

Mulley, J.C., Staples, A., Donnelly, A., Gedeon, A.K., Hecht, B.K., Nicholoson, G.A., Haan, E.A. and Sutherland, G.R. (1993) Explanation for exclusive maternal origin for congenital form of myotonic dystrophy. Lancet **341**: 236-237.

Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esumi, E., Li, S., Nonaka, I. and Nabeshima, Y. (1993) Myogenin gene disruption results in perinatal lethality because of severe muscle defect. Nature **364**: 532-535.

Nagafuchi, S., Yanagisawa, H., Sato, K., Shirayama, T., Ohsaki, E., Bundo, M., Takeda, T., Tadokoro, K., Kondo, I., Murayama, N., Tanaka, Y., Kikushima, H., Umino, K., Kurosawa, H., Furukawa, T., Nihei, K., Inoue, T., Sano, A., Komure, O., Takahashi, M., Yoshizawa, T., Kanazawa, I. and Yamada, M. (1994) Dentatorubral and pallidoluysian atrophy expansion of an unstable CAG trinucleotide on chromosome 12p. Nat. Genet. **6**: 14-18.

Nawrotski, R., Blake, D.J. and Davies, K.E. (1996) The genetic basis of neuromuscular disorders. Trends Genet. **8**: 294-298.

Nazir, J., Floresco, S.B., O'Kusky, J.R., Diewert, V.M., Richman, J.M., Zeisler, J., Borowski, A., Marth, J.D., Phillips, A.G. and Hayden, M.R. (1995) Targeted disruption of the Huntington's disease gene results in embryonic lethality and behavioural and morphological changes in heterozygotes. Cell **81**: 811-823.

Nazir, J., Goldberg, Y.P. and Hayden, M.R. (1996) Huntington disease: new insights into the relationship between CAG expansion and disease. Hum. Mol. Genet. **5**: 1431-1435.

Neville, H.E. (1979) in *Handbook of Clinical Neurology. Diseases of Muscle, Part I, Volume 40.* Edited by Vinken, P.J. and Bruyn, G.W. North Holland Publishing, New York. pp. 63-123.

Nickells, R.W. (1996) Retinal ganglion cell death in glaucoma: the how, the why and they maybe. J. Glaucoma **5**: 345-356.

Novelli, G., Gennarelli, M., Zelano, G., Pizzuti, A., Fattorini, C., Caskey, C.T., and Dallapiccola, B. (1993) Failure in detecting mRNA transcripts from the mutated allele in myotonic dystrophy muscle. Biochem. Mol. Biol. Int. **29**: 291-297.

Oberle, I., Rousseau, F., Heitz, D., Kretz, C., Devys, D., Hanauer, A., Boue, J., Bertheas, M.F. and Mandel, J.L. (1991) Instability of a 550-base pair DNA segment and abnormal methylation in Fragile X syndrome. Science **252**: 1097-1102.

O'Hoy, K.L., Tsilfidis, C., Mahadevan, M.S., Neville, C.E., Barceló, J., Hunter, A.G.W. and Korneluk, R.G. (1993) Reduction in size of the myotonic dystrophy trinucleotide repeat mutation during transmission. Science **259**: 809-812.

Olson, E.N. and Klein, W.H. (1994) bHLH factors in muscle development: dead lines and commitments, what to leave in and what to leave out. Genes and Dev. **8**: 1-8.

Olsson, J.E., Gordon, J.W., Pawlyk, B.S., Roof, D., Hayes, A., Molday, R.S., Mukai, S., Cowley, G.S., Berson, E.L. and Dryja, T.P. (1992) Transgenic mice with a rhodopsin mutation (pro23his): a mouse model of autosomal dominant retinitis pigmentosa. Neuron **9**: 815-830.

Orr, H.T., Chung, M-Y., Banfi, S., Kwaitkowski Jr, T.J., Servadio, A., Beaudet, A.L., McCall, A.E., Duvick, L.A., Ranum, L.P.W. and Zoghbi, H.Y. (1993) Expansion of an unstable trinucleotide CAG repeat in spinocerebellar ataxia type 1. Nat. Genet. **4**: 221-226.

Otten, A.D. and Tapscott, S.J. (1995) Triplet repeat expansion in myotonic dystrophy alters the adjacent chromatin structure. Proc. Natl. Acad. Sci. USA **92**: 5465-5469.

Phi-Van, L., von Kries, J.P., Ostertag, W. and Stratling, W.H. (1990) The chicken lysozyme 5' matrix attachment region increases transcription from a heterogeneous promoter in heterologous cells and dampens position effects on the expression of transfected genes. Mol. Cell. Biol. **10**: 2302-2307.

Pinol-Roma, S. and Dreyfuss, G. (1992) Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm. Nature **355**: 730-732.

Portera-Cailliau, C., Sung, C-H., Nathans, J. and Adler, R. (1994) Apoptotic photoreceptor cell death in mouse models of retinitis pigmentosa. Proc. Natl. Acad. Sci. USA **91**: 974-978.

Portwood, M.M., Wicks, J.J., Lieberman, J.S. and Fowler, W.M. (1984) Psychometric evaluation in myotonic muscular dystrophy. Arch. Phys. Med. Rehab. **65:** 533-536.

Portwood, M.M., Wicks, J.J., Lieberman, J.S. and Duveneck, M.J. (1986) Intellectual and cognitive function in adults with myotonic muscular dystrophy. Arch. Phys. Med. Rehab. **67**: 299-303.

Puissant, C. and Houdebine, L-M. (1990) An improvement of the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Biotechniques **8**: 148-149.

Pulst, S-M., Nechiporuk, A., Nechiporuk, T., Gispert, S., Chen, X-N., Lopes-Cendes, I., Pearlman, S., Starkman, S., Orozco-Diaz, G., Lunkes, A., de Jong, P., Rouleau, G.A., Auburger, G., Korenberg, J.R., Figueroa, C. and Sahba, S. (1996) Moderate expansion of a normally biallelic trinucleotide repeat in spinocerebellar ataxia type 2. Nat. Genet. **14**: 268-276.

Rastinejad, F. and Blau, H.M. (1993) Genetic complementation reveals a novel regulatory role for 3' untranslated regions in growth and differentiation. Cell **72**: 903-917.

Reddy, S., Smith, D.B.J., Rich, M.M., Leferovich, J.M., Reilly, P., Davis, B.M., Tran, K., Rayburn, H., Bronson, R., Cros, D., Balice-Gordon, R.J. and Housman, D. (1996) Mice lacking the myotonic dystrophy protein kinase develop a late onset progressive myopathy. Nat. Genet. **13**: 325-335.

Redman, J.B., Fenwick Jr., R.G., Fu, Y-H., Pizzuti, A., Caskey, C.T. (1993) Relationship between parental trinucleotide GCT repeat length and severity of myotonic dystrophy in offspring. J. Am. Med. Assoc. **269**: 1960-1965.

Rhodes, S.J. and Konieczny, S.F. (1989) Identification of MRF-4: a new member of the muscle regulatory factor gene family. Genes and Dev. **3**: 2050-2061.

Richards, R.I. and Sutherland, G.L. (1992) Dynamic mutations: a new class of mutations causing human disease. Cell **70**: 709-712.

Roses, A.D., Harper, P.S. and Bossen, E.H. (1979) in *Handbook of Clinical Neurology. Diseases of Muscle, Part I, Volume 40.* Edited by Vinken, P.J. and Bruyn, G.W. North Holland Publishing, New York. pp. 485-532.

Roses, A.D. (1994) Muscle biochemistry and a genetic study of myotonic dystrophy. Science **264**: 587.

Rozmahel, R., Wilschanski, M., Matin, A., Plyte, S., Oliver, M., Auerbach, W., Moore, A., Forstner, J., Durie, P., Nadeau, J., Bear, C. and Tsui, L-C. (1996) Modulation of disease severity in cystic fibrosis transmembrane conductance regulator deficient mice by a secondary genetic factor. Nature Genet. **12**: 280-287.

Rudnicki, M.A., Braun, T., Hinuma, S. and Jaenisch, R. (1992) Inactivation of *MyoD* in mice leads to up-regulation of the myogenic HLH gne Myf-5 and results in apparently normal muscle development, Cell **71**: 383-390.

Rudnicki, M.A., Schnegelsberg, P.N.J., Stead, R.H., Braun, T., Arnold, H-H. and Jaenisch, R. (1993) MyoD or Myf-5 is required for the formation of skeletal muscle. Cell **75**: 1351-1359.

Sabourin, L.A., Mahadevan, M.S., Narang, M., Lee, D.S.C., Surh, L.C. and Korneluk, R.G. (1993) Effect of the myotonic dystrophy (DM) mutation on mRNA levels of the DM gene. Nat. Genet. **4**: 233-238.

Sabourin, L. (1996) in *Myotonic Dystrophy: A Study of the Expression of the Myotonic Dystrophy Gene in Affected Tissues and cells.* Ph.D. Thesis, University of Ottawa.

Salvatori, S., Biral, D., Furlan, S. and Marin, O. (1994) Identification and localization of the myotonic dystrophy gene product in skeletal and cardiac muscles. Biochem. Biophys. Res. Comm. **203**: 1365-1370.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) in *Molecular Cloning: A Laboratory Manual (Second Edition)*. Cold Spring Harbour Publishing, New York.

Sanpei, K., Takano, H., Igarashi, S., Sato, T., Oyake, M., Sasaki, H., Wakisaka, A., Tashiro, K., Ishida, Y., Ikeuchi, T., Koide, R., Saito, M., Sato, A., Tanaka, T., Hanyu, S., Takiyama, Y., Nishizawa, M., Shimizu, N., Nomura, Y., Segawa, M., Iwabuchi, K., Eguchi, I., Takahashi, H. and Tsuji, S. (1996) Identification of the spinocerebellar ataxia type 2 gene using a direct identification of repeat expansion and cloning technique, DIRECT. Nat. Genet. **14**: 277-284.

Sarnat, H.R. and Silbert, S.W. (1976) Maturation arrest of fetal muscle in neonatal myotonic dystrophy: a pathological study of four cases. Arch. Neurol. **33**: 466-474.

Shaw, D.J., McCurrach, M., Rundle, S.A., Harley, H.G., Crow, S.R., Sohn, R., Thirion, J-P., Hamshere, M.G., Buckler, A.J., Harper, P.S., Housman, D.E. and Brook, J.D. (1993) Genomic organization and transcriptional units at the myotonic dystrophy locus. Genomics **18**: 673-679.

Shelbourne, P., Winqvist, R., Kunert, E., Davies, J., Leisti, J., Thiele, H., Bachmann, H., Buxton, J., Williamson, B. and Johnson, K. (1992) Unstable DNA may be responsible for the incomplete penetrance of the myotonic dystrophy phenotype. Hum. Mol. Genet. **1**: 467-473.

Shutler, G., Korneluk, R.G., Tsilfidis, C., Mahadevan, M., Bailly, J., Smeets, H., Jansen, G., Wieringa, B., Lohman, F., Aslandis, C. and de Jong, P.J. (1992) Physical mapping and cloning of the proximal segment of the myotonic dystrophy gene region. Genomics **13**: 518-525.

Skerjanc, I.S., Slack, R.S. and McBurney, M.W. (1994) Cellular aggregation enhances myoD-directed skeletal myogenesis in embryonal carcinoma cells. Mol. Cell. Biol. **14**: 8451-8459.

Sung, C-H., Makino, C., Baylor, D. and Nathans, J. (1994) A rhodopsin gene mutation responsible for autosomal dominant retinitis pigmentosa results in a protein that is defective in localization to the photoreceptor outer segment. J. Neurosci. 14: 5818-5833.

Storbeck, C., Sabourin, L.A. and Korneluk, R.G. (1997) Definition of regulatory sequence elements in the promoter region and the first intron of the myotonic dystrophy kinase (DMK) gene. J. Biol. Chem. (submitted).

Tachi, N., Kozuka, N., Ohya, K., Chiba, S. and Kikuchi, K. (1995) Expression of myotonic dystrophy protein kinase in biopsied muscles. J. Neurol. Sci. **132**: 61-64.

Taneja, K.L., McCurrach, M., Schalling, M., Housman, D. and Singer, R.H. (1995) Foci of trinucleotide repeat transcripts in nuclei of myotonic dystrophy cells and tissues. J. Cell. Biol. **128**: 995-1002.

Thayer, M.J. and Weintraub, H. (1993) A cellular factor stimulates the DNA-binding activity of MyoD and E47. Proc. Natl. Acad. Sci. USA **90**: 6483-6487.

The Dutch-Belgium Fragile X Consortium. (1994) *Fmr1* knockout mice: a model to study fragile X mental retardation. Cell **78**: 23-33.

The Huntington's Disease Collaborative Research Group. (1993) A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. Cell **72**: 971-983.

Thibault M.C., Mathieu, J., Perusse, L. and Korneluk, R.G. (1993) A closer look into the relationship between the degree of allelic expansion of the CTG repeat and the severity of myotonic dystrophy. Am. J. Hum. Genet. **53A**: 1239.

Thornton, C.A., Johnson, K. and Moxley III, R.T. (1994) Myotonic dystrophy patients have larger CTG expansions in skeletal muscle than in leukocytes. Ann. Neurol. **35**: 104-107.

Tsang, S.H., Gouras, P., Yamashita, C.K., Kjeldbye, H., Fisher, J., Farber, D.B. and Goff, S.P. (1996) Retinal degeneration in mice lacking the γ subunit of the rod cGMP phosphodiesterase. Science 272: 1026-1029.

Tsilfidis, C., MacKenzie, A.E., Mettler, G., Barceló, J. and Korneluk, R.G. (1992) Correlation between CTG trinucleotide repeat length and frequency of severe congenital myotonic dystrophy. Nat. Genet. **1**: 192-195.

Timchenko, L.T., Nastainczyk, W., Schneider, T., Patel, B., Hofmann, F. and Caskey, C.T. (1995) Full-length myotonin protein kinase (72 kDa) displays serine kinase activity. Proc. Natl. Acad. Sci. USA **92**: 5366-5370.

Timchenko, L.T., Miller, J.W., Timchenko, N.A., DeVore, D.R., Datar, K.V., Lin, L., Roberts, R., Caskey, C.T. and Swanson, M.S. (1996) Identification of a $(CUG)_n$ triplet repeat RNA-binding protein and its expression in myotonic dystrophy. Nucleic Acids Res. **24**: 4407-4414.

Tohgi, H., Kawamorita, A., Utsugisawa, K., Yamagata, M. and Sano, M. (1994) Muscle histopathology in myotonic dystrophy in relation to age and muscular weakness. Muscle and Nerve **17**: 1037-1043.

Turnpenny, P., Clark, C. and Kelly, K. (1993) Intelligence quotient profile in myotonic dystrophy, intergenerational deficit, and correlation with CTG amplification. J. Med. Genet. **31**: 300-305.

Vanier, T.M. (1960) Dystrophia myotonica in childhood. Br. Med. J. **2**: 1284.

van der Ven, P.F.M., Jansen, G., van Kuppevelt, T.H.M.S.M., Perryman, M.B., Lupa, M., Dunne, P.W., ter Laak, H.J., Jap, P.H.K., Veerkamp, J.H., Epstein, H.J. and Wieringa, B. (1993) Myotonic dystrophy kinase is a component of neuromuscular junctions. Hum. Mol. Genet. **2**: 1889-1894.

Verkerk, A.J.M.H., de Graaff, E., De Boulle, K., Eichler, E.E., Konecki, D.S., Reyniers, E., Manca, A., Poustka, A., Willems, P.J., Nelson, D.L. and Oostra, B.A. (1993) Alternative splicing in the fragile gene FMR1. Hum. Mol. Genet. **2**: 399-404.

Wang, J., Pegoraro, E., Menegazzo, E., Gennarelli, M., Hoop, R.C., Angelini, C. and Hoffman, E.P. (1995a) Myotonic dystrophy: evidence for a possible dominant-negative RNA mutation. Hum. Mol. Genet. **4**: 599-606.

Wang, Y-H. and Griffith, J. (1995b) Expanded CTG triplet blocks from the myotonic dystrophy gene create the strongest known natural nucleosome positioning elements. Genomics **25**: 570-573.

Waring, J.D., Haq, R., Tamai, K., Sabourin, L.A., Ikeda, J-E. and Korneluk, R.G. (1996) Investigation of myotonic dystrophy kinase isoform translocation and membrane association. J. Biol. Chem. **271**: 15187-15193.

Weintraub, H., Tapscott, S.J., Davis, R.L., Thayer, M.J., Adam, M.A., Lassar, A.B. and Miller, A.D. (1989) Activation of muscle-specific genes in pigment, nerve, fat, liver and fibroblast cell lines by forced expression of MyoD. Proc. Natl. Acad. Sci. USA **86**: 5434-5438.

Weintraub, H. (1993) The MyoD family and myogenesis: redundancy, networks and thresholds. Cell **75**: 1241-1244.

Whiting, E.J., Waring, J.D., Tamai, K., Somerville, M.J., Hincke, M., Staines, W.A., Ikeda, J-E. and Korneluk, R.G. (1995) Characterization of myotonic dystrophy kinase (DMK) protein in human and rodent muscle and central nervous tissue. Hum. Mol. Genet. **4**: 1063-1072.

Wieringa, B. (1994) Myotonic dystrophy reviewed: back to the future? Hum. Mol. Genet. **3**: 1-7.

Wischmeyer, E., Nolte, E., Klocke, R., Jockusch, H. and Brinkmeier, H. (1993) Development of electrical myotonia in the ADR mouse: role of chloride conductance in myotubes and neonatal animals. Neuromusc. Disord. **3**: 267-274.

Wissman, A., Ingles, J., McGhee, J.D. and Mains, P.E. (1997) *Caenorhabditis elegans* LET-502 is related to rho-binding kinases and human myotonic dystrophy kinase and interacts genetically with a homolog of the regulatory subunit of smooth muscle myosin phosphatase to affect cell shape. Genes and Dev. **11**: 409-422.

Wong, L-CJ., Ashizawa, T., Monckton, D.G., Caskey, C.T. and Richards, C.S. (1995) Somatic heterogeneity of the CTG repeat in myotonic dystrophy is age and size dependent. Am. J. Hum. Genet. **56**: 114-122.

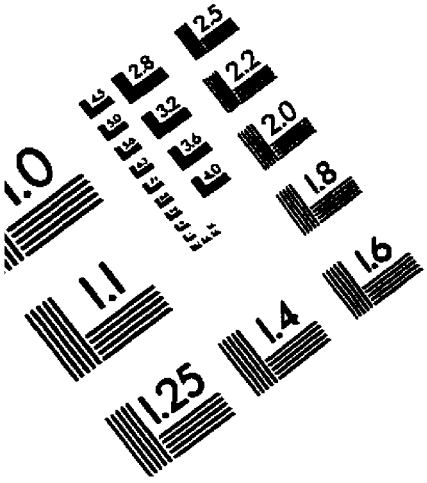
Wright, W.E., Sassoon, D.A. and Lin, V.K. (1989) Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. Cell **56**: 607-617.

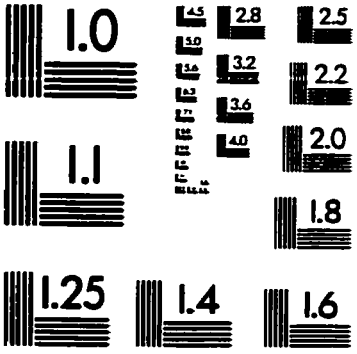
Yarden, O., Plamann, M., Ebbole, D.J. and Yanofsky, C. (1992) *cot-1*, a gene required for hyphal elongation in *Neurospora crassa*, encodes a protein kinase. EMBO J. **11**: 2159-2166.

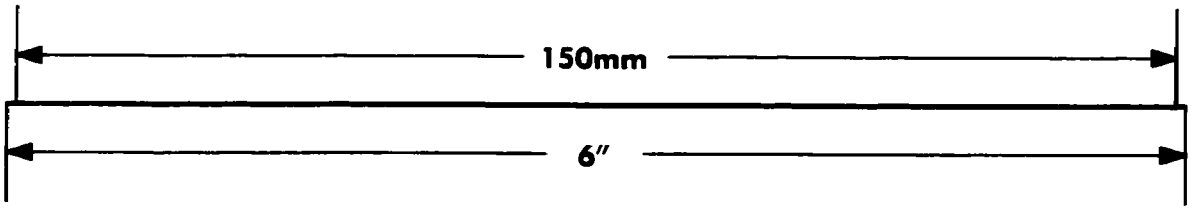
Zatz, M., Passos-Bueno, M.R., Cerqueira, A., Marie, S.K., Vainzof, M. and Pavanello, R.C.M. (1995) Analysis of the CTG repeat in skeletal muscle of young and adult myotonic dystrophy patients: when does the expansion occur? Hum. Mol. Genet. **4**: 401-406.

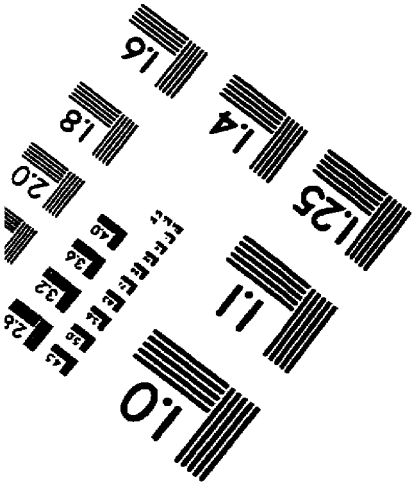
Zhang, W., Behringer, R.R. and Olson, E.N. (1995) Inactivation of the myogenic bHLH gene MRF4 results in up-regulation of myogenin and rib anomalies. Genes and Dev. **9**: 1388-1399.

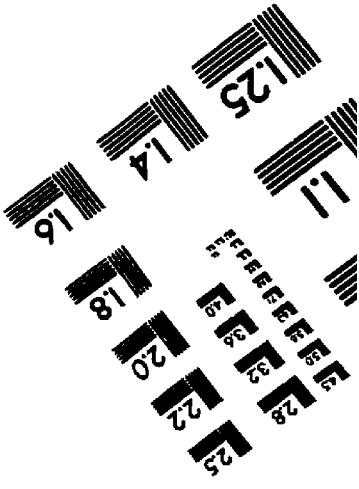
IMAGE EVALUATION TEST TARGET (QA-3)











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