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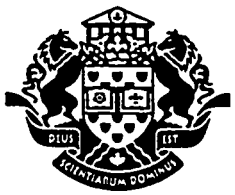

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EFFECTS OF THE MYOTONIC DYSTROPHY MUTATION IN MUSCLE DIFFERENTIATION AND APOPTOSIS

A Thesis Submitted to the School of Graduate Studies

University of Ottawa

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

Department of Biochemistry, Microbiology and Immunology

Faculty of Medicine

By

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Abstract

Myotonic dystrophy (DM) is the most common inherited neuromuscular disorder of adult life. DM also strikes in childhood and displays incredible variability in penetrance and age of onset. Adult DM is characterized mainly by myotonia, progressive distal muscle weakness and wasting, cardiac conduction abnormalities, testicular atrophy and premature balding. Congenital (c)DM is the most severe form of DM. The main features of cDM include hypotonia, or profound muscle immaturity, respiratory distress, talipes, increased numbers of satellite myoblasts and mental retardation. The genetic defect for DM was identified as an unstable CTG trinucleotide repeat found in the 3' untranslated region (UTR) of a serine threonine protein kinase, DMPK. Normal individuals possess 5-35 CTG repeats, typical adult DM patients have repeat sizes ranging from 80 to 1000 while cDM patients have from 1000 to several thousand CTG repeats. This discovery provided a molecular basis to account for large variability of penetrance and age of onset in DM.

The presence of the mutation in the 3' UTR of the DMPK gene precluded any effect it might have on the polypeptide sequence of the translated gene product. At the time this work was initiated, two hypotheses were proposed explaining how the CTG expansion might affect DMPK. The first hypothesis, based on the observation of reduced DMPK mRNA in patient muscle, speculated that the CTG repeat in the 3' UTR of the DMPK gene interfered with transcription of DMPK leading to very low levels or an absence of DMPK. The second hypothesis put forward by our laboratory following observation of elevated DMPK mRNA in patient skeletal muscle and brain samples

suggested that elevated DMPK protein levels and activity might be the molecular basis of DM. In addition, it was suggested that the CUG repeat at the RNA level might be acting in a dominant gain of function fashion by sequestering RNA binding proteins. Over time, the first hypothesis was disproved, mainly because mutant DMPK mRNA isolated from patients was readily detected by standard techniques while the latter part of the second hypothesis, that pertaining to an RNA-mediated effect, gained momentum.

DMPK mRNA is found most abundantly in heart, then skeletal muscle, smooth muscle and brain. The DMPK protein was localized to intercalated disks in heart, neuromuscular junctions in skeletal muscle and within ventricular ependymal and choroid plexus cells in the brain. Given the specificity of this localization pattern and the increasing importance of *in vivo* models for genetic disease, identifying endogenous DMPK transcription regulatory elements was undertaken. Initially, we sought to identify transcription control elements governing DMPK transcription in skeletal muscle *in vitro*. Various deletion fragments of the DMPK 5' promoter region and first intron were fused to the chloramphenicol acetyl transferase (CAT) gene and assayed for promoter or enhancer strength in TE32 myoblasts or NIH 3T3 fibroblasts. In addition, it was determined if the first intron contained functional E-boxes by fusing this element downstream of the CAT reporter with the thymidine kinase (TK) core promoter. These constructs were co-transfected with a MyoD expression vector into C3H10T1/2 cells and assayed for MyoD mediated activation. We identified a ubiquitously active 189 bp promoter within the DMPK 5' region. We also found that the DMPK first intron can function as an enhancer element in myoblasts but not in fibroblasts. This first intron

fragment was responsive to MyoD and the responsive sequences mapped to a stretch of 55 bp that contains four conserved E-box elements.

Work in our laboratory progressed from ascertaining mRNA levels in patient tissues to testing the hypothesis that overexpression of DMPK might cause features of DM. The C2C12 mouse myoblast system was employed in these studies. Sabourin *et al.* discovered that overexpression of the DMPK cDNA in this cell line inhibited differentiation of these cells. The portion of the cDNA responsible for this inhibition was found to be the 3' UTR, more specifically, the 5' region of the 3' UTR, upstream of the CTG repeat sequence. Concurrently, a transgenic model was constructed by Narang *et al.* whereby the entire 15 Kb human DMPK gene was used in the construction of transgenic mice. These animals expressed the human DMPK mRNA and protein in the appropriate tissues and had many features of DM including type I fibre atrophy, central nuclei and ringed fibres.

Based on *in vitro* and *in vivo* DMPK overexpression studies described above, we posed the following question. If the DMPK 3' UTR can inhibit myogenesis *in vitro* and a transgenic animal expressing the whole DMPK gene can result in DM like pathology, can overexpression of the DMPK 3' UTR, without attached DMPK coding sequences, inhibit myogenesis and cause DM like pathology *in vivo*?

To answer this question, a transgene was constructed whereby the DMPK promoter/enhancer drove expression of the GFP cDNA with a wild type (11 CTG repeats) or mutant (99 CTG repeats) DMPK 3' UTR. Before the constructs were injected, they were tested for their ability to produce GFP protein *in vitro* by transfection into C2C12 myoblasts. Stable clones were generated and initial empirical observations

suggested that the CTG 99 cell lines were generally less healthy than CTG 11 cell lines. We found that CTG 11 and CTG 99 cell lines were more susceptible to cell death during differentiation than control cell lines with a Pgk 3' UTR. In addition, CTG 11 and 99 cell lines were more susceptible to staurosporine induced apoptosis compared with the control cell lines. Interestingly, CTG 99 cell lines were found to express about ten-fold less transgene mRNA than CTG 11 cell lines. We also developed inducible cell lines using the same GFP reporter and 3' UTR elements. Induction of expression resulted in about three fold higher levels of CTG 99 mRNA over CTG 11 mRNA at 48 hours post induction. Levels of cell death were assayed following induction and CTG 99 cell lines showed a marked level of cell death while CTG 11 cell lines did not. The presence of CTG repeats within mRNA therefore appears to be very problematic for the cell. It is known that CUG repeats in mRNA can form hairpin structures. Interestingly, the double stranded RNA activated protein kinase (PKR) binds double stranded RNA, usually produced by viruses, and can repress translation or induce apoptosis in response. We found that in the presence of the DMPK 3' UTR, particularly when the CTG repeat is longer, PKR becomes activated and autophophorylates. We also found that inhibition of this enzyme can reduce the death susceptibility of some CTG repeat stable cell lines. Furthermore, we found that patient amniocytes and myoblasts are susceptible to staurosporine induced cell death. Taken together, this data suggests that myoblasts expressing the DMPK 3' UTR are prone to cell death in an expression level and repeat length dependent manner. In addition, patient cells were found to be susceptible in much the same way.

Finally, the same constructs outlined above were used to create transgenic mice. We found by northern and western blot analysis that the transgene was expressed in the appropriate tissues confirming the *in vivo* utility of the DMPK regulatory elements employed. Further investigation of transgene expression by *in situ* hybridization revealed that the transgene was co-expressed with myogenin in the developing mouse embryo at day 12 and 19. These experiments also revealed that myogenin levels *in vivo* were reduced in transgenic embryos compared with wild type embryos suggesting that expression of the DMPK 3' UTR *in vivo* inhibits accumulation of myogenin and perhaps myogenesis. In adult mice there was consistent muscle atrophy in CTG 91 but not in CTG 11 mice despite much lower expression levels of the CTG 91 transgene. Together, these results indicate that expression of the DMPK 3' UTR by itself may inhibit myogenesis *in vivo* and contribute to pathological features of DM like muscle atrophy.

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DEDICATION

To my family: Suzana, Paula, Karen, Jurgen and Norma.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1. GENERAL INTRODUCTION AND LITERATURE REVIEW

CHAPTER 3. CONTROL OF DMPK EXPRESSION *IN VITRO*.

LIST OF ABBREVIATIONS

bp	base pair
°C	degree Celsius
cDNA	complementary DNA
Ci	curies
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dTTP	deoxythymidine triphosphate
dGTP	deoxyguanosine triphosphate
DM	dystrophia myotonica/myotonic dystrophy
DNA	deoxyribonucleic acid
dpc	days post coitum
dpm	disintegration per minute
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
h	hour
kb	kilobase
l	litre
LB	Luria-Bertani medium
min	minute
ml	millilitre
mM	millimolar
mmol	millimole

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

Contribution of Candidate

The candidate was the driving force behind all work presented in this thesis. Chapter 3 was performed in its entirety by the candidate. Other authors appearing on the publication resulting from this work besides the candidate and the principal investigator (Dr. Korneluk) provided technical and experimental design input only. Chapters four and five were assisted by several summer students and technicians, all trained by the candidate. More specifically, stable cell line maintenance was assisted by Heather McLeod, Ameneh Mirizae and Kate Daniel. Kate Daniel, Rene Carriere and Ameneh Mirizae assisted in collecting data on cell death studies. Connie Craig and Gaby Cherton-Horvat provided input in design of staurosporine cell death assays. Nazim Ahmed contributed with western blot studies carried out on transgenic mice. Suzana Drmanic performed *in situ* hybridization on the transgenic mice. Kate Daniel assisted in transgenic mouse colony maintenance and provided technical support in fibre area measurement studies. Chapters four and five are being prepared for publication as separate papers.

CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

The past fifteen years in the field of molecular genetics have marked the beginnings of a revolution in treating genetic disease. The power of isolating a disease gene by positional cloning gave way to the challenge of deciphering entire pathways with a single protein as a starting point. The transition from gene to protein to pathway and eventually to therapeutic intervention will not be a trivial task, however advances in technology and input from many disciplines will make this goal a reality sooner than we think.

The gene found to be mutated in myotonic dystrophy (DM) was cloned in 1992. Only now, after years of investigation into several candidate mechanistic theories is the field beginning to converge on the probable molecular mechanism that gives rise to this unique disorder. This convergence will no doubt speed the field toward therapeutic interventions for DM, yet great challenges remain.

Building on previous work in our laboratory, we set out to identify a molecular explanation for myotonic dystrophy. In order to target the appropriate tissues, we first characterized regulatory elements governing DMPK expression in myoblasts *in vitro* and employed this information in the construction of model systems to investigate putative disease mechanisms. Using stable cell lines, patient cells and transgenic mice, we attempted to demonstrate a role in disease pathogenesis for the DM mutation, an expanded CTG repeat in the 3'UTR of the DM protein kinase (DMPK) gene. This putative mechanism involves activation of the double stranded RNA activated protein kinase (PKR) leading to a susceptibility to apoptosis of mutant cells. Such a mechanism

implicates the mutation as a direct mediator of pathogenesis and is completely consistent with the dominant mode of inheritance of DM.

1.1 CLINICAL FEATURES OF MYOTONIC DYSTROPHY

Dystrophica Myotonica (DM), or Myotonic dystrophy as it is commonly known is the most common inherited adult neuromuscular disorder and affects approximately 1/8000, however, the frequency in certain regions can be as high as 1/450 as a result of genetic founder effects (Bouchard et al 1989). Interestingly, two forms of the disease exist, adult DM and the more severe congenital DM (cDM). Often, a mother with adult DM will give birth to a congenitally affected child. This intriguing feature of DM is known as genetic anticipation - the apparent worsening of symptoms accompanied by earlier onset through the generations of affected families.

In order to fully appreciate the aims of this thesis it will be of service to review some of the clinical features of this remarkable disease.

1.1.2 ADULT MYOTONIC DYSTROPHY

The adult form of myotonic dystrophy is characterized by myotonia and dystrophic changes to muscle, or wasting of distal muscle groups leading to progressive weakness (Harper 1989). Adult DM affects several other systems in addition to muscle making it unique among muscular dystrophies. Patients with DM experience endocrine dysfunction such as hyperinsulinaemia and testicular atrophy, cataracts and other ocular manifestations, mental disturbances, and premature hair loss. In addition to effects seen in skeletal muscle, cardiac conduction abnormalities are common in DM (Harper 1989). Adult DM is thus a complex disorder both clinically and pathologically.

Congenital myotonic dystrophy is more severe than adult DM. Hypotonia, respiratory distress, feeding difficulties, talipes and mental retardation comprise the main

clinical features (Harper 1989). Initially, cDM was not recognized as a form of Myotonic dystrophy, however, it has subsequently become clear that it is an acute form of the adult disease and surviving cDM patients develop features of adult DM as they mature (Harper 1989).

More detailed clinical observations in various organs and tissues affected by DM can be found in Harper (Harper 1989). Here I will focus mainly on clinical observations of DM in skeletal muscle.

1.1.3 MUSCLE INVOLVEMENT IN DM

The observation of myotonia and progressive muscle weakness and wasting in a patient is usually sufficient for a diagnosis of DM (Harper 1989). A unique combination of muscles is affected in typical adult DM, which can differentiate this disorder from other adult myopathies. Face and jaw muscles, anterior neck muscles, and distal limb muscles are primarily affected (Harper 1989). Facial muscle involvement is manifest as a lack of expression and sagging eyelids (ptosis). In addition, jaw muscle weakness can cause the mouth to hang open, particularly in cDM. Furthermore, tongue, palate and esophageal muscular weakness contributes to difficulty in swallowing and can result in the aspiration of material into the bronchiae leading to pneumonia. Distal limb weakness results in loss of power in the wrist and weakness of dorsoflexion of the foot. In addition, wasting is observed in the forearm and muscles of the hand. Weakness of the sternomastoid muscles of the neck can impair some patients' ability to raise their head. It is important to note that unlike other muscular dystrophies that affect proximal muscles like Duchenne's or Limb girdle muscular dystrophy, DM patients rarely become immobile due to the selective involvement of distal muscles (Harper 1989).

1.1.4 MYOTONIA

The most revealing feature of adult DM is myotonia. This is the inability to relax a muscle immediately after it has contracted. Evidence of myotonia is the inability to release an object immediately after it is grasped. A physician's test for clinical myotonia is simply to have the patient squeeze the physician's index and middle finger with their hand with considerable force and then release the grip (Harper 1989). A positive result is the delayed straightening of the patient's fingers from a clenched position. Myotonia can also be detected using electromyography or EMG. This technique records action potentials resulting from the flow of electrolytic ions through channels located in muscle membranes. In addition to detecting myotonia, EMG can indicate dystrophic changes which include reduced amplitude and polyphasic action potentials (Harper 1989). EMG tracings on DM patient muscles can reveal what is known as a "myotonic run" which is a rapid rise in amplitude of the action potential followed by a gradual decline of electrical activity lasting a period of 2 to 11 seconds (Ricker & Meinck 1972).

Clinical myotonia is only observed in DM patients with limited muscle weakness. In adult DM patients with severe muscle weakness, or in neonatal congenital DM cases where the initial problem is muscle immaturity, no clinical myotonia is observed (Harper 1989). Myotonia is detectable by EMG in cDM patients in the neonatal period and also in adult DM patients with severe muscle involvement if performed on muscles that are relatively spared from wasting.

Myotonia can be debilitating to the point where an individual can no longer do his/her job, particularly in cold conditions. DM patients often do not acknowledge

myotonia as a defect but rather consider it to be normal "stiffness" or another malady altogether such as arthritis (Harper 1989).

1.1.5 CONGENITAL MYOTONIC DYSTROPHY

Congenital DM was first overlooked and high levels of spontaneous abortions, still births and infant deaths were attributed to obstetrical difficulties (Harper 1989). In 1960, Vanier described the main features of cDM which include hypotonia, difficulty in sucking and swallowing, facial weakness, and talipes. Mental retardation and respiratory distress are also integral features of cDM. There is a lack of clinical myotonia in cDM due to the paucity of muscle development, however electrical myotonia can often be seen (Harper 1989). Almost exclusively the mother is the affected parent.

Hypotonia is usually absent by the age of 3-4 years. Facial weakness, which worsens with age, is seen in about 80 % of cDM cases and results in tenting of the upper lip and an open jaw.

Respiratory distress is the most life threatening aspect of cDM. There are several contributing factors including diaphragmatic and intercostal muscle problems, pulmonary immaturity, aspiration pneumonia and failure of cerebral respiratory control (Harper 1989). All are involved in causing respiratory distress, however the relative contribution of each is unclear. Severely affected cDM neonates will not survive without assisted respiration. However, cDM patients often gain strength and once past the neonatal respiratory distress continue to improve throughout childhood.

There is a general delay in motor development in cDM. Motor landmarks such as crawling, talking and walking are all delayed and the length of delay correlates with the degree of hypotonia. This feature affects about 85% of cDM patients, however, there is

steady improvement throughout childhood and cDM patients almost always walk. Death in cDM most often results from a failure to establish respiration and occurs within the first hours or days of life. Prognosis for life is good beyond the prenatal period as only 3 % of cDM infants die after this period (Harper 1989), however, these patients will invariably develop features of adult DM.

1.1.6 MUSCLE PATHOLOGY IN DM

When observed together, a collection of skeletal muscle pathologies are considered telling of DM. These features include increased central nuclei, nuclear chains, ringed fibres, sarcoplasmic masses, typel fibre atrophy, increased fibre splitting and increased arborization of innervation. Less prominent features are small angular fibres, moth eaten fibres, type II hypertrophy and increased fibrosis (Harper 1989). Even in mild cases, increased fibre size variation and a high proportion of "moth eaten" fibres can be seen. In addition, small angular fibres are frequently observed.

Central nuclei within muscle fibres are a characteristic feature of DM. This occurs early in pathogenesis before any major changes are seen. Initially there are only a few central nuclei but increases in numbers are seen as the severity of muscle involvement increases. On longitudinal section, rows of as many as 20 nuclei are observed which vary in size, some appearing pale and others pyknotic. Irregularities of the nuclear membrane and inclusion bodies within the nuclei are also evident.

Ringed fibres are the result of a fibre that is wrapped spirally around another muscle fibre to make it appear as though there is a ring surrounding the fibre when cross-sectioned. These are seen in about 70 % of DM patient muscle samples, but much less frequently in other muscle disorders and very rarely in normal muscle.

Sarcoplasmic masses are homogenous regions of the sarcoplasm containing disorganized intermyofibrillary material but no myofibrils (Harper 1989). Analysis by electron microscopy showed that bundles of tubules, free ribosomes and some myofilaments were also present (Fardeau 1964; Mussini et al 1970).

Ultrastructural analysis by electron microscopy reveals degenerative changes in myofibrils affecting the Z line and I bands specifically (Harper 1989). In addition, thinning of muscle capillary basement membranes and degenerating mitochondria of unusual shape have been described (Harper 1989).

Defects within muscle spindles have been observed. In particular, the intrafusal fibres are abnormally small and more numerous. On longitudinal section, the intrafusal fibres are tangled and contain fibrous tissue (Harper 1989). In addition, abnormal nerve terminal endings were noted with unusually shaped endplates and nerve endings covering a significant portion of the intrafusal fibre (Swash & Fox 1972). The observed increased number of intrafusal fibres is due to splitting and refusing. These fibres appeared to be degenerating when analyzed by electron microscopy (Swash & Fox 1975). Fibre splitting is thought to be the result of mechanical stress from repetitive myotonic discharges while increased terminal nerve branching is likely a secondary response to fibre splitting and an attempt to reinnervate the split fibres (Harper 1989).

In congenital DM, muscle fibres are reduced in number and size and when cross-sectioned are round rather than polygonal in shape. There are no signs of degeneration, necrosis of fibres or fibre splitting. A lack of fibre type distinction is seen in cDM muscle and like adult DM there is an abundance of central nuclei and type 1 fibre atrophy. Electron microscopy reveals disorganization in the Z-line region and a dilated

tubular system. A unique feature seen in cDM and not in other congenital myopathies are an abundance of satellite cells. Finally, muscle from a 27 week pre-term fetus was composed of 80% myotubes when in fact no myotubes should be present after 22 weeks (Sahgal et al 1983). This illustrates a profound lack of maturity of cDM muscle, which is seen as hypotonia in the cDM infant.

1.2 GENETICS OF DM

In 1992, the causative mutation for DM was identified as an unstable CTG, providing a molecular explanation for the observed phenomenon of genetic anticipation. To date, no other inactivating mutation within the DM gene has been found strongly suggesting that the presence of the CTG repeat expansion is the only genetic cause for DM and that no other polymorphism or mutation leading to reduced levels of DMPK can result in DM.

1.3 MESSENGER RNA LEVELS IN PATIENT MUSCLE

The position of the CTG repeats within the 3'UTR of the DMPK gene suggested an unorthodox molecular pathology for DM. A number of initial studies showed DMPK mRNA levels in adult patient tissues to be reduced (Carango et al 1993; Fu et al 1993; Hofmann-Radvanyi et al 1993; Novelli et al 1993). These studies showed that DMPK mRNA levels correlated inversely with the length of the CTG repeat suggesting that haploinsufficiency was the mechanism of DM. One leading hypothesis was that a block in transcription resulted from the presence of the CTG expansion in DNA. It was reported that the DMPK CTG repeat is the strongest known nucleosome positioning element (Wang et al 1994) and that the CTG repeat in DNA alters adjacent chromatin structure (Otten & Tapscott 1995). However, a study by Sabourin and colleagues

(Sabourin et al 1993) using northern blot analysis clearly demonstrated that normal and mutant transcripts were present in patient muscle. In addition, using congenital DM tissues and slot blot, northern blotting and competitive RT-PCR assays, DMPK mRNA levels were found increased. A dominant gain of function exerted by the CTG repeat present in the 3'UTR of the DMPK mRNA was suggested as the molecular mechanism for DM. Another study using RT-PCR found no difference in mature mRNA levels from normal and patient samples regardless of repeat size (Bhagwati et al 1996). Other RT-PCR studies examined DMPK normal and mutant RNA in the total or fully processed form. Wang *et al.* (Wang et al 1995) found DMPK mRNA levels in total RNA from DM patients to be 50% that of normal control levels, however, a similar reduction of DMPK mRNA was seen in myopathic controls. In mature mRNA (poly A+), the mutant DMPK message was found to be 25% of normal message. Krahe *et al.* (Krahe et al 1995) found no difference between normal and mutant DMPK RNA levels when unspliced message was examined but found a 50% decrease in mutant message compared with normal when spliced message was examined suggesting that processing of the DMPK transcript is altered in DM.

A landmark paper in 1995 utilized *in situ* hybridization with oligonucleotide probes to localize mutant transcript in normal and patient fibroblasts and myoblasts and found the mutant message to be sequestered in foci within the nucleus (Taneja et al 1995). In addition, these foci were found associated with the nuclear matrix, were resistant to purification by most mRNA isolation techniques and could only be liberated using cesium chloride gradient centrifugation (Davis et al 1997). This explained to some degree reductions of mutant DMPK mRNA observed in some earlier studies.

Furthermore, by Northern blotting, it was found that total wild type levels of DMPK mRNA from patient cells were no different from controls however, the mutant message ranged from 32% to 71% of control levels (Davis et al 1997).

1.4 INVOLVEMENT OF A DOWNSTREAM GENE, SIX5

Although generation of primary transcripts from the DMPK locus is not altered by the CTG expansion, abnormal chromatin structure around the expanded CTG repeat was reported (Otten & Tapscott 1995; Wang et al 1994). Furthermore, the CTG repeat is located within a CpG island (Boucher et al 1995). Together, these observations suggest that transcription of a gene downstream of DMPK may be compromised. To this end, a homeobox gene, originally termed DM associated homeodomain protein (DMAHP), now known as Six5 based on its homology to the Six family of homeodomain transcription factors (Kawakami et al 2000) was identified downstream of the DMPK gene (Boucher et al 1995). It was proposed that the CTG repeat disrupted transcription control elements of Six5 (Boucher et al 1995). Subsequently it was shown that the CTG repeat interrupted an enhancer element for Six5 and it was found that Six5 mRNA specifically from the mutant allele was greatly reduced (Klesert et al 1997) (Thornton et al 1997). Interestingly, two reports based on RT-PCR of the Six5 and DMPK genes indicate that while the DMPK mRNA is reduced, no change in Six5 mRNA levels are seen (Eriksson et al 1999) (Hamshere et al 1997). Six5 null mice however, developed cataracts which may be reflective of the role this gene plays in the developing eye and may also suggest a minor role for this gene in DM pathogenesis (Klesert et al 2000; Sarkar et al 2000).

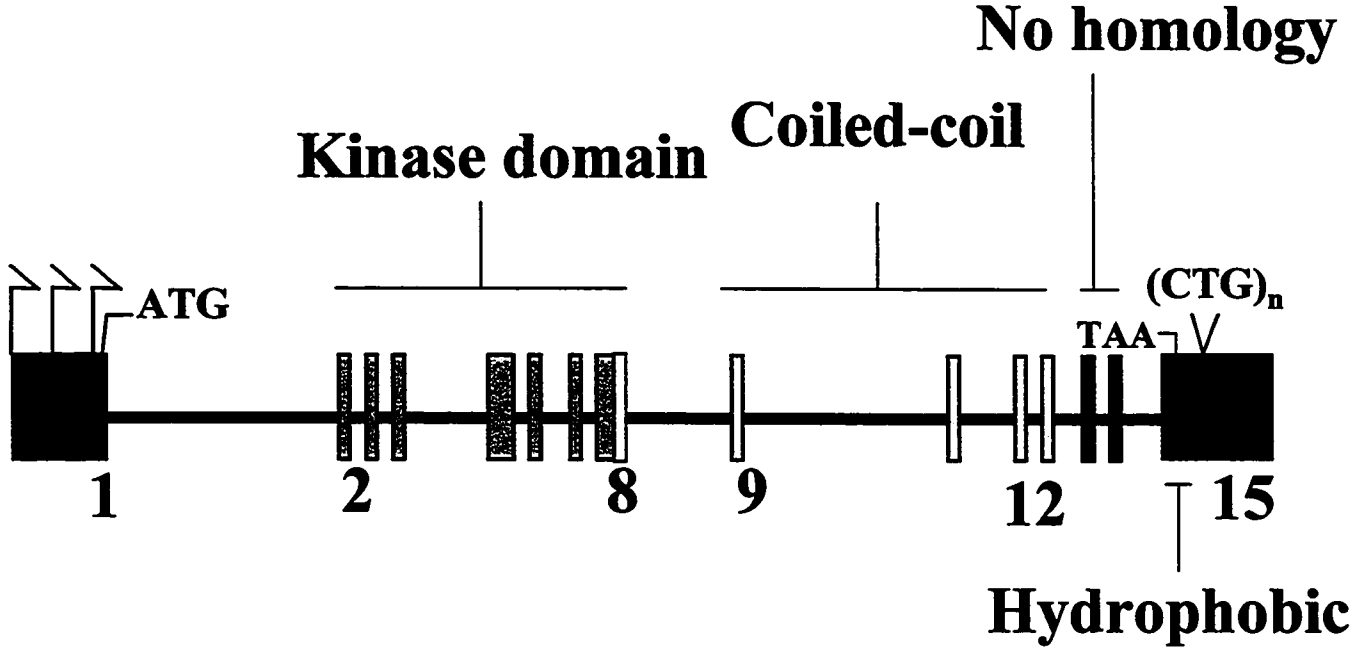
1.5 MYOTONIC DYSTROPHY KINASE (DMPK)

The Myotonic Dystrophy protein kinase, DMPK, is a serine threonine protein kinase. Its structure is divided into four domains (figure 1-1). The first domain, which is located at the N-terminus is 29 amino acids long and contains a leucine rich repeat corresponding to exon 1 of the gene. This region of the protein is 92.8% identical between mouse and human suggesting functional importance (Epstein & S. 1998). The protein kinase domain, which is also amino terminal, is comprised of 279 amino acids and encompasses exons 2 to 8 of the gene. Initially this domain had most homology to the kinase domains of SP6 kinase, PKA and PKC but it now appears that DMPK is the founding member of a new family of kinases related to the Rho binding kinases. The closest human family member is PK428, a kinase of unknown function with 78.4% similarity and 70.9% identity to DMPK (Epstein & S. 1998). A C. elegans kinase CEESP52F has 76.6% similarity and 65.1% identity to DMPK however it is more closely related to human PK428 than DMPK. A human Rho kinase, $p160^{ROCK}$ has 66.1% similarity and 52% identity while rat ROKα has 63.3% similarity and 49.3% identity to DMPK (Epstein & S. 1998). The rho kinases are known to phosphorylate myosin light chain thus mediating events leading to and requiring cell shape changes like stress fibre formation, smooth muscle contraction and cell motility, which are induced by Rho activity (Kosako et al 2000).

The third domain of DMPK is an α-helical coiled-coil domain 79 amino acids in length as predicted by the PAIRCOIL algorithm and is 88.6% identical between mouse

Figure 1-1. Structure of the Myotonic Dystrophy Kinase (DMK). DMPK contains 15 exons and is comprised of 15 Kb of genomic DNA. The $(CTG)_n$ mutation was mapped to exon 15 within the 3'UTR. Exon 1 contains a leucine zipper. Exons 2-8 comprise the protein kinase domain. Exons 9-12 encode an α-helical coiled coil domain. Exons 13-14 have no homology to any proteins and are often spliced out of the mRNA. Exon 15 contains a hydophobic stretch of amino acids.

DMPK Structure



and human DMPK (Epstein & S. 1998). Within the helical region are sequences which are very similar to domains within kinases and binding proteins known to associate with RHO A, however, it has yet to be determined if DMPK can bind RHO A (Epstein & S. 1998).The fourth domain of DMPK, although only 62.5% identical between mouse and human DMPK, is predicted to encode a hydrophobic α-helix and has similarities to sequence in other proteins known to anchor to the cytoplasmic face of the endoplasmic reticulum (Epstein & S. 1998). The function of DMPK is not known, however, DMPK is able to autophosporylate (Dunne et al 1994) and transphosphorylate recombinant histones (Dunne et al 1994; Waring et al 1996).

Inferences into DMPK function can be made from immunolocalization studies. DMPK was localized at sites important for cell-cell communication including neuromuscular junctions and myotendinous junctions in skeletal muscle, intercalated disks in heart, and Purkinje cells, hippocampal neurons, ependymal and choroid plexus cells in the brain (van der Ven et al 1993; Whiting et al 1995). Similar results were obtained by several laboratories indicating a general agreement of DMPK localization (Dunne et al 1996b; Maeda et al 1995; Salvatori et al 1997).

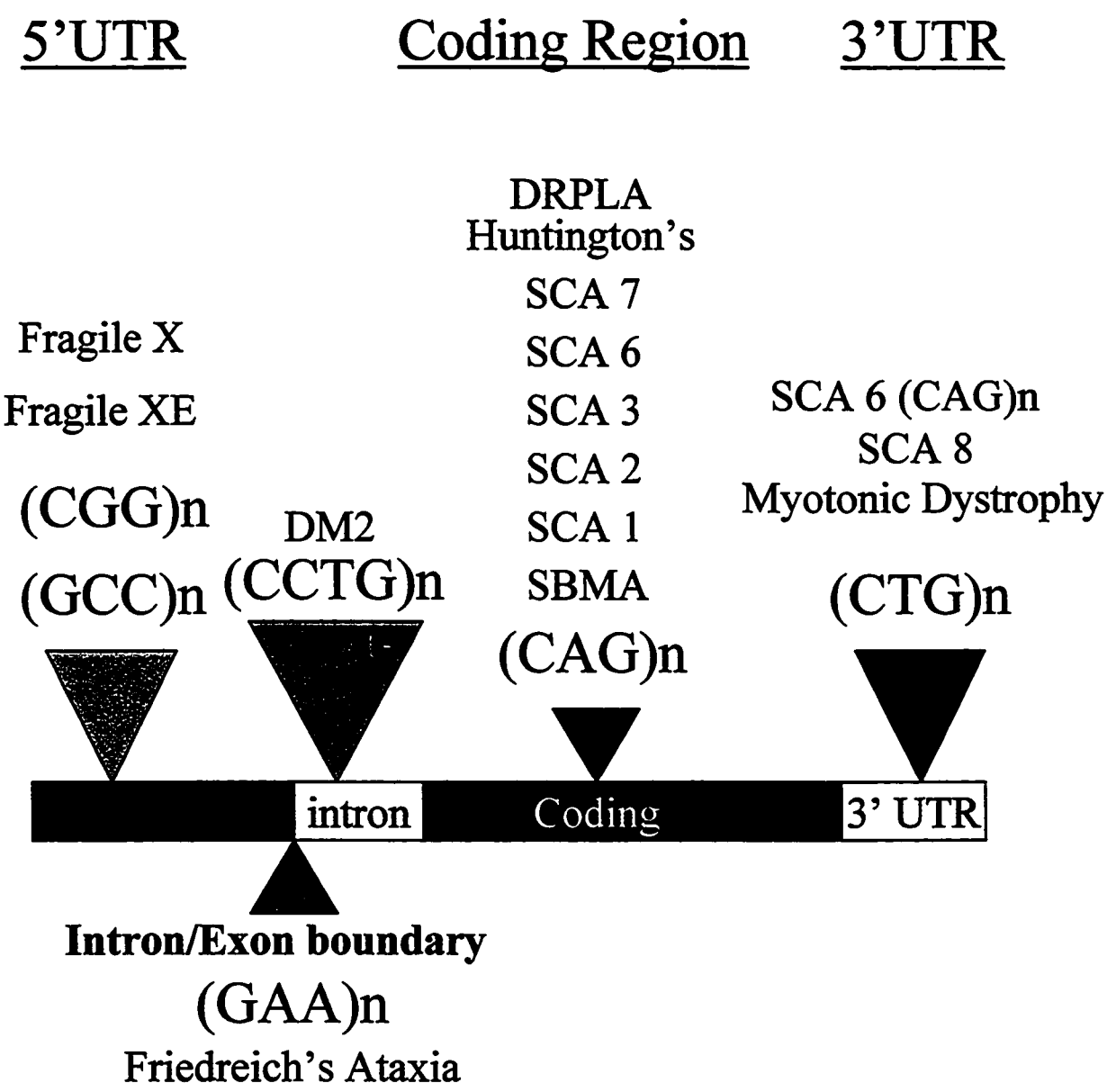
1.6 TRINUCLEOTIDE REPEAT (TNR) DISORDERS

Myotonic dystrophy is one of several genetic diseases caused by expansion of a trinucleotide repeat. This novel type of mutation, known as a dynamic mutation, influences disease severity depending on the degree of expansion and is the molecular explanation for genetic anticipation in DM (Harper 1996) and Fragile X (Szot 1997). About a dozen TNR disorders have been discovered to date (Cummings & Zoghbi 2000). In 1991, the mutations responsible for Fragile X and Spinobulbar muscular atrophy

(SBMA) were identified followed soon after in 1992 by the identification of myotonic dystrophy (DM) mutation. The mutations responsible for Huntington's disease (HD), the Spino cerebellar ataxias (SCA), Dentatorubral Pallidoluysian atrophy (DRPLA) and Friedrich's ataxia followed in quick succession. All of these mutations were identified as trinucleotide repeat expansions of varying nucleotide composition. In all cases, normal individuals possess repeat sizes within a certain range at the locus in question and all affected individuals have repeat size ranges significantly larger than this (Cummings & Zoghbi 2000). Repeat composition was originally thought to be GC rich sequences. For example, the Fragile X mutation is CGG, the DM mutation is CTG, the Huntington, SBMA, DRPLA and Spino cerebellar ataxias mutant sequence is CAG. The exception to this is Friedrich's ataxia, which was identified as a GAA repeat (Sarmiento et al 1998).

The TNR diseases also differ in terms of the location of the trinucleotide repeat, and related to this, the expansion sizes tolerated and disease mechanism (Figure 1-2). The mutation for Fragile X is located in the 5' untranslated region of the FMR1 gene, expansions can reach into the thousands and the disease mechanism is the loss of FMR1 protein due to transcriptional silencing as a result of hypermethylation of the CGG repeat (Warren & Ashley 1995). HD, SBMA, DRPLA and the SCAs are all the result of a CAG expansion in the coding region of their respective genes (Warren 1996). Interestingly, mutant repeat sizes rarely exceed 100 CAG repeats while the normal range is between 10 and 35 CAG repeats. All of these diseases result from a dominant gain of function of the mutant protein containing a novel stretch of glutamine residues leading to neurodegeneration of some form. In the case of DM, the mutation lies within the 3' UTR of the DMPK gene, expansions can reach into the thousands and the mechanism remains

Figure 1-2. The location of trinucleotide repeat expansions within the gene unit. Of the twelve trinucleotide repeat disorders, eight are found within the coding region of the genes affected. In addition, the eight coding region disorders are CAG polyglutamine tracts and result in translation of a polypeptide toxic to a sub-population of brain cells. In the most severe cases of polyglutamine disorders, CAG tracts expand to a maximum size of only about 150. Fragile X and Fragile XE are the only two expansions to occur in the 5' UTR. These repeats are CGG and GCC respectively and in severe cases can be greater than one thousand repeats in length. Hypermethylation accompanies expansion of the Fragile X repeats resulting in transcriptional inhibition. Friedrich's ataxia is the only trinucleotide repeat disorder to be recessive, the only one to have a GAA repeat and the only trinucleotide mutation present in an intron. In the most severe cases of Friedrich's ataxia, GAA repeats can expand from a normal size of around 40 repeats to greater than 1000. To date, loss of protein due to transcriptional interference is thought to be the cause of this disease. Of the fourteen trinucleotide repeat disorders, Myotonic dystrophy and SCA 8 are the only CTG trinucleotide repeat expansions and with the exception of certain SCA 6 isoforms, the only repeat expansions located in the 3' UTR. A second form of DM, termed DM2 was recently found to be caused by a tetranucleotide repeat within the first intron of the ZNF 9 gene.


5'UTR
Coding Region
3'UTR
DRPLA
Huntington's
SCA 7
SCA 6
SCA 3
SCA 2
SCA 1
SBMA
(CAG)n
Fragile X
Fragile XE
(CGG)n
(GCC)n
DM2
(CCTG)n
SCA 6 (CAG)n
SCA 8
Myotonic Dystrophy
(CTG)n
intron
Coding
3' UTR
Intron/Exon boundary
(GAA)n
Friedreich's Ataxia

unresolved. Finally, Friedrich's ataxia is another example of a non-coding mutation but in this instance is found within the first intron of the Frataxin gene. The disease mechanism is a repeat length dependent reduction in Frataxin protein levels indicating that Friedrich's ataxia is a loss of function disorder.

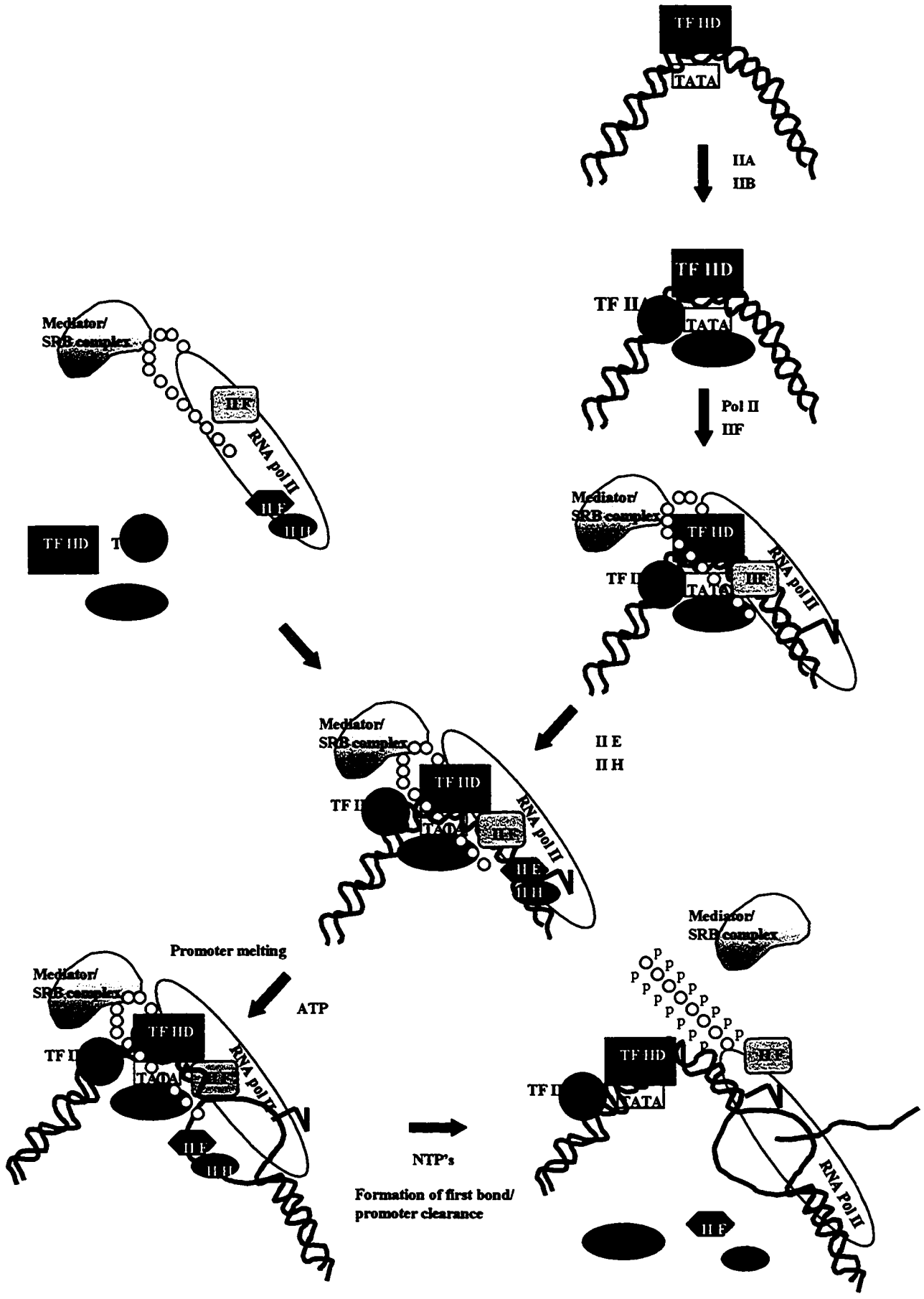
1.7 TRANSCRIPTION

Fundamental to gene expression, transcription is the complex process whereby a DNA dependent RNA polymerase (RNA pol II) binds to a promoter region resulting in production of an RNA copy of that particular gene.

There are three distinct nuclear RNA polymerases each responsible for transcribing a particular class of genes (Roeder 1996). Class I genes encoding large ribosomal RNAs are transcribed by RNA polymerase I, class II genes which include precursors to mRNA and small nuclear RNAs (sn RNAs) U1-U5 are transcribed by RNA polymerase II. In addition, small structural RNAs like 5S RNA, tRNA and U6 RNA, or class III RNAs, are transcribed by RNA polymerase III (Roeder 1996).

Early experiments revealed that RNA polymerase II was not sufficient for specific initiation of transcription in vitro (Roeder et al 1976; Weil et al 1979). Additional factors subsequently identified augment RNA polymerase II (RNA pol II or pol II) in selective initiation of transcription. These factors, termed "General Transcription Factors" or GTFs, include TF IIA, TF IIB, TF IID, TF IIE, TF IIF, and TF IIH (Zawel & Reinberg 1993) (Figure 1-3). The GTFs are a group of about 30 polypeptides that assemble on promoter regions and form the pre-initiation complex (PIC). The function of this complex is to recruit RNA pol II to the precise location for initiation of transcription. Typical class II genes contain a core promoter element, which facilitates accurate

Figure 1-3. Two models of transcription pre-initiation complex (PIC) assembly. The stepwise model (top right) assumes components assemble one at a time beginning with TFIID binding the TATA box followed by TFIIA and TFIIB binding. Subsequently, RNA pol II, TFIIF and the mediator/SRB complex are incorporated. The holoenzyme model of PIC assembly (top left) predicts that RNA pol II bound to TFIIE, TFIIF and TFIIH together with the mediator/SRB complex exists pre-assembled in solution. This holoenzyme combines with TFIIA, TFIID and TFIIB to complete the PIC. In both cases, PIC formation is followed by promoter melting, formation of the first diester bond, promoter clearance and transcript elongation.


Holoenzyme Model
Stepwise Model of Assembly
TF IID
TATA
IIA
IIB
TF IIF
Pol II
IIF
Mediator/
SRB complex
RNA pol II
IIE
IIH
TF IIB
TAFs
Promoter melting
ATP
NTP's
Formation of first bond/
promoter clearance

initiation by RNA pol II, and distal regulatory elements which allow integration of activator or repressor functions with the core promoter (Roeder 1996). The TATA box (ATATAA) and the initiator sequence (Py Py A + 1 N T/A Py Py) are the two known core promoter elements. Some promoters contain both of these elements, some contain one while others contain none (Roeder 1996).

Assembly of the GTFs was initially thought to occur in a stepwise manner whereby one factor binds to the complex and serves to attract and facilitate binding of the next factor. However, recent evidence suggests that RNA pol II exists already bound to several GTFs in a "holoenzyme" form (Orphanides et al 1996).

1.7.1 STEPWISE MODEL OF PIC ASSEMBLY:

The stepwise model begins with the binding of TF IID to the promoter (Figure 1-3). TF IID is the only GTF with site specific DNA binding activity that occurs through TATA binding protein (TBP) (Orphanides et al 1996). TF IIA binding follows, which mainly functions to facilitate TF IIB association with the TF IID – DNA complex, but can also act as a transcription anti-repressor (Ma et al 1996; Merino et al 1993);(Auble et al 1994), and play a role in transcriptional activation (Ma et al 1996). The role of TF IIB is to attract RNA Pol II to the complex and, in association with Pol II, specify the exact start site of transcription (Orphanides et al 1996). Interestingly, crystallographic data shows that each successive GTF associating with the TF IID-(TBP)/TATA complex occupies minimal interaction area on the surface of TBP while adding considerably to the total protein interaction surface available (Orphanides et al 1996). Next, TF IIF and RNA polymerase II associate with the TATA/TF IID/TF IIA/TF IIB (DB) complex. RNA pol II requires TF IIF to associate with the complex. This heterotetrameric factor can also

increase the rate of transcription elongation (Flores et al 1989; Price et al 1989); and ensures accurate initiation by circumventing interactions of RNA pol II with non-promoter sites (Orphanides et al 1996). Although TF IIF brings pol II into the PIC, transcription still cannot proceed without additional GTFs TF IIE and TF IIH, which are thought to initiate elongation (Flores et al 1990; Orphanides et al 1996). TF IIE recruitment to the PIC precedes TF IIH incorporation and it has been shown that TF IIE can associate with pol II in solution suggesting it may join the PIC concomitantly with TF IIF and pol II. TF IIE, once part of the PIC, attracts TF IIH (Flores et al 1990) which has ATP dependent helicase and kinase activity thought to be crucial for rendering pol II competent for transcription (Drapkin & Reinberg 1994). TF IIH is thought to mediate pol II transcription by separating the DNA strands (promoter melting) via its helicase activity, a process that is required for elongation of the transcript (Orphanides et al 1996).

Once the PIC is complete, the DNA is separated allowing pol II access to the nucleotides of the template. Pol II catalyzes the first diester bond in the RNA molecule, dissociates from the PIC (promoter clearance) and moves down the template (elongation) thus creating the RNA copy. The pol II carboxy terminal domain (CTD) is transformed from a hypo to a hyperphosphorylated state between the PIC and elongation phase. The role of phosphorylation of the CTD is emerging and seems to be dispensable if the promoter contains a TATA box.

1.7.2 HOLOENZYME MODEL OF PIC ASSEMBLY:

Biochemical and genetic experiments led to belief in a pre-existing complex of GTFs and pol II, or "pol II holoenzyme", poised for PIC formation given the appropriate cues. SRB genetic screen for suppressors of a mutant yeast proteins, identified through a

strain resulting from partial deletion of the CTD of pol II, are part of a large complex which includes GTFs and pol II (Koleske & Young 1994). A similar complex termed "mediator" was discovered biochemically following a search for proteins allowing a purified yeast transcription system to respond to acidic activators (Kim et al 1994). It became clear that the SRB and mediator complexes shared many of the same constituents and were linked to the pol II holoenzyme through the CTD of pol II (Orphanides et al 1996) (Figure 1-3). This complex couples transcriptional activators and repressors to the PIC and thus is the link between modifiers of transcription and the transcription machinery (Orphanides et al 1996). To achieve promoter clearance, pol II must lose contact with the SRB/mediator complex as well as the other GTFs. Phosphorlyation of the CTD by protein kinases within the SRB/mediator complex is now believed to inhibit the association of pol II with this complex so that pol II can clear the promoter and continue with elongation of the transcript (Orphanides et al 1996).

1.7.3 TATA-LESS INITIATION COMPLEX

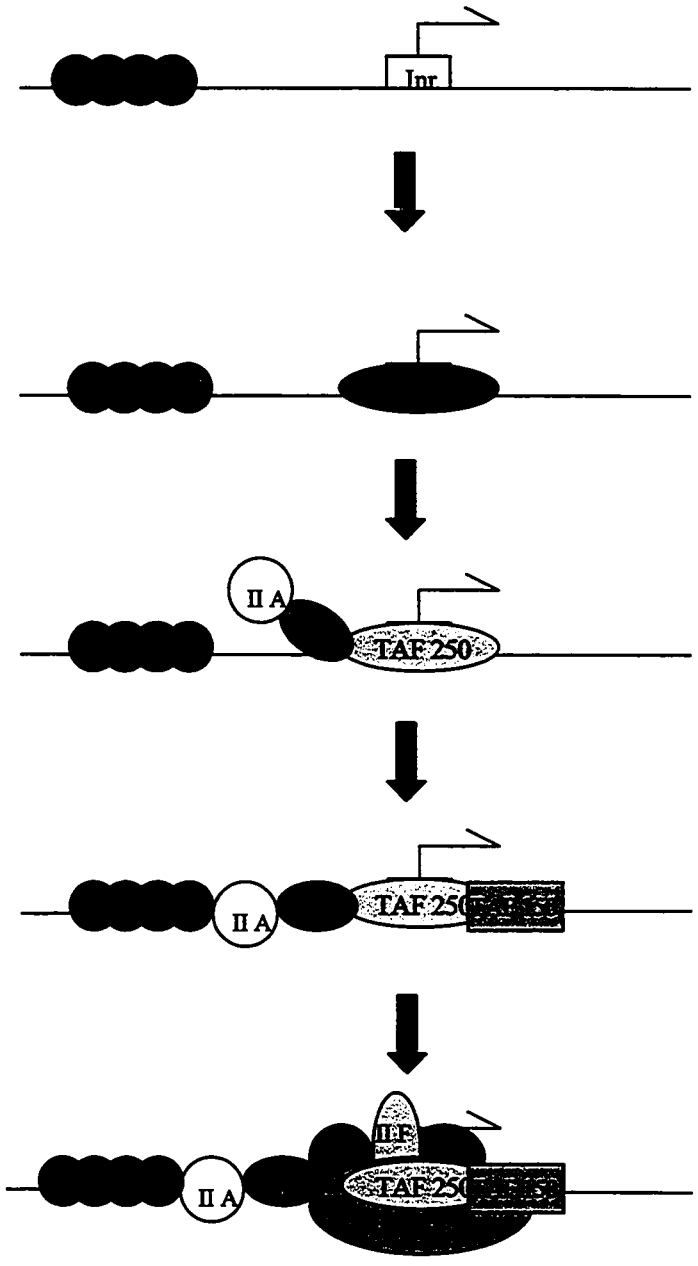
The TATA box (TATAA) was the first consensus DNA element within promoter sequences found to interact with the transcription machinery and thus has become the best known. The first genes to be cloned were abundantly expressed and all contained a TATA box at position –20 to –30 relative to the transcription initiation site. Now, however, a growing number of promoters do not contain TATA boxes and it is apparent that only abundantly expressed genes have this sequence (Smale 1997). Some promoters have a sequence known as "initiator" (Inr) positioned at the transcription start site. This element, (Py Py A +1 N T/A Py Py) functions in an analogous fashion to the TATA box by attracting RNA polymerase to the start site of transcription so that accurate initiation

takes place (Smale 1997). Typically, TATA containing promoters have one or a few transcription start sites in close proximity whereas TATA-less promoters have from several to more than a dozen transcription start sites spanning several hundred base pairs (Smale 1997). Interestingly, in promoters with a TATA box and Inr element in the presence of an upstream activator, a mutated Inr element has no effect on promoter strength. In contrast, in a similar promoter lacking a TATA-box, this mutation abolishes promoter activity indicating that at least one of these elements must be present for transcription to take place (Javahery et al 1994).

Several proteins have been found to bind the initiator element including the GTFs TF IID, TF II I, YY1 and RNA polymerase II (Smale 1997). TF IID has been found to contact the TATA box, the Inr and sequences downstream of the transcription start site (Smale 1997). The TAF responsible for the TF IID activity is TAF 250 which has been found to crosslink to the AdML Inr sequence (Oelgeschlager et al 1996) (figure 1-4). Gel shift studies have revealed that TF IID interaction with the TATA box is stronger than its association with the Inr element (Kaufmann & Smale 1994). However, in the context of artificial promoters, Inr activity is as strong as the TATA box (Emami et al 1995; Smale et al 1990). This suggests that other factors may be required to stabilize the TF IID association with the Inr element, or, alternatively, the TF IID complex that binds the Inr sequence is different from the one that binds the TATA box (Smale 1997).

A second factor found to bind the Inr element is TF II I (Roy et al 1991). This factor contacts the Inr sequence and also sequences downstream of the transcription start site at +45 and +55 of the AdML promoter (Roy et al 1991). TF II I is homologous to

Figure 1-4. Assembly of the transcription pre-initiation complex at the initiator. Initiator elements are often employed in the absence of a TATA box. These elements are located at the transcription start site. Transcription activators (red circles) bind upstream of the start site. TAF 250 then binds the initiator element followed by TBP and TFIIA. TBP and TFIIA only make contacts with the DNA upstream of the initiator after TAF 150 binds to sequence downstream of the initiator. RNA pol II, TFIIB, IIE and IIF then associate with the other factors to complete formation of the PIC on the initiator element.


Inr
IIA
TAF 250
IIA
IIA

helix loop helix transcription factors and may sequester TF II I activity by forming heterodimers (Roy et al 1993). In addition, *in vitro* assays demonstrate that TF II I and TBP are sufficient to activate transcription without requirement for any other TAFs (Roy et al 1991). However, it is apparent that this combination of factors is not sufficient for initiation from all Inr promoters and that TF II I may only interact with a subset of Inr elements (Smale 1997). RNA polymerase II has been found to directly interact with the Inr element and to initiate a low level of transcription (Carcamo et al 1991). However, the relevance of this observation is unclear as this interaction might be expected given the requirement of pol II to contact this sequence in the course of transcription initiation and elongation (Smale 1997).

Lastly, another transcription factor found to interact with the Inr element is YY1 (Seto et al 1991; Shi et al 1991; Usheva & Shenk 1994), which differs from the other factors in that it only recognizes a specific subset of Inr sequences and also sequences which diverge from the consensus (Javahery et al 1994). YY1 is a zinc finger transcription factor related to the drosophila gap protein Kruppel (Shi et al 1991) and recognizes the core sequence CCAT, present in only some Inr sequences (Javahery et al 1994). The role of YY1 has been questioned since it has been shown that if its binding site is mutated and binding abolished, Inr function and transcription proceeds normally (Javahery et al 1994; Lo & Smale 1996).

Formation of the PIC on TATA-less promoters likely begins with recognition of the Inr by the TAF 250 component of TF IID (figure 1-4). Included in the TFII D complex is TF II A and TBP, which may or may not make contact with sequences upstream depending on whether or not a TATA box is present or if the sequence is rich with A/T

bases. Next, TAF 150, which has been found to be crucial for Inr dependent transcription (Kaufmann & Smale 1994; Verrijzer et al 1994) likely contacts DNA downstream of the transcription start site. Subsequently, other GTFs assemble along with pol II much like TATA dependent initiation to complete the PIC.

The TATA box and Inr perform very similar functions and so it is curious that both exist. It is thought that specific interactions with transcriptional regulators might explain the existence of two core transcription initiation elements (Aso et al 1994; Emami et al 1995; Mack et al 1993; Merino et al 1993).

1.7.4 MUSCLE SPECIFIC TRANSCRIPTION

Transcription in muscle is mediated mainly by the muscle specific regulatory factor (MRF) family of transcription factors (Olson & Klein 1994). These helix-loop-helix (HLH) factors include MyoD, Myf 5, myogenin and MRF4, all of which heterodimerize with members of the E family HLH proteins and bind the cognate sequence CANNTG (Olson & Klein 1994). In addition to the MRF family, the MEF2 family of MADS box transcription factors play a key role in muscle specific transcription. These factors, which bind A/T rich sequences, are capable of interacting with MyoD at a single DNA binding site within muscle specific promoter and enhancer elements to synergistically activate myogenesis (Molkentin et al 1995). Together, these transcription factors activate transcription of a myriad of muscle specific genes during the process of myogenesis (Olson & Klein 1994).

Interestingly, the MRF proteins are expressed only in skeletal muscle whereas MEF2 family members are expressed in skeletal muscle, heart, smooth muscle and brain (Molkentin & Olson 1996). Linking muscle specific transcription factors to the basal

transcription machinery occurs through interactions of MyoD and myogenin with the CREB binding protein family member p300, which is known to interact with TBP (Puri et al 1997; Yuan et al 1996).

The muscle creatine kinase (MCK) gene promoter/enhancer is the most extensively studied muscle specific regulatory unit. The MCK regulatory sequences consist of a ~350 bp proximal promoter and a 206 bp enhancer located at –1050 to –1256 relative to the transcription start site (Shield et al 1996). One E-box is located in the proximal promoter while two MEF2 sites, another two E-boxes, a TREX1 binding site, an AP2 element and a CARG box are found within the upstream enhancer sequence (Firulli & Olson 1997). Deletion of all E-boxes in the MCK upstream regulatory units results in loss of reporter expression in cultured myoblasts (Shield et al 1996). However, in transgenic animals, loss of reporter expression was observed in cardiac muscle and tongue while expression was compromised in slow type I muscle (soleus) but was unaffected in fast muscles (EDL, abdominals, quadriceps) (Shield et al 1996). This indicates that binding sites for various transcription factors are utilized differently depending on muscle type and that reporter analysis in tissue culture does not always translate to transgenic mice (Shield et al 1996) (Firulli & Olson 1997).

1.8 APOPTOSIS

Apoptosis is a genetically controlled form of cell death initiated by external or internal signals designed to discard cells that have outlived their usefulness or that may be detrimental to the organism (Vaux 1993). Clearly apoptosis is a major device employed by cells and tissues to maintain homeostasis as evidenced by the multitude of cellular components capable of integrating with the core machinery (Evan & Littlewood

1998). This process differs markedly from necrosis, which is the other known form of cell death (Vaux 1993). Typically, apoptosis is a physiological form of cell death (Kerr et al 1972; Wyllie et al 1980) occurring in otherwise healthy tissues resulting in the loss of individual cells (Vaux 1993). In contrast, necrosis is the result of severe trauma (Barr & Tomei 1994) and involves loss of whole areas of tissue (Vaux 1993). In addition, necrosis attracts professional phagocytic cells, whereas apoptotic cells are ingested by neighbouring cells *in situ* (Vaux 1993). During necrosis, the cell membrane ruptures, releasing the cell contents and evoking an immune response whereas the cell membrane remains intact as the dismantling and packaging of cellular contents proceeds in apoptosis, sequestering the cellular debris in small membrane packets called "blebs" thereby precluding an immune response (Barr & Tomei 1994). Initially, apoptosis is marked by shrinkage of the nucleus, followed later by loss of organelles and membrane integrity (Vaux 1993). The cellular DNA is rapidly degraded into oligonucleosomal sized fragments which can be visualized by gel electrophoresis (Barr & Tomei 1994).

1.8.1 APOPTOSIS DURING DEVELOPMENT

Apoptosis occurs during metamorphosis in insects and amphibians when larval tissues are lost and adult forms emerge (Schwartz et al 1990; Truman et al 1992; Weeks et al 1992). Also, during early development, the blastocyst implants into the uterine lining and cells of the endometrium die by apoptosis (Parr et al 1987). In addition, cells die by apoptosis during gut formation (Williams & Bell 1991), remodeling of the limb buds (Antalikova et al 1989) and during neural development, where a significant proportion of neurons fail to make productive connections and as a result die by apoptosis (Kimura & Truman 1990). Furthermore, during selection of T and B cells in the immune

system, clones producing T-cell receptors or antibodies that are self-reactive are eliminated by apoptosis (Ekert & Vaux 1997).

1.8.2 APOPTOSIS AND DISEASE

The importance of cell death to the organism is illustrated by many examples of human disease which result from inappropriate regulation of this process. In some cases, inappropriate apoptosis results in pathology. For example, in chronic neurodegenerative disorders like Alzheimer's disease, prion infection, ataxia telangeictasia and more acute conditions like stroke, it has been demonstrated that apoptosis is at least part of the pathogenic mechanism (Barr & Tomei 1994). In addition, AIDS patients have reduced numbers of CD4+ T-cells because of a viral induced increase in sensitivity to apoptosis (Gougeon & Montagnier 1993).

Conversely, there are diseases of deficient apoptosis. For example, human malignancies may arise from a cell failing, due to genetic lesion, to execute its cell death program often triggered in response to DNA damage or an elevated growth rate (Carson & Ribeiro 1993; Martin et al 1994).

1.8.3 APOPTOSIS EFFECTORS

Several stimuli result in activation of apoptotic pathways. Some stimuli engage the apoptotic machinery on the cell surface (eg. TNF, FasL, serum withdrawal) whereas other stimuli can enter the cell directly (eg. UV radiation, radiation, hormones, chemotherapeutic agents). Regardless of how it is triggered, once committed to apoptosis, every facet of the molecular machinery is activated to rapidly dispose of the cell.

1.8.4 THE DEATH RECEPTORS

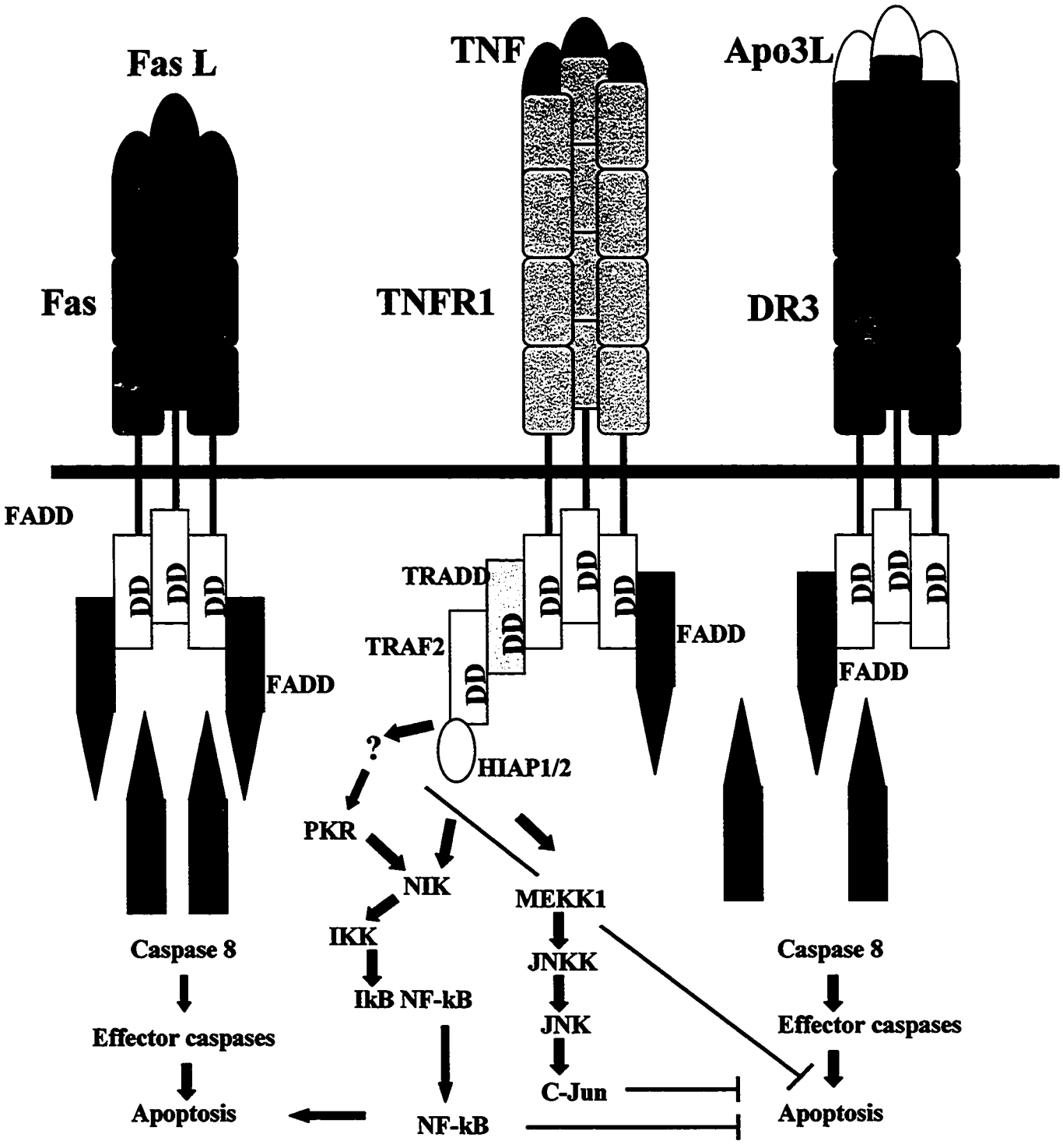
The death receptor complexes include the TNF receptor (TNFR1), Fas (CD95, Apo1) and DR3 (Apo3, Wsl1) among others (Ashkenazi & Dixit 1998) (figure 1-5). Each of these receptors, which have an extracellular ligand binding domain and an intracellular "death domain", homotrimerize when bound by their respective ligands (TNF, FasL and Apo3L respectively). This results in assembly of the DISC (death inducing signaling complex), a complex of signal transducers on the cytoplasmic tail of the receptor (Ashkenazi & Dixit 1998). Fas activation by binding of Fas L results in activation of apoptosis and is a means for clearing activated T-cells following an immune response and killing of target cells such as cancerous cells or virally infected cells (Nagata 1997). TNFR1 activation by TNF leads to apoptosis occasionally but mostly leads to activation of proinflammatory and immunomodulatory genes through activation of transcription factors AP-1 and NF-kB. DR3 activation leads to activities similar to those downstream of TNFR1 activation but mostly in separate biological compartments than TNF (Ashkenazi & Dixit 1998).

1.8.5 DEATH SIGNALLING FROM FAS

Upon ligation of Fas by FasL, FADD (Fas associated death domain) associates with the death domain of Fas (Chinnaiyan et al 1995). Within FADD is a motif known as the death effector domain (DED) which recruits the unprocessed form of a cysteine protease termed pro-caspase-8 through a pair of DEDs in its amino terminus (Cryns & Yuan 1998).

Clustering of pro-caspase 8 molecules into close proximity at the DISC is thought to stimulate the auto-catalytic activity of this protease (Cryns & Yuan 1998). The cleavage events within caspase 8 liberate a long and short portion of the enzyme that

Figure 1-5. Molecular control of apoptosis. The homotrimeric receptors Fas, TNFR1 and DR3 are bound by their respective ligands, FasL, TNF and Apo3L. This results in the cytoplasmic death domains (DD) to come into close proximity providing an interaction surface to cytoplasmic death domain proteins . The recruitment of caspases to these complexes via their death domains results in their oligomerization, proteolytic activation and induction of the apoptotic cascade. Kinase cascades like the MAP kinase pathway can feed into apoptotic signaling events and influence the fate of the cell. In addition, activation of NF-κB can result in cell death or survival, depending on the context and PKR has been shown to participate in activation of NF-κB resulting in cell death.


Fas L
TNF
Apo3L
Fas
TNFR1
DR3
FADD
TRADD
TRAF2
FADD
FADD
FADD
DD
?
HIAP1/2
PKR
NIK
MEKK1
IKK
JNKK
Caspase 8
Caspase 8
IkB NF-kB
JNK
Effector caspases
Effector caspases
C-Jun
Apoptosis
Apoptosis
NF-kB

associate to form the protease, from the DED domain allowing the active molecule access to its downstream targets, specifically the effector pro-caspase 3 (Kumar & Colussi 1999). Processed caspase 8 then dimerizes, cleaves pro-caspase 3, resulting in its activation and ultimately cleavage of a multitude of cellular proteins leading to the demise of the cell (Cryns & Yuan 1998).

1.8.6 SIGNALING FROM TNFR1

Signaling events from TNFR1 are similar to Fas signaling. The main difference is that TNFR1 can signal downstream to activate transcription of genes involved in inflammation and immunity in addition to connecting the receptor to the apoptosis machinery. Fas however, functions exclusively to kill the cell (Ashkenazi & Dixit 1998). Upon receptor ligation, TRADDs (TNF receptor associated death domain) associate via their death domains with the cytoplasmic tail of TNFR1 (figure 1-5). Another group of proteins also containing death domains known as TRAFs (TNF receptor associated factors) interact with TRADDs through death domain contacts and serve as adaptors to signal transduction cascades leading to NF-kB and AP-1 activation (Ashkenazi & Dixit 1998). FADD can also interact with the TRADDs leading to caspase 8 activation and cell death as described above.

1.8.7 DEATH SIGNALS FROM THE MITOCHONDRIA

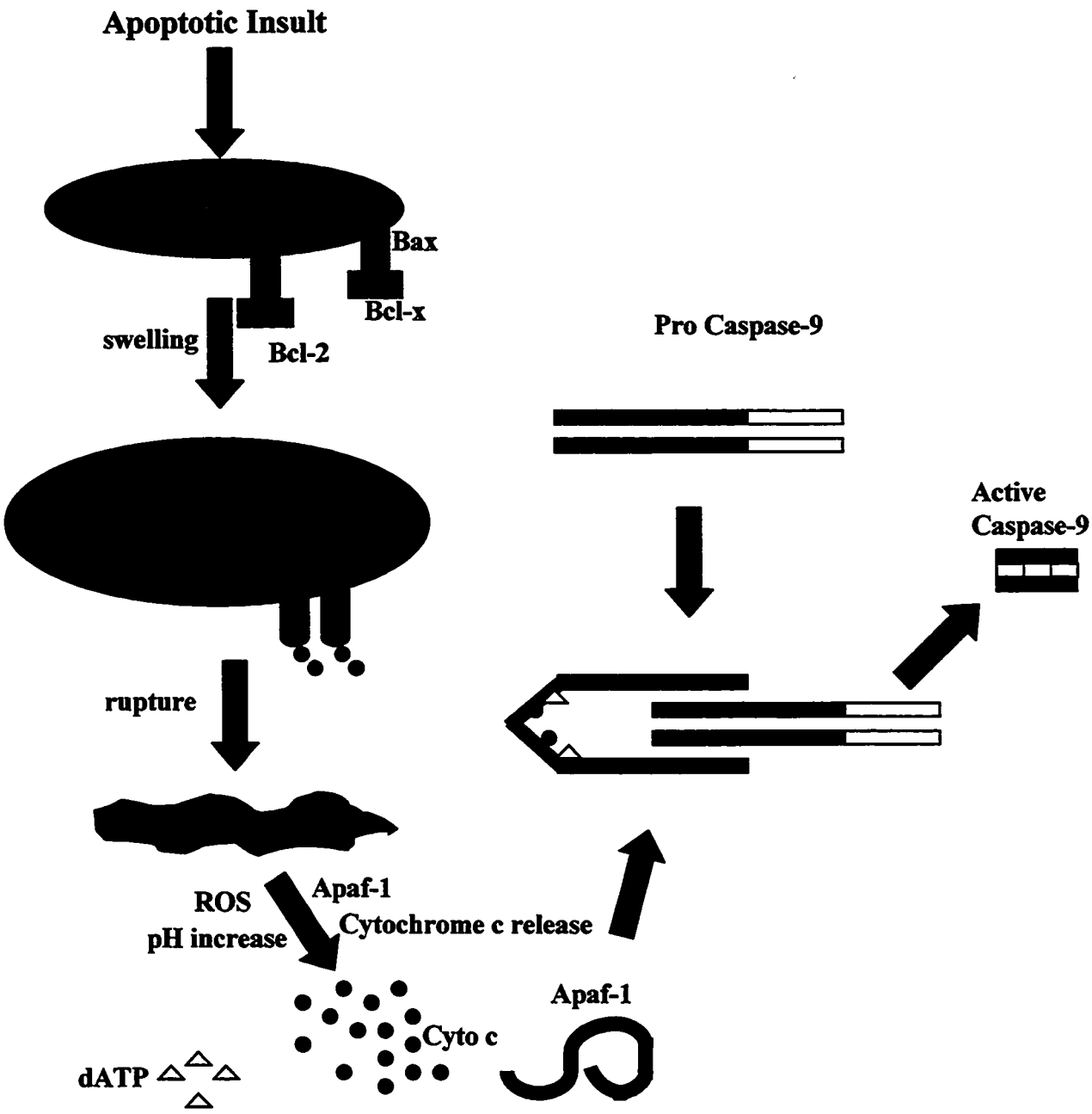
The role of mitochondria in apoptosis is only partially understood. In response to various apoptotic stimuli like growth factor withdrawal, UV radiation, glucocorticoids and various chemotherapeutic agents and protein kinase inhibitors like staurosporine, mitochondria release cyotochrome c (cyto c) into the cytoplasm (Liu et al 1996). This

can be blocked by anti-apoptotic proteins Bcl-2 and Bcl-x and enhanced by the pro-apoptotic protein Bax (Green & Reed 1998) presumably by Bax forming pores in the mitochondria allowing cyto c release and Bcl-2 and Bcl-x preventing this (Cryns & Yuan 1998) (figure 1-6). In some cell types, apoptotic insults result in swelling of the mitochondrial outer membrane which eventually burst releasing a large quantity of cyto c into the cytoplasm (Cryns & Yuan 1998). It is possible that cyto c release occurs via depolarization of the mitochondrial PT pore, however, cyto c release during apoptosis has been observed in the absence of PT pore depolarization and cyto c release sometimes precedes changes in mitochondrial membrane potential (Yang et al 1997).

The importance of cyto c release to the cytoplasm rests in its role in catalyzing the formation of the apoptosome, a pre-apoptotic complex composed of Apaf-1, cyto c, dATP and pro-caspase 9 (Liu et al 1996). The end result is activation of pro-caspase 9 which in turn activates pro-caspase 3 to initiate cleavage events necessary for apoptosis (Liu et al 1996) (figure 1-6). Apaf-1 is a caspase like protein that contains an amino terminal caspase recruitment domain (CARD) that functions as an activator of pro-caspase 9 (Li et al 1997). However, in non-apoptotic conditions, this protein is in a conformation that masks the CARD domain preventing its association with caspase 9 (Li et al 1997). Upon release of cyto c in the presence of dATP, Apaf-1 undergoes a conformation change and associates with and activates caspase 9 leading to apoptosis (Li et al 1997).

In addition to releasing cyto c, it has been reported that in some cell types a subset of pro-caspase 3 is found in the mitochondria which is released upon apoptotic insult (Mancini et al 1998). Further, another pro-apoptotic protein, apoptosis-inducing factor (AIF) is

Figure 1-6. Apoptotic signals initiated at the cell surface are amplified through involvement of the mitochondria. Pro-apoptotic members of the Bcl-2 family such as Bax, form pores in the mitochondrial membrane in response to signals originating from receptors at the cell surface. Anti-apoptotic Bcl-2 family members such as Bcl-2 itself or Bcl-x can heterdimerize with pro-apoptotic family members and essentially block the pores. If however, the apoptotic signal is strong enough, the pro-apoptotic proteins will overcome the effects of the anti-apoptotic proteins and a variety of factors will escape from the mitochondria. Ultimately, the mitochondria may rupture in response to the converging apoptotic signals. Among the released factors are Apaf-1, cytochrome c, dATP and reactive oxygen species (ROS). This outflux is accompanied by an increase in cellular pH. Apaf-1, in collaboration with dATP, interacts with pro-caspase 9 and facilitates auto-catalytic processing of this pro-enzyme to its active form. Activated caspase 9 is capable of activating pro-caspase 3, a key apoptotic effector protease resulting in amplification of the apoptotic process.


Apoptotic Insult
Bax
Bcl-x
swelling
Bcl-2
Pro Caspase-9
Active
Caspase-9
rupture
ROS
pH increase
Apaf-1
Cytochrome c release
Apaf-1
Cyto c
dATP

conformation change and associates with and activates caspase 9 leading to apoptosis (Li et al 1997).

In addition to releasing cyto c, it has been reported that in some cell types a subset of pro-caspase 3 is found in the mitochondria which is released upon apoptotic insult (Mancini et al 1998). Further, another pro-apoptotic protein, apoptosis-inducing factor (AIF) is released from the mitochondria and can process pro-caspase 3 in vitro (Susin et al 1997; Susin et al 1996).

Clearly the mitochondria plays a key role in cell death. Cleverly, the cell can efficiently integrate the apoptotic machinery as evidenced by recruitment of mitochondrial signals during receptor mediated apoptosis through caspase 8 action, which serves to amplify the apoptotic response and speed the disposal of the offending cell (Kuwana et al 1998).

1.9 DOUBLE STRANDED RNA ACTIVATED PROTEIN KINASE (PKR)

PKR is an interferon inducible protein kinase of 68 kD in humans and 65 kD in mouse and comprises one arm of the antiviral effects of interferon (Williams 1999). It exists as a dimer upon activation and it's most noted function is phosphorylation of the translation factor eukaryotic initiation factor 2 alpha (eIF2α) (de Haro et al 1996) resulting in translation inhibition of both viral and cellular messages (Williams 1999). In addition to its antiviral role, PKR has also been shown to participate in stress signalling pathways, regulate cell growth (Clemens & Elia 1997; Tan & Katze 1999), differentiation (Kronfeld-Kinar et al 1999; Salzberg et al 2000) and cell death (Gil & Esteban 2000).

PKR has two double stranded RNA binding domains (dsRBD) at the amino terminus and a serine/threonine kinase domain at the carboxy terminus (Williams 1999).

In the presence of ds RNA, PKR binds to the RNA via its dsRBDs, dimerizes, changes conformation and autophosphorylates resulting in activation (Carpick et al 1997; Manche et al 1992). This occurs in response to viral infection where often an abundance of double stranded (ds) RNA resulting from bi-directional viral transcription accumulates within the cell. The profile of PKR activation in response to dsRNA is bell curve shaped with PKR kinase activity increasing with ds RNA concentration to a point after which ds RNA is inhibitory and PKR activity is reduced (Williams 1999). Adenoviridae employ the strategy of dsRNA overproduction through the VAI gene to ablate PKR activity and escape its antiviral effects during infection (Kaufman 1999b). Other strategies utilized by viruses to dampen PKR activity include producing proteins that antagonize ds RNA, prevent PKR dimerization, cleave PKR, interfere with PKR substrate binding, activate phosphatases that dephosphorylate eIF-2α and PKR and finally inhibit PKR expression (Kaufman 1999a). Some viruses utilize several of these strategies indicating the importance and potency of PKR antiviral activity (Kaufman 1999a).

Antiviral mechanisms of PKR include shutdown of viral and host protein synthesis via phosphorylation of eIF-2α at serine 51. Furthermore, PKR action results in induction of IFN and leads to a variety of responses including activation of RNAse L, which cleaves RNA non-specifically and promotes apoptosis. PKR can also directly induce apoptosis (Stark et al 1998). Interestingly, apoptosis can result simply by inhibiting protein synthesis via eIF-2α phosphorylation presumably by reducing levels of anti-apoptotic polypeptides (Kaufman 1999a) or allowing translation of specific pro-apoptotic messages such as Bax analogous to the GCN4 message in yeast (Gil & Esteban 2000). In addition, PKR can induce apoptosis by activating NF-κB (Der et al 1997)

through activation of Iκ-B kinase complex (Bonnet et al 2000; Zamanian-Daryoush et al 2000) components leading to increased Fas (Der et al 1997) and Bax expression (Balachandran et al 1998). However, signaling through the Fas associated death domain protein (FADD) leading to activation of caspase 8 was found to occur but was not dependent on Fas or FasL (Balachandran et al 1998) (Gil & Esteban 2000). Other potential apoptotic genes that respond to NF-κB include interferon responsive factor-1 (IRF-1) (Kumar et al 1997), caspase-1 (Casano et al 1994) and p53 (Wu & Lozano 1994). A resistance to TNFα induced apoptosis is seen in PKR null fibroblasts indicating the importance of PKR in apoptosis (Der et al 1997). Furthermore, apoptosis levels are affected when expression of PKR inhibitory proteins are altered (Datta & Datta 1999; Tang et al 1999).

In addition to PKR action through NF-κB, PKR interacts with and phosphorylates p53 on serine 392 (Cuddihy et al 1999b) and serine 18 leading to increased p53 transcriptional activity (Cuddihy et al 1999a). PKR has also been shown to upregulate p53 at the transcriptional level through an NF-κB mechanism and it is suggested that p53 functions downstream of PKR in TNFα induced PKR mediated apoptosis (Yeung & Lau 1998). Interestingly, p53 activates Bax expression at the transcriptional level indicating that p53 may play a role in PKR mediated apoptosis by activating the expression of this gene (Thornborrow & Manfredi 1999).

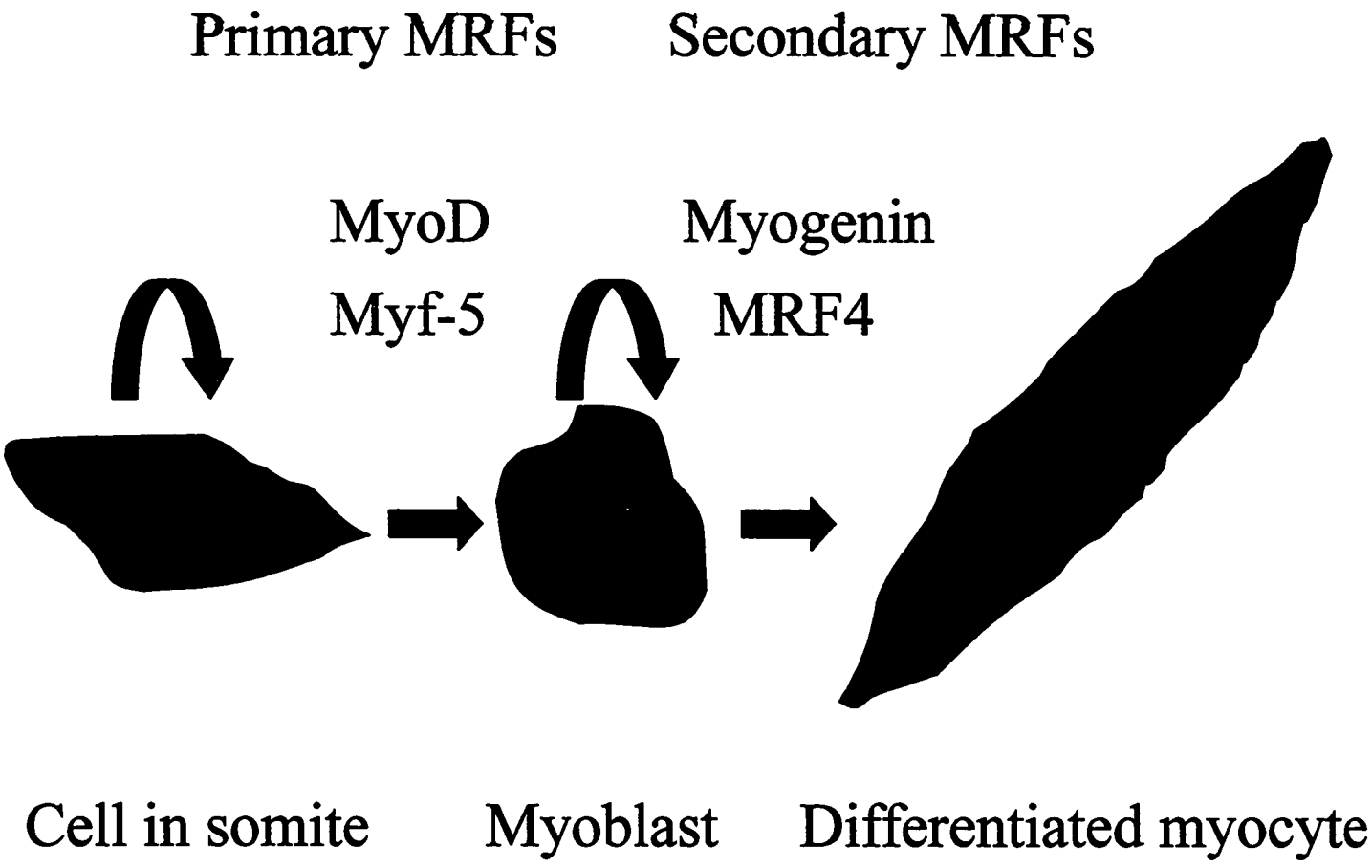
1.10 MYOGENESIS

The process of myogenesis entails the formation of specialized multi-celled syncytia, or myotubes, which ultimately combine to form skeletal muscle, from individual precursor cells during embryonic development or recovery from injury (figure 1-7). Most of what is known about myogenesis has been learned from studying cell culture models capable of differentiating from individual myoblasts to multinucleated myotubes following growth factor withdrawal (Silberstein et al 1986). The cell cycle arrest triggered by removal of growth factors results in transcriptional upregulation of genes involved in muscle formation, particularly those coding for proteins involved in muscle contraction (Olson & Klein 1994). Work from the last decade has revealed some of the major molecular events governing this process.

1.11 MYOD AND MOLECULAR CONTROL OF MYOGENESIS

A key protein involved in triggering myogenesis is myogenic determination factor 1 or MyoD. This protein was identified as a myoblast specific factor by subtraction of mRNA from pre-myogenic fibroblasts from myogenic myoblasts (Davis et al 1987). MyoD was the first of a family of four myogenic regulatory factors (MRFs) to be cloned. MRFs are helix-loop-helix (HLH) transcription factors belonging to the acheate-scute c-myc family (Olson & Klein 1994; Rudnicki & Jaenisch 1995; Weintraub et al 1991), their defining feature being the ability to convert certain non-muscle cell lines to the myogenic lineage when ectopically expressed (Olson & Klein 1994). The three other MRFs in addition to MyoD include myogenin (Myf-1) (Edmondson & Olson 1989), Myf-5 (Braun et al 1989) and MRF4 (Myf-6/Herculin) (Braun et al 1990; Miner & Wold 1990; Rhodes & Konieczny 1989). These factors all contain a conserved

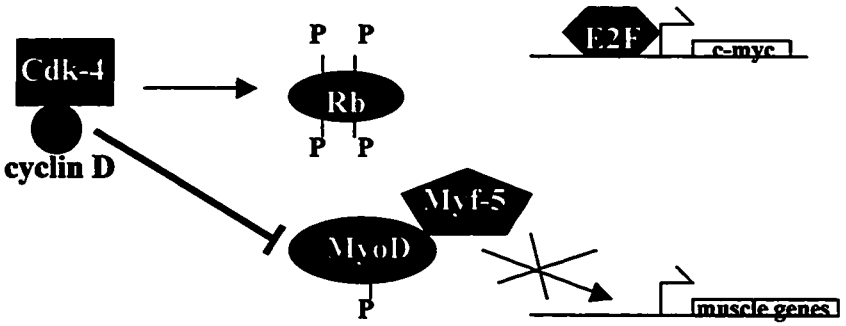
Figure 1-7. Muscle regulatory factors (MRFs) and their role in myogenesis. The process of myogenesis in vivo occurs when cells in the somites of a developing animal become committed to the myogenic lineage. This is accomplished by induction of the primary myogenic regulators MyoD and Myf-5. The somitic myoblast populations expand and individual myoblasts migrate to the limbs where they eventually will become muscle. Myoblasts in the somites and limbs differentiate further into myotubes. For this process, the secondary myogenic regulators, myogenin MRF4 are required.


Primary MRFs
Secondary MRFs
MyoD
Myf-5
Myogenin
MRF4
Cell in somite
Myoblast
Differentiated myocyte

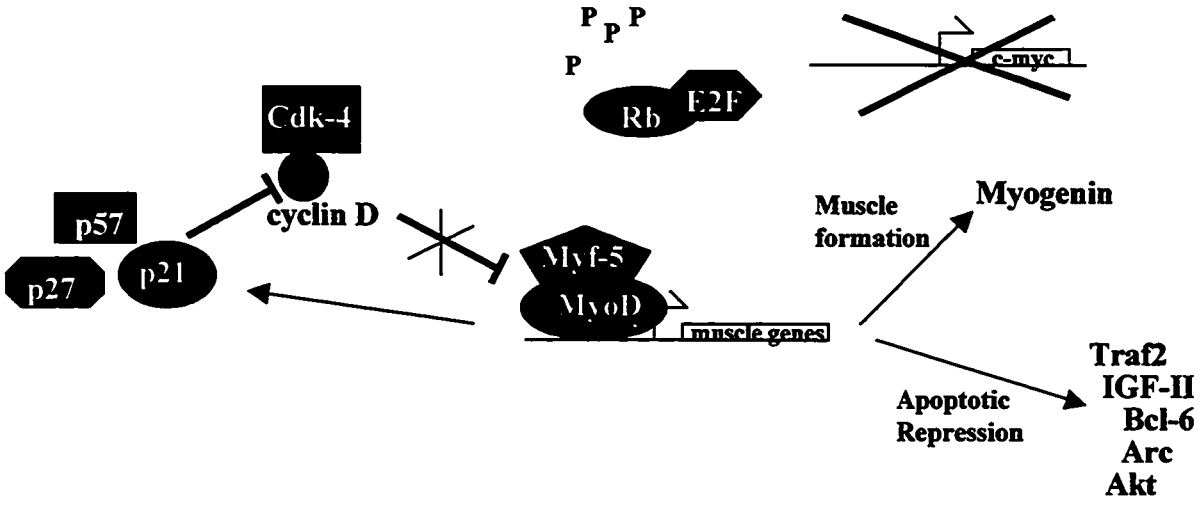
basic domain essential for binding DNA and a HLH domain which is reqired for dimerization (Olson & Klein 1994) (Rudnicki & Jaenisch 1995; Weintraub et al 1991). MRFs preferentially heterodimerize with E proteins. E-proteins include the products of the E2-2 gene (ITF2) and the E2-5 gene (ITF1, E12 and E47) which interact with the MRFs through their HLH motifs. The resulting dimers bind to the hexanucleotide sequence CANNTG which is commonly found in promoter and enhancer elements of muscle specific genes (Olson & Klein 1994) (Rudnicki & Jaenisch 1995; Weintraub et al 1991). In cycling myoblasts however, the E proteins dimerize with a protein that lacks a DNA binding domain known as Id, that is abundant in myoblasts but not expressed in differentiated myotubes (Benezra et al 1990b); (Benezra et al 1990a). In a similar manner, murine twist (mTwist) sequesters E-proteins from the MRFs (Spicer et al 1996) and another HLH protein called Mist 1 which lacks a transcriptional activation domain binds to and inhibits MyoD activity (Lemercier et al 1998). The association between Id and mTwist with the E-proteins prevents MyoD from forming functional heterodimers with E proteins and is one of several mechanisms used to sequester MyoD activity within cycling myoblasts in order to prevent premature differentiation. Another mechanism for preventing MyoD function in cycling myoblasts is selective association with the retinoblastoma gene product (Rb) (Lassar et al 1994; Olson 1992). This protein is pivotal in controlling the cell cycle by determining the availability of the transcription factor E2F, which activates expression of early growth genes thereby promoting cell cycle progression. In proliferating cells, E2F is free of Rb and fully functional because Rb is maintained in a hyper-phosphorylated state by cyclin dependent kinases (Sabourin & Rudnicki 2000) (figure 1-8). However, when myoblasts are induced to differentiate,

Figure 1-8. Molecular control of homeostasis in myoblasts and muscle. The proliferating myoblast state is maintained by phosphorylation of Rb, leaving E2F free to transactivate genes required for cell division. MyoD is sequestered and prevented from activating myogenesis by phosphorylation in the DNA binding domain. During myoblast differentiation, Rb becomes hypophosphorylated as a result of inhibition of cyclin dependent kinases by p21 and p57. Repression of Myo D is relieved and its binding activity increases leading to activation of muscle specific genes and the myogenic program. If myoblasts fail to exit the cell cycle during differentiation, they will die by apoptosis. For example, myoblasts overexpressing large T antigen fail to exit the cell cycle and die by apoptosis. Animals lacking p57/p21 have no skeletal muscle, display elevated rates of myoblast apoptosis and continue DNA replication in myotubes. In both cases, Rb is not able to sequester E2F resulting in expression of pro-apoptotic genes and in the absence of muscle differentiation, pro-apoptotic genes remain uninduced.

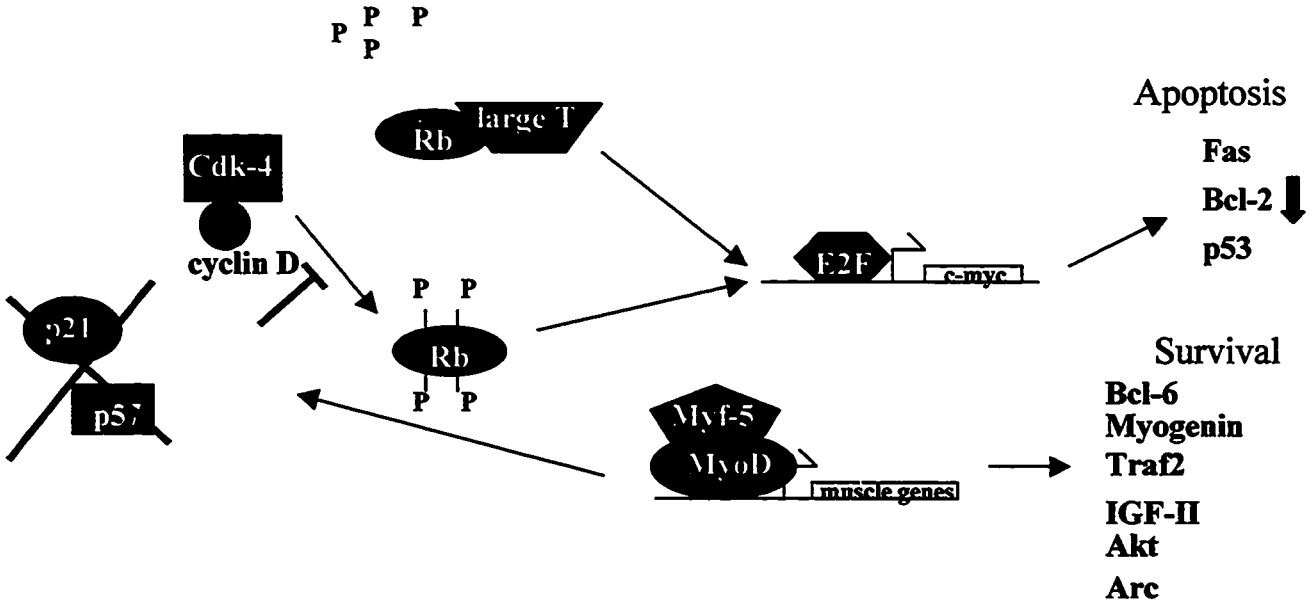
Proliferation



Differentiation



Apoptosis



the cyclin dependent kinase inhibitor p21 is upregulated and Rb phosphorylation is reduced resulting in sequestration of E2F, growth arrest and the onset of terminal differentiation (Guo et al 1995; Halevy et al 1995). MyoD associates with hypo-phosphorylated Rb, which acts as a co-factor during MyoD mediated transcriptional activation (Gu et al 1993).

1.11.1 MRF EXPRESSION DURING EMBRYOGENESIS

In situ hybridization studies with mouse embryos have shown distinct spatial and temporal expression patterns for each of the four MRFs. Myf-5 appears at 8.5 days post coitum (dpc) in the rostral somites but expression is reduced after day 14 (Ott et al 1991). Myogenin is seen at day 8.5 while MyoD is expressed at day 10.5, which corresponds with the expression of markers of terminal differentiation (Sassoon et al 1989). MRF4 appears between embryonic day 9 and 12 but is then downregulated until after birth (Bober et al 1991). In the developing limb bud, Myf-5 is expressed between days 10 and 12 while MyoD and myogenin are activated after day 10. In contrast, MRF4 expression appears only after day 16 (Ott et al 1991) (Sassoon et al 1989) (Bober et al 1991).

Interestingly, the MRFs are activated during development through a combination of autoactivation and input from other developmentally important transcription factors. For example, myogenin expression in the somites and limb buds of the developing mouse is controlled by a myocyte enhancer factor 2 (MEF 2) site and an E-box element indicating that its expression is at least partially MRF dependent (Cheng et al 1993). MyoD has two upstream enhancer elements, one 6Kb and one 20 Kb upstream of the transcription start site (Asakura et al 1995; Goldhamer et al 1992). When these

elements are utilized together to direct expression of a reporter gene in vivo, a pattern indistinguishable from the expression pattern of the endogenous gene is observed (Sabourin & Rudnicki 2000). The upstream enhancer is E-box independent (Kucharczuk et al 1999), however, it has been demonstrated *in vivo* that Myf-5, and a paired box domain protein, Pax-3, act upstream of MyoD in the myogenic hierarchy, the latter being a candidate for activation of this transcriptional element (Tajbakhsh et al 1997).

1.11.2 TARGETED INACTIVATION OF MRF GENES

Gene deletion of the MRFs in mice has identified a hierarchy of function of these factors during embryonic development. Interestingly, when MyoD is absent, muscle forms normally, however, the expression of Myf-5 is increased (Rudnicki et al 1992). If the Myf-5 gene is deleted, again, muscle forms normally, however, a rib defect results in perinatal lethality (Braun et al 1992). When both MyoD and Myf-5 are absent from the mouse, the mice die at birth due to a lack of myoblasts and skeletal muscle indicating that these two genes are required for myoblast and muscle formation (Rudnicki et al 1993). Mice deficient in myogenin have the appropriate number of myoblasts but die at birth due to a lack of myofibres (Hasty et al 1993; Nabeshima et al 1993). Finally, when MRF4 is deleted, the mice are viable with apparently normal skeletal muscle, however, myogenin expression is increased four fold (Braun & Arnold 1995; Patapoutian et al 1995; Zhang et al 1995). Therefore, gene targetting experiments have uncovered a partial redundancy built into muscle development and reveals two groups of MRFs. The primary MRFs – MyoD and Myf-5 are crucial for myogenic determination while the secondary MRFs - myogenin and MRF4 are required for myogenic terminal differentiation (Sabourin & Rudnicki 2000) (figure 1-7).

1.12 APOPTOSIS IN MUSCLE

The fact that skeletal muscle can undergo apoptotic cell death has been known for more than thirty years but is only now being recognized as an important process in various human dystrophic conditions. The first examples of apoptosis studied were dying muscle cells observed during metamorphosis of insects when muscles undergo massive degeneration (Lockshin & Williams 1965). Cell death is now an accepted component of several neuromuscular diseases including Duchenne muscular dystrophy (DMD) (Sandri et al 1998a), Spinal muscular atrophy (SMA) (Tews & Goebel 1996) and Limb girdle muscular dystrophy type 2A (Baghdiguian et al 1999). It is still unclear whether apoptosis is involved in myotonic dystrophy pathology with some evidence for (Sandri & Carraro 1999) and against (Inukai & Sobue 1997; Migheli et al 1997).

1.12.1 APOPTOSIS IN MYOBLASTS AND MYOGENESIS

Apoptosis has been well studied in cell free and the usual cell systems but is poorly understood in myoblasts (Sandri & Carraro 1999). As expected, apoptosis can be initiated in myoblasts using well characterized triggers such as serum withdrawal, DNA damage, oxidative stress, and by disturbing the cell cycle (Sandri & Carraro 1999). In myoblast cell lines (eg. C2C12) however, serum withdrawal also triggers differentiation making this model system unique. Differentiation is initiated by low serum medium leading to exit from the cell cycle (Sabourin & Rudnicki 2000).

In myoblasts, a delicate balance exists between cell growth, apoptosis and differentiation. In order for myoblasts to terminally differentiate, they must exit from the cell cycle (Sandri & Carraro 1999). Four hours after induction of differentiation, a proportion of myoblasts die by apoptosis (Mampuru et al 1996). This wave of apoptosis

is followed by a transient increase in cell proliferation accompanied by increased expression of transcription factors c-myc, fos and SRF. A second wave of apoptosis follows 7-14 hours following serum withdrawal (Mampuru et al 1996). In differentiating myoblasts, p21, a cyclin dependent kinase inhibitor is induced leading to decreased Rb phosphorylation resulting in sequestration of E2F transcription factor, precluding mitosis, and imposing exit from the cell cycle (figure 1-8). Induction of p21 correlates with an apoptosis resistant phenotype (Wang & Walsh 1996). Several anti-apoptotic signaling factors are induced upon myoblast differentiation including Akt (Fujio et al 1999), IGF II (Smith 1995), Bcl-6 (Kumagai et al 1999), Traf2 (MacLachlan & Giordano 1998) and ARC (Koseki et al 1998) (figure 1-8). This is consistent with the long-term survival of multinucleate myotubes cultured in low serum medium (Wang et al 1997). Despite the gradual accumulation of survival factors during differentiation, myoblasts remaining in the cell cycle in the presence of differentiation signals die by apoptosis. For example, when myoblasts expressing Polyoma virus large T antigen are induced to differentiate, they cannot exit the cell cycle because Rb fails to sequester E2F resulting in large scale apoptosis (Fimia et al 1998) (figure 1-8). In addition, myoblasts from Rb deficient mice induced to differentiate undergo increased apoptosis compared to myoblasts from wild type mice (Wang et al 1997). This phenomenon is observed in mice lacking cdk inhibitors p27 and p57. These mice have myoblasts with an elevated growth rate that fail to fuse to form myotubes and muscle and die by apoptosis (Zhang et al 1998).

The Fas/Fas L system is a possible mechanism for killing myoblasts as Fas is expressed on the surface of this cell type (Kang et al 1997) and overexpression of Fas L induces apoptosis in these cells (Sandri & Carraro 1999). In addition, p53 is capable of

inducing cell death in myoblasts upon DNA damage (Soddu et al 1996) and Bcl-2 levels decline as differentiation proceeds perhaps leaving cells susceptible to apoptosis during this process (Dominov et al 1998).

Apoptosis of developing muscle is a normal aspect of development. This is observed in the tadpole tail (Nishikawa & Hayashi 1995), in birds (McClearn et al 1995) and also in humans (Webb 1972). In humans, between 20 and 24 weeks gestation, some myotubes are eliminated by apoptosis in order to give the future muscle its proper size and shape (Sandri & Carraro 1999).

Apoptosis is a pathological feature observed in the muscle of mouse models of muscular diseases. The dystrophin complex is comprised of a group of several proteins at the sarcolemma providing a link between the extracellular matrix and the sarcolemmal actin cytoskeleton (Sandri & Carraro 1999). This complex is thought to buffer muscle against the shear forces generated during muscle contraction (Straub & Campbell 1997). Mdx mice are deficient in dystrophin (Sicinski et al 1989). These mice undergo a period of muscle degeneration at 2-3 weeks of age followed by a phase of regeneration completed by about 6 weeks of age (McArdle et al 1995). Degeneration was initially thought to be a necrotic process, however, recent studies demonstrating TUNEL labeling of roughly 0.5-2% of total nuclei and DNA laddering suggest an apoptotic component precedes necrosis (Sandri et al 1995) (Tidball et al 1995) (Matsuda et al 1995). In addition, mice deficient in the basement membrane protein merosin die before 5 weeks of age with myofibre degeneration beginning as early as 9 days after birth. These mice exhibit growth retardation, severe muscular dystrophy, TUNEL positive staining of 1%

of all nuclei and oligonucleosomal DNA laddering both at day 11, when the most severe degeneration is observed (Miyagoe et al 1997).

In the absence of disease, myonuclei from mature muscle have been shown to undergo apoptosis. Following space flight, rat soleus muscle was found to atrophy considerably yet the nucleus to mass ratio of the muscle had not changed suggesting a regulated reduction in nuclear number had occurred (Hikida et al 1997). Following hind limb unloading, muscle atrophy was found to coincide with myonuclear apoptosis in the absence of significant immune cell infiltration indicating that myonuclear loss by apoptosis is a normal adaptive response in muscle (Allen et al 1997). However, this atrophy associated myonuclear apoptosis could only be seen in one nucleus for every four hundred examined suggesting that it is a gradual process (Allen et al 1997). Myonuclear apoptosis is more prevalent in denervated muscle which can be reversed upon re-inervation (Tews et al 1997) and following intense exercise (Haas et al 1988; Podhorska-Okolow et al 1998; Thompson & Scordilis 1994).

1.12.2 APOPTOSIS IN HUMAN MUSCLE DISEASE

Evidence of apoptosis in muscle disease is not conclusive in all cases but most studies suggest that this process plays some role in pathogenesis in many disorders. Muscle from patients with DMD were analyzed and 5% of fibres were found to stain positive for Fas but no TUNEL positive myonuclei were observed (Behrens et al 1997). In a study that analyzed DMD and DM patient muscle, 17% of fibres were positive for Fas but the TUNEL reaction showed no apoptotic nuclei in the interstitium nor in muscle (Inukai et al 1997). These studies however, analyzed relatively few (<500) myofibres (Sandri & Carraro 1999). Another study also looking at DMD and DM patients found

TUNEL positive nuclei only in the interstitium of DMD and DM patients. However, electron microscopy demonstrated myonuclei with compact and electron dense chromatin deposited around the periphery in DM patients suggesting the presence of activated nucleases (Migheli et al., 1997). Tews and Goebel (Tews and Goebel, 1997) found apoptotic nuclei in the interstitium and in myofibres of DMD and Adhalin deficiency patient muscle. In addition, in human DMD, Sandri et al. (Sandri et al., 1998) found a low level of TUNEL positive myofibre nuclei and demonstrated that some interstitial TUNEL positive nuclei were muscle satellite cells undergoing apoptosis. Finally, apoptosis of myonuclei has been confirmed in limb girdle muscular dystrophy type 2A (Baghdiguian et al., 1999).

In SMA, apoptotic bodies and abnormal myonuclei with condensed chromatin were identified using electron microscopy (Fidzianska et al., 1990). In the most severe form of SMA (type I), most nuclei of atrophic fibres were positive for TUNEL staining while some myonuclei within normal appearing fibres also stained positive for TUNEL. In type III SMA, TUNEL positive myonuclei were frequently seen (2.3-3% of all myonuclei) but were not necessarily confined to atrophic fibres (Migheli et al., 1997). Interestingly, Bax is expressed in both normal and early atrophic myofibres whereas ICE, Bcl-2 and Bcl-x are only expressed in atrophic fibres (Tews and Goebel, 1997) suggesting that atrophic fibres are attempting to rescue themselves from apoptosis. It is clear that apoptosis is a response to insult in fully formed skeletal muscle and that detection of this process may be elusive, particularly in progressive disorders like DM where atrophy related loss of myonuclei may be a slow and gradual process.

1.13 THESIS OBJECTIVE

The objective of this thesis was to investigate possible molecular mechanisms responsible for myotonic dystrophy. Initial efforts focussed on identification of muscle specific regulatory elements within the DMPK gene for use in transgenic studies. At the time these studies were initiated, the DMPK 3' UTR was known to inhibit differentiation of cultured myoblasts and it was suspected that the expanded CTG repeat within this element, in the absence of its associated coding region, might be responsible for causing the disease. It was also known that overexpression of the entire DMPK gene with 22 CTG repeats in mice could cause a number of features of DM. By constructing stable cell lines in mouse myoblasts, we determined the DMPK promoter/enhancer could correctly target expression of a reporter gene in this cell type. These cell lines expressed the DMPK 3' UTR with a wild type (11) and mutant (91) number of CTG repeats linked to the reporter gene GFP utilizing DMPK regulatory elements and were used in an effort to understand molecular mechanisms underlying DM. Using the same DNA constructs, a transgenic approach was employed to determine if some or all features of DM could be recapitulated *in vivo* by overexpression of just the DMPK 3' UTR containing a wild type or mutant number of CTG repeats, in the absence of DMPK coding sequence. In addition, we could ascertain the utility of the DMPK promoter/enhancer elements *in vivo*.

CHAPTER 2. GENERAL MATERIALS AND METHODS

2.1 PCR AND CLONING

Many DNA fragments were cloned utilizing polymerase chain reaction (PCR). Approximately 125 ng (2.5 µl) of gene specific upstream and downstream primers were combined with 100 ng (1 µl) of plasmid DNA template, 5X PCR buffer (Gibco-BRL, 200 mM Tris-HCl pH 8.4, 500 mM KCl), 1.5 mM $MgCl_2$, 2.5 µl (5%) DMSO, 1 µl (5 U) taq DNA polymerase (Gibco-BRL) and made up to 50 µl with sterile dH_2O. Reactions were topped with mineral oil and amplified in a PCR machine (Perkin-Elmer) for 30 cycles of 96°C for 1 minute, 55-65°C for 1 minute and 72°C for 1.5 minute. Following 30 amplification cycles, PCR products were incubated at 72°C for 8 minutes then at 4°C until analysis. Often PCR products were cloned immediately following amplification. Ligation reactions were performed as described in Maniatis (Sambrook 1989) (pp.1.63-1.67). Briefly, 2 µl of PCR product was mixed with 1 µl of pCR 2.1 vector (Invitrogen), 2 µl of 5X ligase buffer (Gibco-BRL, 250 mM Tris HCl pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% PEG), 1 µl of T4 DNA ligase (Gibco-BRL), 1 µl of 10 mM ATP and 3 µl of sterile dH_2O, quickly spun and incubated at room temperature for 30 minutes to overnight. Ligation products were incubated with 100 µl of competent E. coli DH5α in a 15 ml polypropylene tube on ice for 30 minutes then heat shocked at 42°C for 90 seconds. LB broth (800 µl) was added and this mixture was incubated at 37°C with shaking for 1 hour. A portion of the transformation mixture (300 µl) was added to a LB agar plate containing 50 µg/ml ampicillen, spread with a sterilized hockey stick and incubated inverted overnight in a 37°C incubator. Individual colonies were picked and analyzed.

2.2 GENERATION OF BLUNT ENDS

To facilitate cloning, blunting of restriction enzyme generated 5' DNA overhangs was sometimes performed. In a 50 µl reaction, DNA (5 µg) digested with a restriction enzyme producing 5' overhangs was incubated with 5U of Klenow fragment (Gibco-BRL) in Gibco buffer React 2 (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl) and 3 µl of 0.5M dNTP mix at 30°C for 15 minutes.

2.3 PREPARATION OF COMPETENT E.COLI

Competent E.coli were produced using the method of Hanahan (Hanahan 1985). E. coli strain DH5α were grown overnight in 5 ml of Luria broth (LB). The following day, two flasks containing 50 ml of LB were innoculated with 500 μl each from the overnight DH5α culture and grown for 2 hours at 37°C with shaking. Optical density of the culture was measured using a spectrophotometer at 550 nm until an OD of 0.5 was achieved. Cells were transferred to 50 ml conical tubes and pelleted at 3200 rpm in a floor model Hettich centrifuge. Cells were resuspended in 33 % of the original culture volume (16 ml total per tube) of RF1 (100 mM RbCl, 50 mM $MnCl_2 \cdot 6H_20$, 30 mM potassium acetate, 10 mM $CaCl_2 \cdot 2H_2O$, 15 % glycerol). Cells were incubated on ice for 15 minutes and centrifuged as described. The cells were resuspended in 4 ml per tube of RF2 (8 % of original culture volume) (10 mM MOPS pH 6.8, 10 mM RbCl, 75 mM $CaCl_2 \cdot 2H_2O$, 15 % glycerol), aliquoted into eppendorf tubes, flash frozen in dry ice methanol and stored at –70°C.

2.4 PLASMID MINIPREPS AND DNA DIGESTS

Individual ampicillin resistant colonies were picked with sterile pipette tips, added to 2 ml of LB broth containing 50 µg/ml ampicillin in a 15 ml polypropylene tube and

incubated at 37°C with shaking overnight. Plasmid DNA was prepared using the boiling method (pp. 1.29-1.30, Maniatis (Sambrook 1989)). Approximately 1 ml of culture was transferred to an eppendorf tube and spun at room temperature for 5 minutes at 9000 rpm. The supernatant was aspirated and the pellet resuspended in 100ul of STET buffer (8% sucrose, 50 mM Tris HCl pH 8.0, 50 mM EDTA, 5% Triton X-100) and 15 µl of lysozyme solution (10 mg/ml lysozyme in Tris-HCl, EDTA, NaCl) by vigourous vortexing. Tubes containing this mixture were immersed in boiling water for 45 seconds and spun at room temperature for 10 minutes at 10000 rpm. Following the spin, the pellet was removed using a disposable wooden stick. Plasmid DNA was precipitated by adding 100 µl of isopropanol, vortexing briefly and centrifugation at 10000 rpm for 10 minutes at room temperature. Pelleted DNA was resuspended in 50 µl of TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 7.5) and analyzed by restriction enzyme digestion. Briefly, in an eppendorf tube, 5µl of plasmid DNA was mixed with 2 µl of 10X digestion buffer (Gibco-BRL), 1 µl of RNase A (10 mg/ml), 1 µl of restriction enzyme and 11 µl of sterile dH_2O. The digest was incubated at 37°C for 1 hour and loaded on a 0.8 % TAE agarose gel containing 0.1 µg/ml ethidium bromide. The gel was immersed in 1X TAE buffer and run for 2 hours at 80 volts. Following electrophoresis, a Polaroid photograph of the gel was taken using a Polaroid MP-4 Land Camera.

2.5 DNA PREPARATION

All DNA was prepared using the alkaline lysis method (Birnboim & Doly 1979) from 200 ml of Luria Broth culture containing 50 µg/ml ampicilin and 0.1% glucose innoculated from frozen 25% glycerol stock culture. Cultures were grown overnight at 37°C with shaking. Following overnight growth, bacterial cells were pelleted by

centrifugation at 3000 rpm in a floor model Beckman centrifuge. Plasmid DNA was harvested from the bacterial pellet by alkaline lysis (Birnboim & Doly 1979). Briefly, the pellet was resuspended in 1.6ml of lysozyme solution (50mM glucose, 10 mM EDTA, 25 mM Tris HCl pH 8.0 and 50 mg of lysozyme). To this, 5 ml of NaOH/SDS (0.2 N NaOH, 1% SDS) solution was added for 5 minutes followed by neutralization with 3 ml of 3M sodium acetate for 10 minutes. This was mixed, spun for 10 minutes at 10000 rpm and the supernatant transferred to a new tube. To precipitate the DNA, 4.5 ml of isopropanol was added, the sample was mixed and incubated at RT for 10 minutes. The DNA was then recovered by centrifugation at 10000 rpm and washed in 70% ethanol. Once dried, the DNA was resuspended in 2.4 ml of TE (10mM Tris HCl pH 7.5, 1mM EDTA pH 7.5), 2.75 g of CsCl was added followed by addition of 250 µl of ethidium bromide (10mg/ml). This mixture was loaded into an ultricentrifuge tube and spun at 80000 rpm in a TL 100 Beckman rotor for at least 15 hours. Supercoiled plasmid DNA was recovered using a 23 ½ guage needle, ethidium bromide removed with four extractions of water saturated butanol and brought up to a volume of 2.5 ml with dH_2O. The DNA was precipitated by adding 5 ml of 100 % ethanol, incubating on ice for 1 hour and spinning at 12000 rpm for 10 minutes at 4°C. The DNA was resuspended in 150 µl of TE, 150 µl of 7.5 M ammonium acetate and 600 ul of 100% ethanol, incubated overnight at –20 °C and then pelleted at 14000 rpm in a microfuge at 4°C. The DNA was resuspended in 200 µl of TE and the concentration determined using a spectrophotometer (Beckman).

2.6 OLIGONUCLEOTIDE SYNTHESIS

Oligonucleotides used in PCR and sequencing reactions were synthesized on an automated DNA synthesizer (PCR-Mate, model 391, Applied Biosystems). The phosphoramidite synthesis method was performed according to manufacturer's instructions. Following synthesis, oligonucleotides were removed from the solid support and deblocked from the protective group with ammonium hydroxide at 55°C overnight. The oligonucleotide solution was dried, resuspended in sterile water and analyzed on a spectrophotometer to determine oligonucleotide yield.

2.7 SEQUENCE ANALYSIS

Following purification of DNA, plasmid clones were subjected to dye deoxy cycle sequence analysis using Taq DNA polymerase (Applied Biosystems) on an ABI 373A automated sequencer according to manufacturer's instructions. Sequence data was analyzed using ABI software on an Apple MacIntosh PC.

2.8 RNA PREPARATION

RNA was prepared using the method of Birnboim (Birnboim 1988). Briefly, confluent monolayers from 2 100 mm plates were scraped using a rubber policeman, pipetted into an eppindorff tube and spun in a microfuge for 4 seconds. The cell pellet was resuspended in 400 µl RES-1 buffer (0.5 M LiCl, 1M urea, 1% SDS, 0.02M tri-sodium citrate and 2.5mM EDTA) plus 100 µg/ml of proteinase K, pipetted up and down to mix and incubated at 55°C for 30 minutes. The RNA was then precipitated by adding 26µl of 2M sodium acetate pH 5.6 and 1 ml of 100% ethanol and incubated at –20°C for 20 minutes. The RNA was pelleted by spinning in a microfuge at 10000 rpm for 10 minutes at room temperature. The RNA was redissolved in 400 µl of RES-1 containing

proteinase K and again incubated at 55°C for 30 minutes. RNA was extracted once with phenol/chloroform (1:1) and once with chloroform followed by addition of 400 µl of 5M LiCl, 2 µl of 2N acetic acid and incubated on ice overnight. The RNA was recovered by spinning at 10000 rpm for 5 minutes, resuspended in 400 µl CES-1 (1mM tri-sodium citrate, 1mM EDTA, 0.1% SDS) and ethanol precipitated twice as described above. RNA was resuspended in 50 µl of CES-1 and concentrations determined using a spectrophotometer.

2.9 NORTHERN BLOTTING

In order to determine mRNA expression levels of transgenes in cell lines and mice, 15 µg of RNA was denatured for 15 minutes at 55°C in formaldehyde, formamide and 1X MOPS buffer. The RNA sample was mixed with gel loading buffer (50 % glycerol, 0.25 % bromphenol blue, 0.25 % xylene cyanol, 1 mM EDTA pH 8.0), loaded on an agarose gel containing 0.3M formaldehyde submerged in 1X MOPS buffer and electrophoresced until the bromphenol blue dye front had migrated 80 % of the gel length. The gel was washed 3X 5 minutes in dH_20, 1X 5 minutes in 1 µg/ml solution of ethidium bromide and then photographed. The RNA was transferred to a neutral nylon membrane (Pall-Biodyne) using a downward capilliary transfer technique (Current Protocols in Molecular Biology) in 10X SSC transfer buffer for 1 hour. The blot was washed for 3 minutes with shaking in 2X SSC, dried for 10 minutes at room temperature on Whatman 3 CHR paper and UV crosslinked (Fisher Biotech FB-UVXL-1000) to covalently link he RNA to the membrane.

2.10 RNA SLOT BLOT

To quantitate mRNA expression levels in cell lines, slot blot analysis was performed. Briefly, 4 μg of RNA was denatured in formaldehyde, formamide and 1X SSC followed by incubation for 15 minutes at 55°C. The denatured RNA was diluted with 500 μl of 10X SSC, immobilized using a slot blot apparatus (Schleicher and Schuell) under vacuum to a neutral nylon membrane (Pall-Biodyne) previously equilibrated in 10X SSC. Each slot was washed once with 500 μl of 10X SSC. The blot was then dried for 10 minutes at room temperature on Whatman 3 CHR paper and the RNA covalently linked to the membrane by UV crosslinking.

2.11 PROBE LABELLING AND HYBRIDIZATION

All nucleic acid blots were probed by denaturing 100 ng of gene fragment isolated using Qiaquick ™ (Qiagen) gel extraction technique according to manufacturer's instructions. Fragments were denatured by boiling for 5 minutes followed by incubation on ice for 5 minutes. Denatured fragment was added to a Rediprime (Amersham) tube containing nucleotides, random primers and DNA polymerase. Label (5 μl of α^{32}P-dCTP-Amersham) was added, the contents mixed and incubated at 37°C for at least 10 minutes. Labelled probe was separated from unincorporated label by passage through a NICK column (Amersham). Briefly, columns were equilibrated by passing 3 ml of TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 7.5). Probe was then added in a 1ml volume of TE and eluted. The column was washed 3X with 1 ml of TE and percent incorporation determined using aliquots of probe and wash eluate in a Wallac 1450 MicroBeta Plus plate reader scintillation counter.

Following boiling for 5 minutes, labelled probe was added to membranes equilibrated in hybridization solution (10% polyethylene glycol [PEG], 7% SDS, 1.5X SSPE + 250 µl boiled [15 minutes] salmon sperm DNA [10 mg/ml]) at 65°C for at least 15 minutes. Labelled probe was hybridized to membranes with shaking at 65°C overnight. Blots were washed the following day for 15 minutes in 2X SSC, 0.1% SDS at room temperature, 2X 15 minutes in 0.1X SSC, 0.1% SDS at room temperature and then 4-10X 15 minutes in 0.1X SSC, 0.1% SDS at 65°C. Membranes were blotted on Whatman 3 CHR paper, wrapped in plastic and subjected to autoradiography using Kodak X-OMAT AR scientific imaging film for 1-24 hours and developed.

2.12 CELL CULTURE

All cell lines were cultured in D-MEM (Gibco-BRL) containing 4.5 ug/ml glucose and L-glutamine. To 450 ml of D-MEM, 50 ml of heat inactivated fetal bovine serum (FBS) (Gibco), 500 U of Penicillin and 500 µg/ml of Streptomycin were added. Patient myoblasts, fibroblasts and amniocytes were cultured in identical medium outlined above except that FBS was added at a concentration of 15 %. All cultures of mammalian cells were maintained in a 5% CO_2 humidified atmosphere at 37°C. Cells were frozen in 10 % DMSO in the medium outlined above. Briefly, approximately 1 X 10^6 cells in a 10 cm culture dish were trypsinized in 2 ml of trypsin (0.25 % trypsin in 1X PBS) for about 2 minutes at 37°C and bumped gently to loosen cells. Fresh medium was added and the cells were triturated several times with a 5 ml pipette and transferred to a 15 ml polystyrene conical tube. Approximately 11 ml of cold serum free D-MEM was added and the cells were pelleted at 1200 rpm for 5-10 minutes in a floor model Hettich centrifuge. The medium was removed and the cells were resuspended in 1.5 ml of

freezing medium (10 % DMSO in FBS containing D-MEM) and transferred to a 2 ml cryogenic vial. The cells were incubated for 30 to 60 minutes on ice, transferred to – 80°C overnight and then to liquid nitrogen the following day.

CHAPTER 3 CONTROL OF DMPK EXPRESSION IN VITRO

3.1 INTRODUCTION.

Myotonic dystrophy (DM) is a dominant multisystemic disorder, which in the adult form shows myotonia, progressive muscle weakness and wasting, and heart conduction defects (Harper 1989). In males, testicular atrophy and premature balding are also observed (Harper 1989). Major features of congenital DM include hypotonia, respiratory distress, mental retardation, and a delay in terminal muscle differentiation (Harper 1989). The genetic defect for DM is an expanding CTG trinucleotide repeat found in the 3'untranslated region of a serine threonine protein kinase (DMPK) (Brook et al 1992; Fu et al 1992; Mahadevan et al 1992). An increase in the severity of the disease correlates with an increase in the number of trinucleotide repeats, which provides a molecular basis for genetic anticipation (Hunter et al 1992; Mahadevan et al 1992; Tsilfidis et al 1992). The consequences of CTG repeat expansion remains uncertain.

Conflicting results have been reported as to the levels of total DMPK transcript and protein in patients (Carango et al 1993; Dunne et al 1996a; Fu et al 1993; Hofmann-Radvanyi et al 1993; Koga et al 1994; Novelli et al 1993; Otten & Tapscott 1995; Sabourin et al 1993). The mutant message can be detected by Northern blot analysis (Davis et al 1997; Otten & Tapscott 1995; Sabourin et al 1993) and appears to accumulate in the nucleus of DM patient myoblasts and fibroblasts (Davis et al 1997; Taneja et al 1995). Reduction of mutant (Krahe et al 1995) and both mutant and normal (Wang et al 1995) DMPK mRNA in the polyadenylated fraction has also been reported. Mice nullizygous for DMPK are virtually asymptomatic (Jansen et al 1996; Reddy et al 1996), indicating that DMPK is not an essential protein in this species. DMPK is

expressed mainly in heart, lens, skeletal muscle, testes, and brain (Brook et al 1992) (Dunne et al 1996a; Jansen et al 1992). Delineation of the biological role of DMPK in these tissues will allow a greater understanding of the pathological process of DM. Accordingly, we have begun analysis of the DMPK gene for *cis* elements that direct its expression in various cultured cell lines. Transgenic mice containing a 15-kb human genomic DNA fragment encompassing the entire DMPK gene express the human mRNA and protein in the appropriate tissues, suggesting at least a subset of tissue specific *cis* elements are contained within the transgene sequence (Jansen et al 1996; Narang 2000). Here we attempt identification of transcriptional regulatory elements required for increased DMPK mRNA production in cultured myoblasts *versus* fibroblasts. A 2-kb fragment of the DMPK upstream sequence driving the chloramphenicol acetyl-transferase (CAT) gene in NIH 3T3 fibroblasts and TE32 myoblasts indicates a ubiquitous, housekeeping-type promoter region with only minor muscle-specific regulatory activity. We also investigated various deletions of the DMPK first intron for transcriptional regulatory sequences, which may enhance expression of CAT driven by endogenous and exogenous promoters in fibroblasts and muscle cells. Results presented indicate that the DMPK gene is regulated by a low level promoter that operates in conjunction with an enhancer element in the first intron. This first intron enhancer element is responsive to MyoD, requires the presence of conserved E-boxes, and in the presence of the DMPK promoter, confers increased expression of CAT in myoblasts.

3.2 MATERIALS AND METHODS

3.2.1 CELL CULTURE, DNA TRANSFECTIONS, CAT, AND B-GALACTOSIDASE ASSAYS

Primary human foreskin fibroblasts (HFSF) were a gift from Dr. Chaim Birnboim (Ottawa Regional Cancer Center, Ottawa, Canada), C2C12 mouse muscle cells, TE32 human myoblasts (McAllister et al 1969), NIH 3T3 mouse fibroblasts, human HeLa cells, and C3H10T1/2 mouse fibroblasts were obtained from the ATCC. Transfections were performed with 5 mg of CsCl-purified test plasmid, 3 mg of β-galactosidase expression construct (pSVβ-gal-Promega) by the calcium phosphate co-precipitate method with a glycerol shock essentially as described [Sambrook, 1989 #1522]. β-Galactosidase assays were carried out by adding 45 ml of cell extract to 155ml of Z-buffer (60 mM $Na_2 HPO_4$,40 mM $NaH_2 PO_4$,10 mM KCl, 1 mM $MgSO_4$,50 mM 2-mercaptoethanol, pH 7) and 40 ml of O-nitrophenyl-β-pyranogalactoside (Sigma) (4 mg/ml in 0.1 M phosphate buffer, pH 7, 77.3 g of $Na_2 HPO_4 7H_2 O$, 29.1 g of NaH_2 PO4 $H_2 O$ in 500 ml of $H_2 O$) and incubating at 30 °C for 2 to 15 h. The reactions were stopped by adding 100 ml of 1M $Na_2 CO_3$ and the optical density measured at 420 nm using a spectrophotometer. CAT assays were performed essentially as described (Sambrook 1989).

3.2.2 PLASMID DNA CONSTRUCTIONS

A series of deletions of the DMPK 5' region were generated by standard methods and sub-cloned into *Pst*I or *Pst*I/*Hin*dIII-digested promoterless pCAT-Basic vector (Promega). All 5' region CAT deletion constructs (figure 3-4) are named according to the position of the 5' end of the inserted sequence relative to the DMPK translation initiation

codon, and all share the same 3' termini, which is 42 bp upstream of the ATG initiation codon. Promoter/first intron combinations -722 CAT IVS, -722 CAT SVI, TK CAT IVS, and TK CAT SVI were constructed by subcloning a 2-kb *Eco*RI/*Bam*HI first intron fragment into *Eco*RI/*Bam*HI-digested pCAT-Basic vector down-stream of the CAT gene. The -722, -940 DMPK promoter and TK promoter fragments were inserted into *Hin*dIII/*Pst*I, *Hin*dIII/*Xba*I, and *Hin*dIII sites, respectively, upstream of the CAT gene. The TK promoter was generated by polymerase chain reaction amplification of the herpes simplex thymidine kinase (TK) promoter from a plasmid (4RTKCAT). To construct TK CAT, this polymerase chain reaction product was subcloned into the *Hin*dIII site upstream of the CAT gene in pCAT-Basic. IVS TK CAT and SVI TK CAT were constructed by fusing the 2-kb intron 1 fragment into the *Pst*I site in both orientations upstream of the TK promoter in TK CAT. First intron deletions were generated by polymerase chain reaction, cloned into pCR 2.1, excised with *Eco*RI, and cloned into pCAT-Basic. Subsequently, TK and DMPK promoter elements were cloned into these constructions as described above. To ensure all clones were properly constructed, all plasmid DNA constructions were analyzed by restriction digest and/or DNA sequencing.

3.2.3 PRIMER EXTENSION ANALYSIS

Approximately 50 ng of DMPK primer 2151 (5'-TTGGACAGGCAGCACCATGGC) was end-labeled with [γ-32 P] ATP (Amersham Pharmacia Biotech) using T4 polynucleotide kinase. Primer extension reactions were set up using 60 mg of TE32 myoblast or HFSF RNA essentially as described (Sambrook 1989), except Superscript II reverse transcriptase (Life Technologies, Inc.) was used in place of murine reverse transcriptase. Standards for sizing primer extension products

were generated from the control sequence of the Sequenase 2 kit (U.S. Biochemical Corp.). These were synthesized according to manufacturers instructions with [γ-33 P] dATP (Amersham Pharmacia Biotech) using single-stranded M13mp18 DNA with the primer provided.

3.2.4 ELECTROPHORETIC MOBILITY SHIFT ASSAYS

Oligonucleotides corresponding to both strands of the DMPK GC box (nucleotides 1947–1975, figure 3-3, see below) were annealed using a thermal ramp in a Perkin-Elmer thermocylcer and end-labeled using [γ-32 P]ATP (Amersham Pharmacia Biotech), T4 kinase, and forward reaction buffer (Life Technologies Inc.) followed by incubation for 45 min to 1 h at 37°C. Nuclear extracts were obtained, using the procedure of Hoppe-Seyler *et al.* (Hoppe-Seyler et al 1991), which were quantitated for total protein concentration using a Pierce Micro-BCA kit according to manufacturer's instructions. Nuclear extract (5mg) was incubated with 2.5 X 10^4 cpm of labeled oligonucleotide, 1 mg of poly(dI:dC), 1 ml of bovine serum albumin (10 mg/ml), 2 ml of gel shift buffer (250 mM HEPES, pH 7.6, 50 mM MgCl2 , 340 mM KCl), and competitor for 20 min on ice. The complexes were then mixed with 2 ml of gel loading buffer (20% Ficoll 400, 0.1% bromphenol blue), and electrophoresed on a 5% nondenaturing polyacrylamide gel at 150 V for 1.3 h. Gels were then exposed to film overnight at -80 °C.

3.2.5 OLIGONUCLEOTIDES

Sequences of oligonucleotides used in electrophoretic mobility shift assay and competition analysis (shown in figure 3-5) were synthesized using an ABI 394 DNA/RNA synthesizer and are as follows: thymidine kinase oligonucleotide, 5'-

ATGACACAAACCCCGC-CCAGCGTCTTG-3' (Jones et al 1985); AP-2 oligonucleotide, 5'-AGTCCCCAGGCTC-CCCAGCAG-3' (Mitchell et al 1987); wild type DMPK oligonucleotide, 5'-TAAGGCT-GGGAGGCGGGAGGGGGGCTGG-3'; mutant DMPK oligonucleotide,5'-TAAGGCTGGGAGG***CT***TGAGGGGGGCTGG-3' (mutated nucleotides are shown in boldface italics).

3.3 RESULTS

The focus of this study was on DMPK transcriptional regulation in muscle cells *versus* fibroblasts. Initially, we characterized expression of the DMPK mRNA in a number of human and mouse cell lines in an effort to identify cell lines suitable for the study of DM protein kinase gene myoblast specific *cis* elements. Northern blot analysis (figure 3-1) using a probe from the DMPK cDNA was performed on RNA isolated from a number of human and mouse cell lines. Relative to a Pgk-1 control, DMPK expression was strongest in TE32 myoblasts, followed by C2C12 myotubes and C2C12 myoblasts. Weak DMPK expression was observed in NIH 3T3, primary HFSF, and C3H10T1/2 cells, while the message was barely detectable in HeLa cells. Given the high expression level of DMPK mRNA in TE32 myoblasts and the lower level in NIH 3T3 fibroblasts, comparison of DMPK-reporter gene fusion construct activities between these cell lines was deemed a suitable system for identifying myoblast specific transcription control elements within the DMPK gene.

Primer extension analysis of the DMPK 59 region mapped two major transcription start sites in both myoblasts and fibroblasts, one at position -421 (figure 3-2A) and the other at position -71 relative to the translation initiation codon (figure 3-2B).

Figure 3-1. Northern blot showing expression of DMPK in various cell lines used in the analysis of DMPK regulatory elements. Total RNA was isolated from C2C12 mouse myoblasts *(lane* 1), C2C12 myotubes *(lane* 2), NIH 3T3 mouse fibroblasts *(lane* 3), C3H10T1/2 mouse fibroblasts *(lane* 4), TE32 human rhabdomyosarcoma cells (myoblasts) *(lane* 5), human foreskin fibroblasts *(lane* 6), and human HeLa cells *(lane* 7), and 20 mg was used for Northern analysis. A Pgk-1 probing is shown as a control for RNA loading. Location of the DMPK mRNA and 28 and 18 S ribosomal RNA bands are indicated. DMPK mRNA is most abundant in the myoblast cell lines.

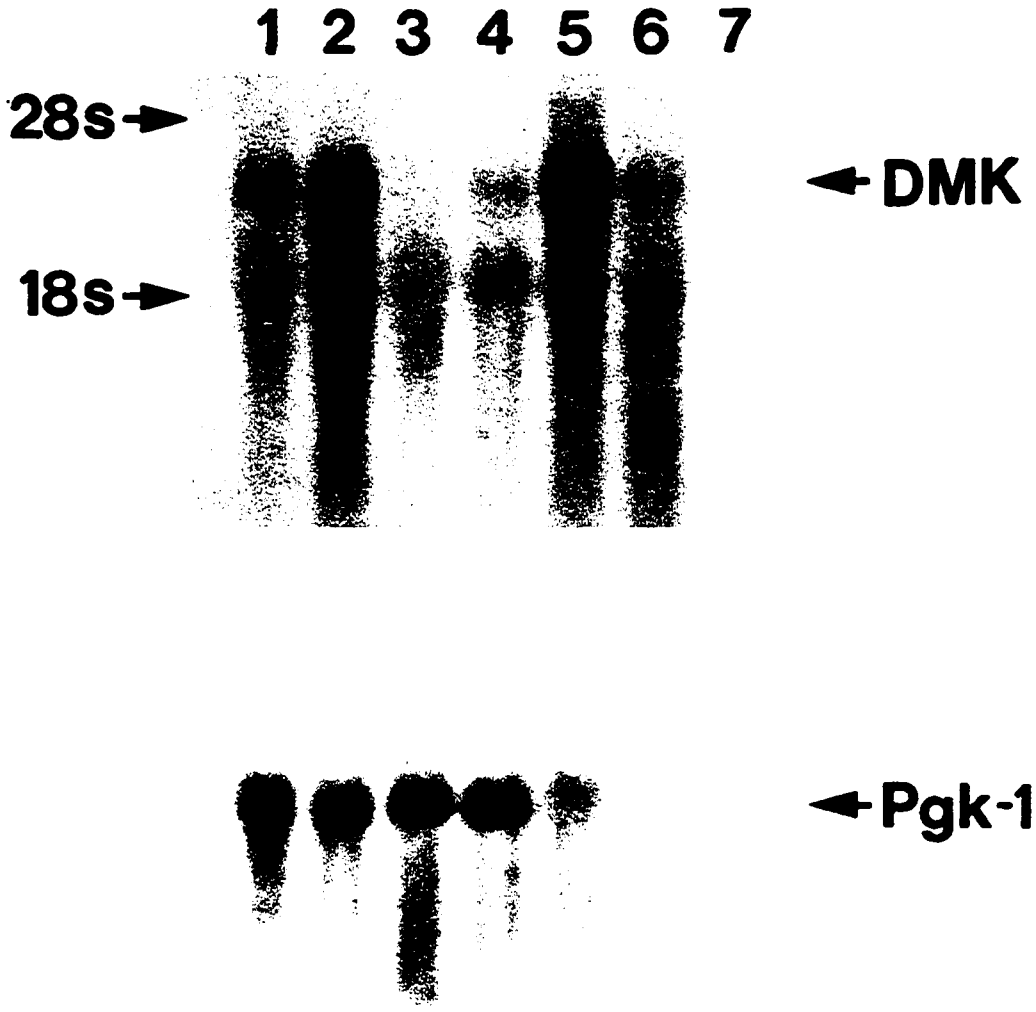
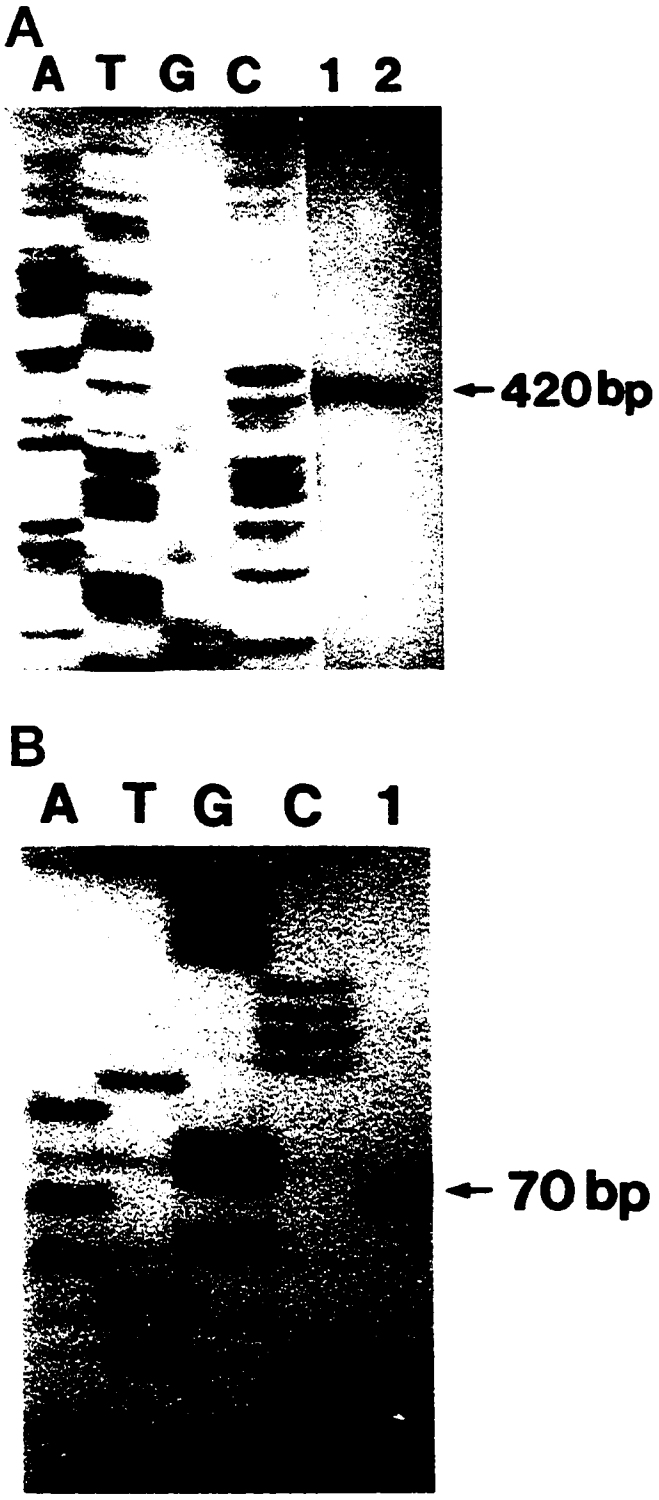

1 2 3 4 5 6 7
28s
18s
DMK
Pgk-1

Figure 3-2. Primer extension analysis was performed mapping two major DMPK transcription start sites with mRNA from TE32 myoblasts and HFSF fibroblasts at positions -71 and –421 relative to the translation initiation codon. (A), upstream primer extension product reverse transcribed using primer 2151 from 60 mg of TE32 RNA *(lane* 1) or from 60 mg of HFSF RNA *(lane* 2). (B), downstream primer extension product also using primer 2151 reverse-transcribed from 60 mg of TE32 RNA *(lane* 1). The DNA size standard in both *A* and *B* is a sequencing ladder generated from single-stranded M13mp18 DNA using the –40 primer (see "Materials and Methods").


A
A T G C 1 2
←420bp
B
A T G C 1
← 70 bp

The sequence products shown in figure *2A* were electrophoresed longer to accurately size the extension products. Longer exposures of primer extension gels revealed other minor start sites, one upstream of -421 consistent with start sites reported earlier (Mahadevan et al 1993) and several between -421 and -71. The location of transcriptional start sites mapped by primer extension is shown schematically in figures 3-3 and 3-4. The 5' region of the DMPK gene contains several well conserved consensus binding sites for known eukaryotic transcription factors, including Sp1 (Kadonaga et al 1988), AP-1 (Lee et al 1987), and AP-2 (Mitchell et al 1987) (figure 3-3). The lack of a TATA and CCAAT box and the high GC content in this region of the DMPK gene are characteristic of housekeeping genes (Herrick et al 1993; Wang et al 1996), growth control genes (Itoh et al 1992), and some kinase gene promoters (Clegg et al 1996; Penn & Benovic 1994). Inspection of mouse and human DMPK 5' regions show strong homology from nucleotide 1900 to the translation initiation codon (nucleotide 2170) (figure 3-3) and includes a conserved GC box upstream of the transcription start site at position -71. Homology between the human and mouse genes is also evident upstream of the transcription start site located at position -421. To identify the location of promoter elements, we constructed promoter deletion mutants of the DMPK 5' region. A 1963-bp fragment of the DMPK 5' region was fused to the bacterial CAT reporter gene and a series of deletion constructs from this parental plasmid were constructed (figure 3-4). To localize possible myoblast specific transcriptional control elements within this sequence, these constructs were transfected into TE32 myoblasts and NIH 3T3 mouse fibroblasts. Relative CAT activities were normalized to promoterless CAT-Basic to compare the

Figure 3-3. Nucleotide alignment of approximately 1 kb of human and mouse DMPK 5'untranslated region. Consensus binding sequences for AP-2, AP-1, and Sp1 are indicated with *boxes*. *Directional arrows* (positions 2097 (-421) and 1747 (-71)) represent the location of the two major transcriptional start sites mapped by primer extension. The *underlined* sequence beginning at nucleotide position -167 represents oligonucleotide 2151, which was used in the primer extension analysis. The *bold* nucleotide position *numbers* (*i.e.* -940) represent the 5' boundaries of promoter deletion constructs and are numbered and positioned relative to the translation initiation codon (ATG), which is *underlined* and indicated by *vertical arrow*. The 3' end of these deletion constructs is marked with a *vertical bar* at position 2125. The *underlined* sequence beginning at position 1948 represents the DMPK probe used in electrophoretic mobility shift assays (below). Several conserved transcription factor consensus binding sequences are located upstream of the mapped transcription start sites, indicating a possible role for these factors in initiation of DMPK transcription.

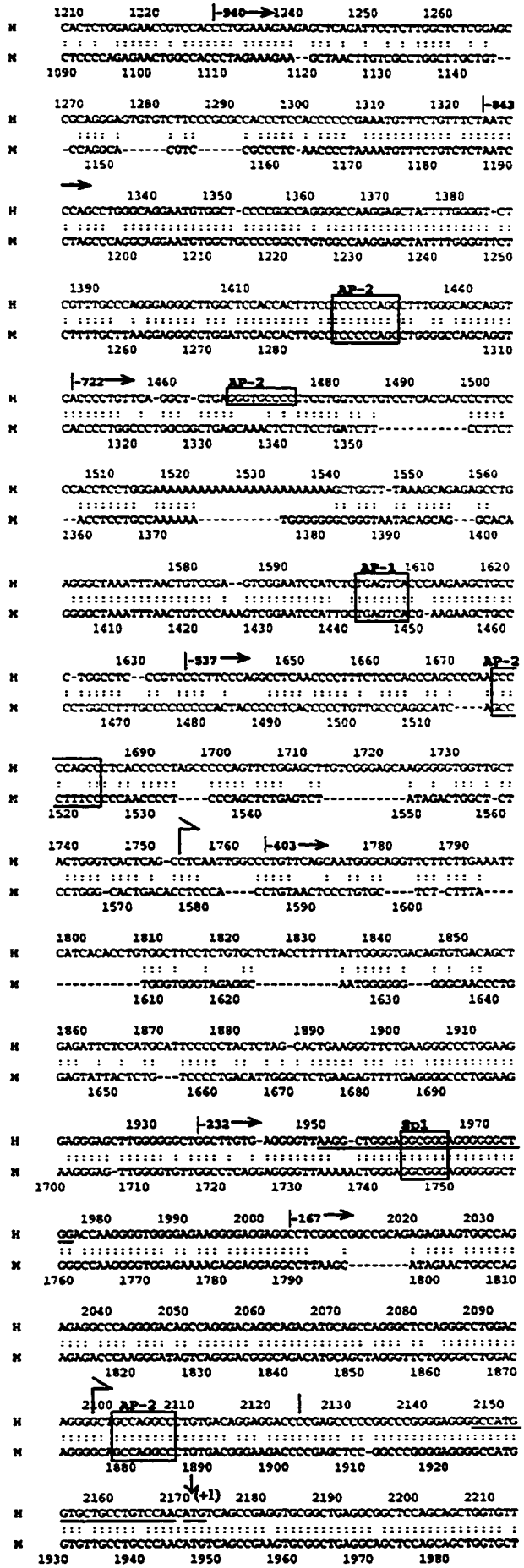
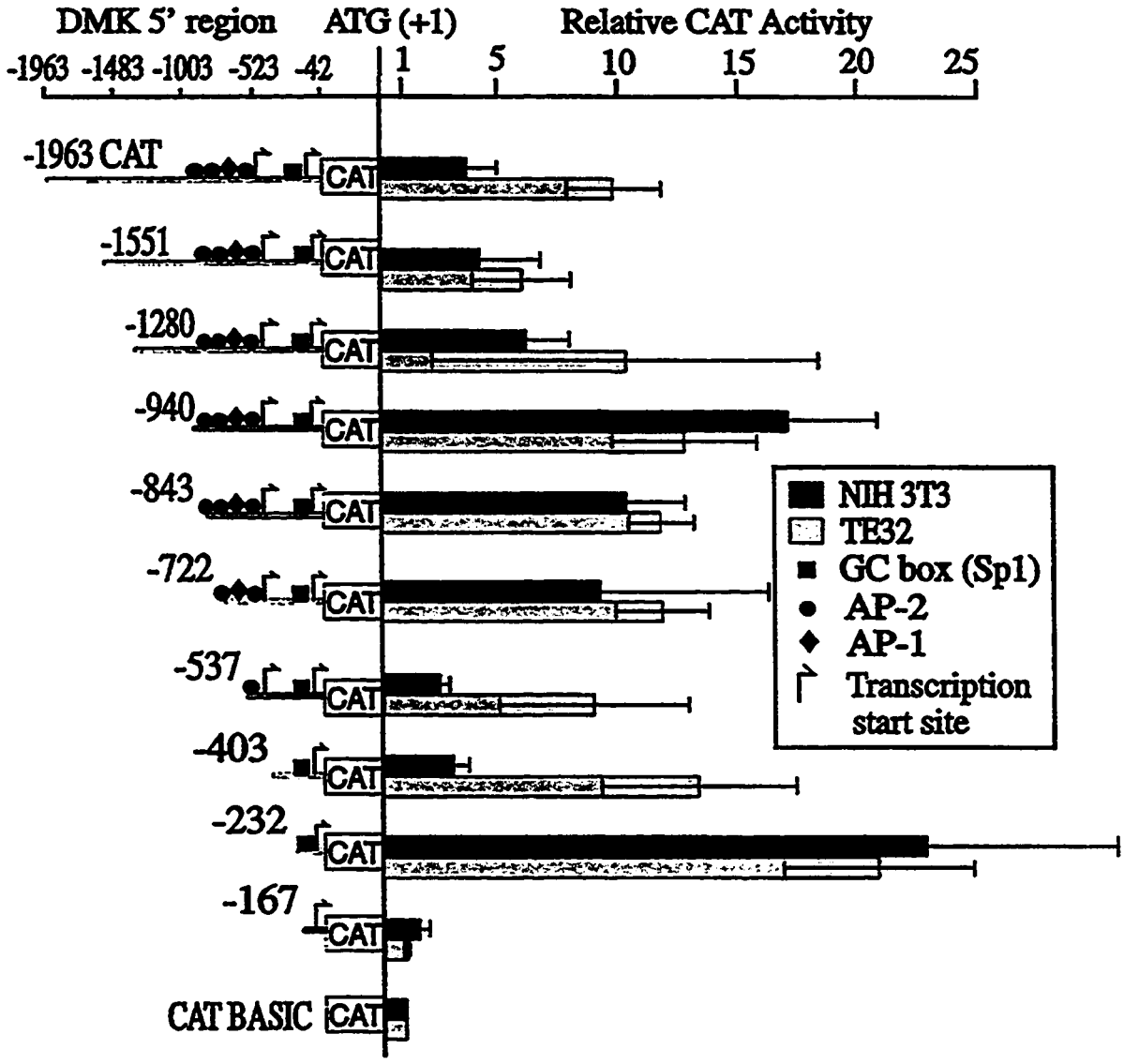
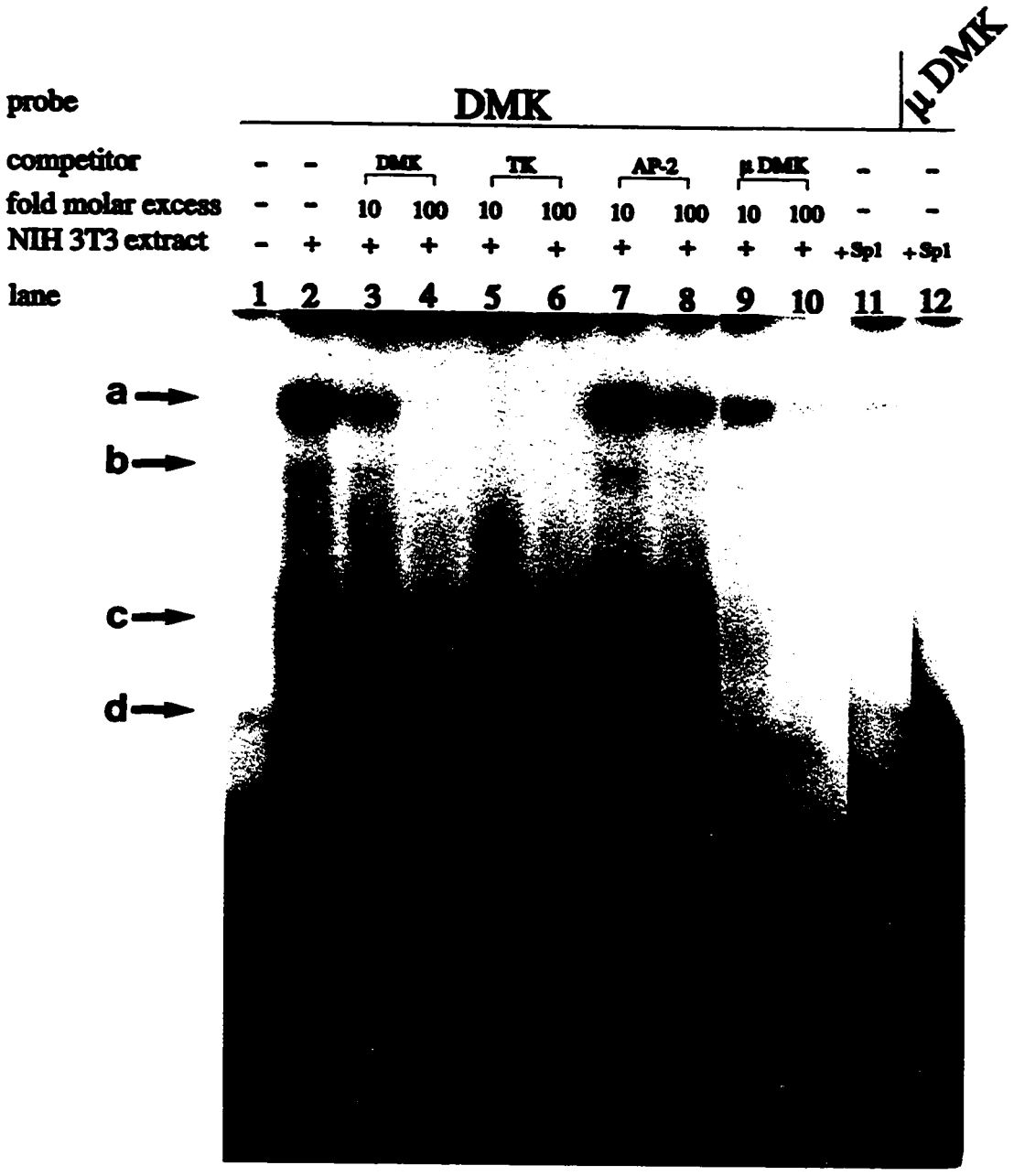

AP-2
AP-1
Sp1
(+1)

Figure 3-4. Activity of 5' end deletion mutants of DMPK promoter sequence in NIH 3T3 fibroblasts and TE32 myoblasts. Successive deletions of the DMPK 5' region were cloned upstream of the CAT reporter gene. The 3' terminus of each deletion construct is nucleotide -42 relative to the DMPK translation initiation codon. These constructs were co-transfected into NIH 3T3 fibroblasts and TE32 myoblasts with pSVβ-gal and CAT and β-galactosidase activities determined. Relative CAT activities are shown as a fold induction over the activity of the promoterless CAT-Basic construct. Symbols above the deletion constructs denote location of conserved consensus transcription factor binding sites, and *directional arrows* above deletion constructs represent locations of mapped transcription start sites. At least two separate experiments were performed with at least two samples for each construct. The averages of two experiments are shown. A minimal promoter was mapped to a 189-bp fragment of DMPK 5'untranslated region, but was not myoblast specific.


DMK 5' region
ATG (+1)
Relative CAT Activity
-1963 -1483 -1003 -523 -42
1 5 10 15 20 25
-1963 CAT
-1551
-1280
-940
-843
-722
-537
-403
-232
-167
CAT BASIC
CAT
NIH 3T3
TE32
GC box (Sp1)
AP-2
AP-1
Transcription start site

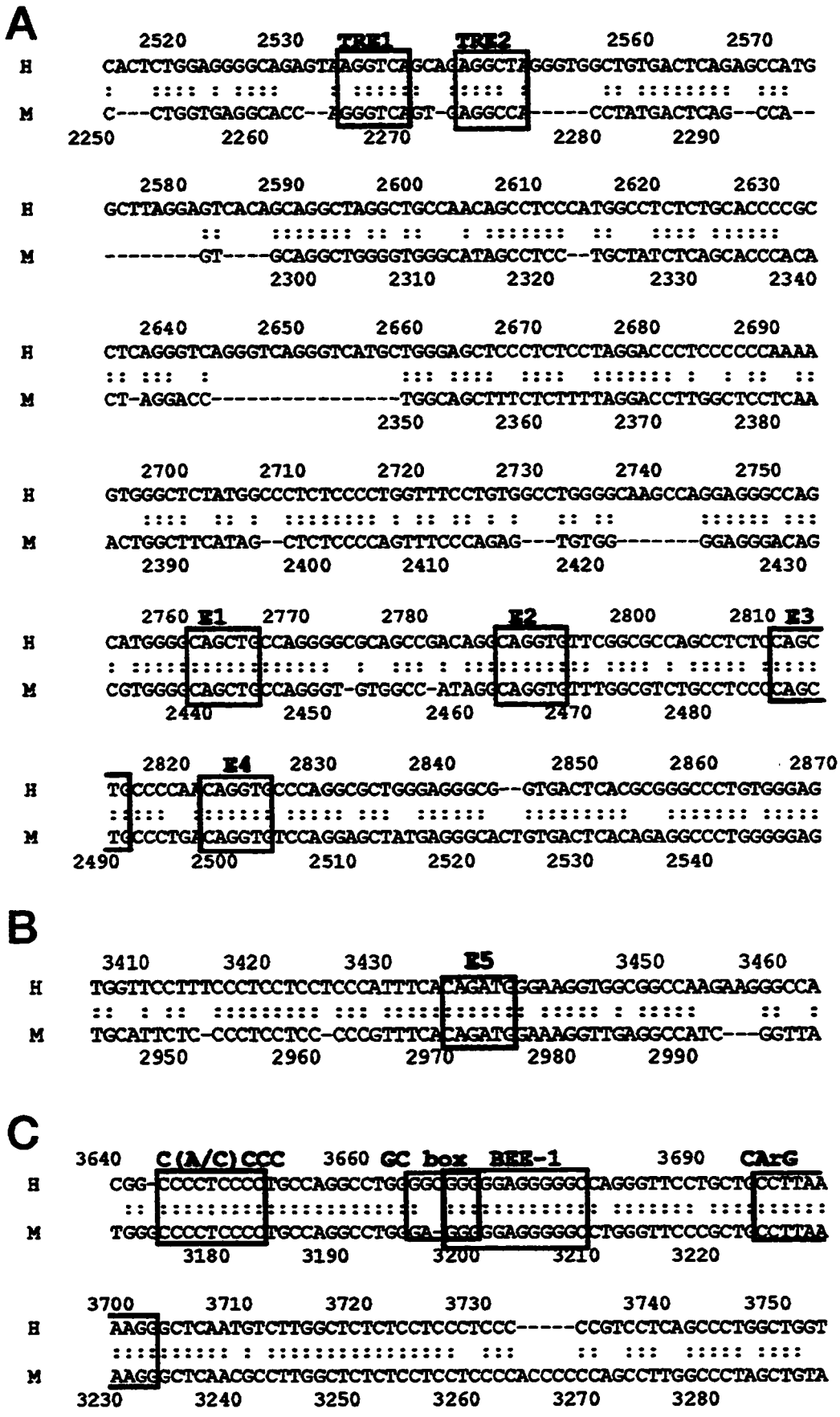
strengths of each construct between the two cell lines (figure 3-4). Modest myoblast-specific expression of CAT was observed for constructs -1963 CAT, -537 CAT, and -403 CAT (figure 3-4); however, most deletion fragments produced similar relative CAT activities in both cell lines, suggesting that only weak myoblast-specific regulatory elements are present in this region of the DMPK gene. A 189-bp deletion fragment of DMPK 5' sequence driving CAT exhibited the highest level of CAT expression in TE32, NIH 3T3, and several other cell lines tested, including HeLa and monkey kidney cells (data not shown). The strength of this promoter fragment was about half the strength of the phosphoglycerate kinase promoter in NIH 3T3 cells and 1 /10 the strength of this promoter in TE32 cells (data not shown). Removal of 66 bp from -232 CAT, which includes a conserved GC box (figure 3-3), resulted in near background CAT expression in all cell lines tested, suggesting important promoter elements reside within this sequence (figure 3-4). To investigate the possibility that Sp1 may bind this region of the DMK promoter and activate transcription, electrophoretic mobility shift assays were performed using a probe to the putative Sp1 binding site. Four apparent protein-DNA complexes were observed (a–d) (figure 3-5). Extracts from several cell lines, including TE32 myoblasts, yield identical banding patterns (data not shown) consistent with the CAT assay data, suggesting that this region of DMPK 5' sequence is part of a ubiquitous promoter element. Complex **d** appears to be probe as it is present in the lane containing only probe (figure 3-5, *lane* 1). Complex **c** can be competed for by wild type DMPK oligonucleotide, an oligonucleotide corresponding to a medium affinity Sp1 site (Jones et al 1985) from the thymidine kinase promoter (TK), an oligonucleotide corresponding to

Figure 3-5. Nuclear protein from NIH 3T3 cells forms a complex with DMPK promoter fragment. (A) 28-bp double-stranded oligonucleotide encompassing a conserved GC box in the DMPK promoter region was incubated with nuclear extracts from NIH 3T3 cells and purified Sp1 protein, subjected to competition analysis with various competitors, and analyzed by non-denaturing polyacrylamide gel electrophoresis. *Lane* 1 is probe by itself, and *lane* 2 is probe incubated with NIH 3T3 nuclear extract. *Lanes 3* and *4* are probe incubated with NIH 3T3 nuclear extract and cold competitor probe added at 10 and 100 fold molar excess, respectively. *Lanes 5* and *6* are the same as *lanes 3* and 4, except the competitor is a double-stranded oligonucleotide corresponding to a medium affinity GC box from the thymidine kinase promoter. In *lanes 7* and 8, a double-stranded oligonucleotide encompassing a binding site for the transcription factor AP-2 is competitor at 10- and 100-fold molar excesses respectively. A double-stranded oligonucleotide identical to the probe except for two nucleotides (see "Materials and Methods") is competitor in *lanes 9* and *10* at 10 and 100-fold molar excesses, respectively. In *lanes 11* and *12*, DMPK probe and mutant DMPK probe (*μDMK*) are incubated with purified Sp1 protein. Purified Sp1 binds to sequence from the DMPK promoter and forms a complex identical to one formed with NIH 3T3 extracts, implicating Sp1 in DMPK transcriptional activation.


probe
DMK
μDMK
competitor
DMK
TK
AP-2
μDMK
fold molar excess
10
100
NIH 3T3 extract
+Sp1
lane
1 2 3 4 5 6 7 8 9 10 11 12
a
b
c
d

GC-rich paired AP-2 sites from the SV40 early promoter (Mitchell et al 1987) and DMPK oligonucleotides mutated in two positions in the GC box *(lanes 3–10)*. This suggests that complex **c** represents a protein binding GC-rich sequences, but not specifically to the Sp1 binding site. The binding of the protein to the DNA in complexes **a** and **b** was specific as a molar excess of both the unlabeled DMPK probe (figure 3-5, *lanes 3* and 4) and TK oligonucleotide (figure 3-5, *lane 5* and 6) could compete for both complexes. Furthermore, only very weak competition was observed when oligonucleotides corresponding to the GC-rich binding site of the unrelated transcription factor AP-2 were used as competitor (figure 3-5, *lanes 7* and 8). In addition, the mutant DMPK oligonucleotide could only compete weakly for complexes **a** and b, suggesting the NIH 3T3 nuclear protein(s) are binding the GC box specifically (figure 3-5, *lane 9* and *10*). Also, this mutant oligonucleotide could not form complexes with NIH 3T3 nuclear extracts (data not shown). Purified Sp1 protein was able to form a complex with the DMPK oligonucleotide of identical mobility to complex **a** seen in *lane 2 (lane 11)*, but not with mutant oligonucleotide *(lane 12)*, suggesting that complex **a** is Sp1-bound to the DMPK oligonucleotide. Taken together, these results demonstrate that Sp1 binds specifically to a GC box located within a minimal region of the DMPK 5' sequence, which behaves as a minimal promoter, suggesting a key role for Sp1 in the basal activity of this DMPK promoter element. Evidence of only minor increases in CAT transcription in myoblasts over fibroblasts found with control elements in the DMPK 5' region prompted us to look within the first intron for other sequences that may increase CAT expression in myoblasts. Mouse and human DMPK sequences were aligned (figure 3-6) and complete conservation of five potential E-boxes, a C(A/T)CCC box, a BEE-1

Figure 3-6. Sequence comparison between mouse and human DMPK first introns. Shown in the figure are: positions and nucleotide comparisons for thyroid hormone response elements and the first four conserved E-boxes (A). (B), position and nucleotide conservation of the fifth conserved (downstream) E-box. (C), location and conservation of a C(A/T)CCC box, BEE-1 element, GC box, and CArG box.


A
TRE1
TRE2
E1
E2
E3
E4
B
E5
C
C(A/C)CCC
GC box
BEE-1
CArG

element (Feo et al 1995), a CArG box (Ernst et al 1991; Sartorelli et al 1990), a GC box (Jones et al 1985), and two thyroid hormone response elements (Muscat et al 1994) was revealed. To explore the possibility that functional muscle-specific regulatory elements are present in the first intron, a 2-kb fragment (Fig. 3-9A) containing most of the first intron was cloned downstream of the CAT gene in both orientations in the presence of a 679-bp fragment of DMPK 5' sequence (figure 3-7A). These constructs were then transfected into TE32 and NIH 3T3 cells. Figure 3-7 shows that the presence of the first intron, irrespective of orientation, in combination with DMPK 5' sequence, can activate transcription of the CAT gene up to 6-fold in TE32 myoblasts, whereas no activation was observed in NIH 3T3 fibroblasts. This demonstrates the existence of an orientation-independent myoblast-specific transcriptional enhancing element within the first intron of the DMPK gene. The muscle-specific transcription factor MyoD has the capability of activating the myogenic program by inducing the expression of muscle-specific genes via E-boxes located in their transcriptional regulatory regions (Davis et al 1987; Olson 1990). To further define a myoblast-specific enhancer role for the first intron, we investigated its responsiveness to MyoD and whether it could function in the context of a heterologous promoter. Constructs in which the first intron was cloned upstream or downstream of the TK promoter driving the CAT gene were generated (figure 3-8A) and co-transfected with a MyoD expression vector (EMSV-MyoD) or with parental vector (EMSV) into C3H10T1/2 fibroblasts. This cell line can be readily differentiated into myoblasts by transfection of members of the MyoD family of myogenic regulators, and this system is used to indicate MyoD responsiveness of co-transfected *cis* sequences (Davis et al 1987). Also, a construct containing the ninth intron (TK CAT I9R), which is similar in

Figure 3-7. Ability of DMPK first intron element to activate muscle specific expression of CAT with a fragment of the DMPK promoter. (A), the DMPK first intron was positioned downstream of CAT in both orientations (IVS + orientation, SVI - orientation) to test for enhancer activity with a 679-bp fragment of DMPK 5' upstream region containing promoter sequence (-722 CAT). (B), these constructs, as well as the parental construct (-722 CAT), were co-transfected with pSV β-gal into NIH 3T3 fibroblasts and TE32 myoblasts, CAT and β-galactosidase activities were assayed, and relative CAT activities determined. The value of 1 was arbitrarily assigned to the relative activities of -722 CAT in both cell lines, and activities of all other constructs were determined relative to these constructs. Three separate experiments were performed with at least three samples for each construct. Data from a representative experiment are shown. The DMPK first intron, independent of orientation, activates expression of CAT via the DMPK promoter specifically in myoblasts.

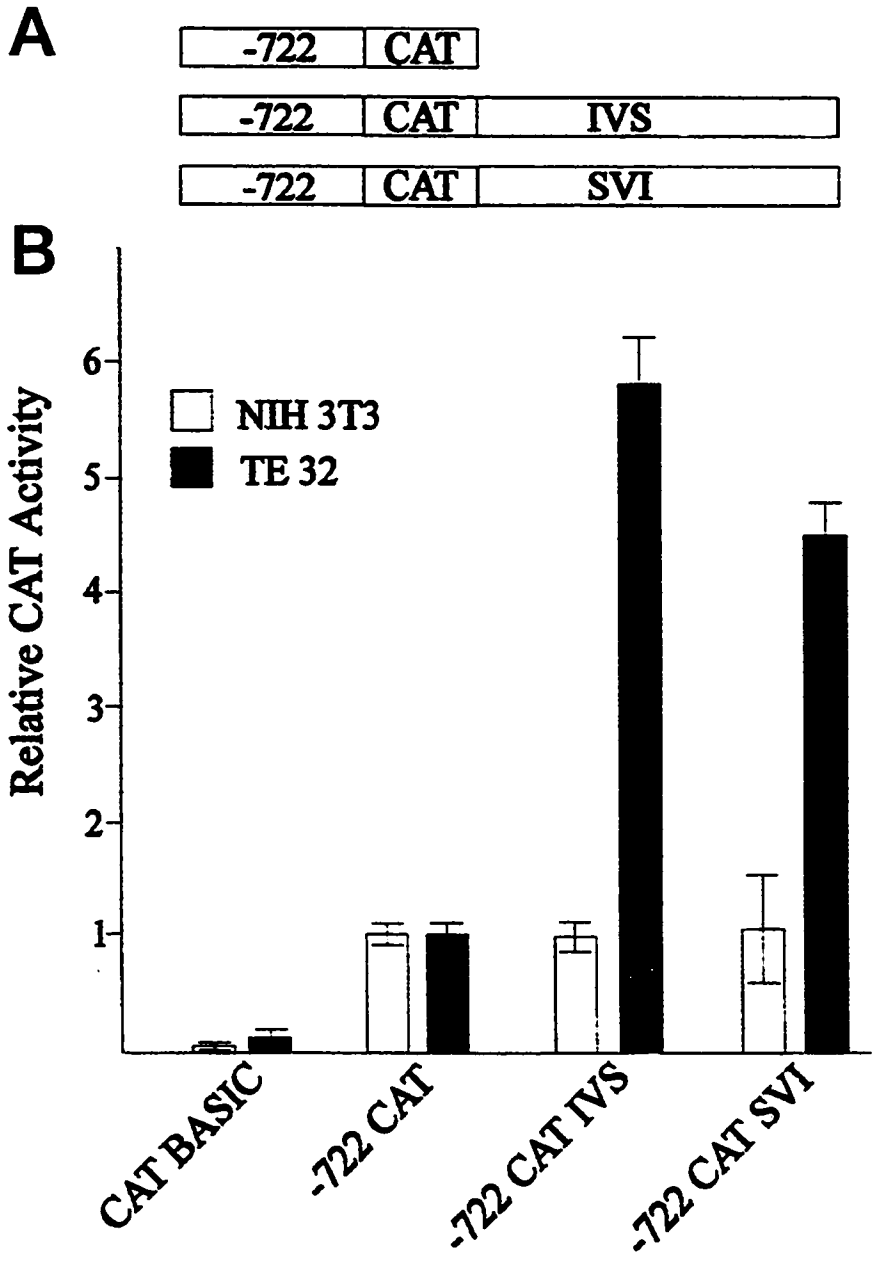

A
-722
CAT
-722
CAT
IVS
-722
CAT
SVI
B
Relative CAT Activity
6
5
4
3
2
1
NIH 3T3
TE 32
CAT BASIC
-722 CAT
-722 CAT IVS
-722 CAT SVI

Figure 3-8. Responsiveness of DMPK first intron element to MyoD. (A), the DMPK first intron was positioned in both orientations either downstream of the CAT gene driven by the TK promoter or upstream of TK driving CAT. As a control, the DMPK ninth intron was positioned in the reverse orientation downstream of the CAT gene driven by the TK promoter. (B), these constructs were co-transfected with pSVβ-gal into C3H10T1/2 cells either with EMSV-MyoD or the EMSV parental vector. CAT and β-galactosidase activities were assayed and relative CAT activities determined. The relative activity of TK CAT in EMSV-trans-fected cells was assigned the value of 1, and all other CAT activities were adjusted relative to this value. Three separate experiments were performed with at least three samples of each construct. Data from a representative experiment are shown. MyoD overexpression can activate expression of a CAT reporter driven by the TK promoter up to 20-fold in the presence of the DMPK first intron, but not the ninth intron.

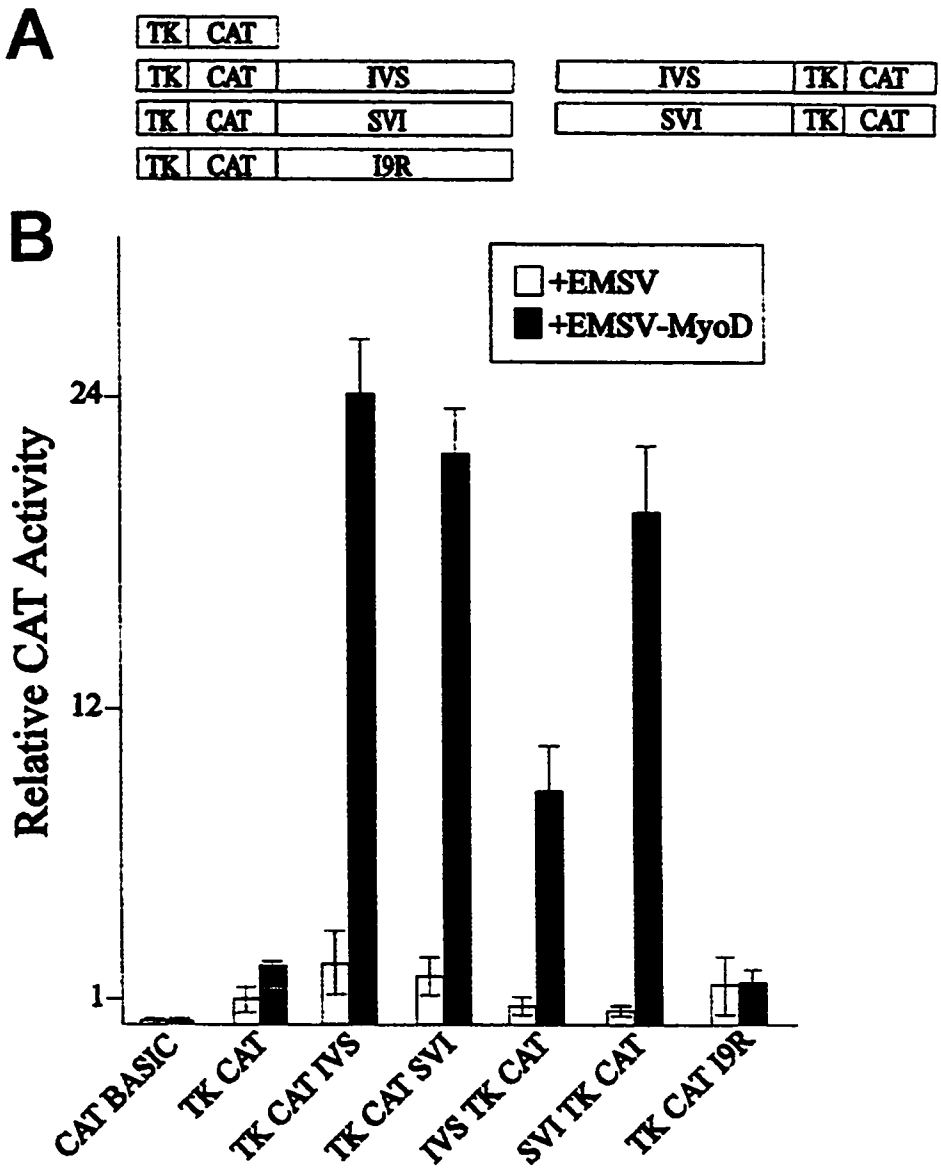
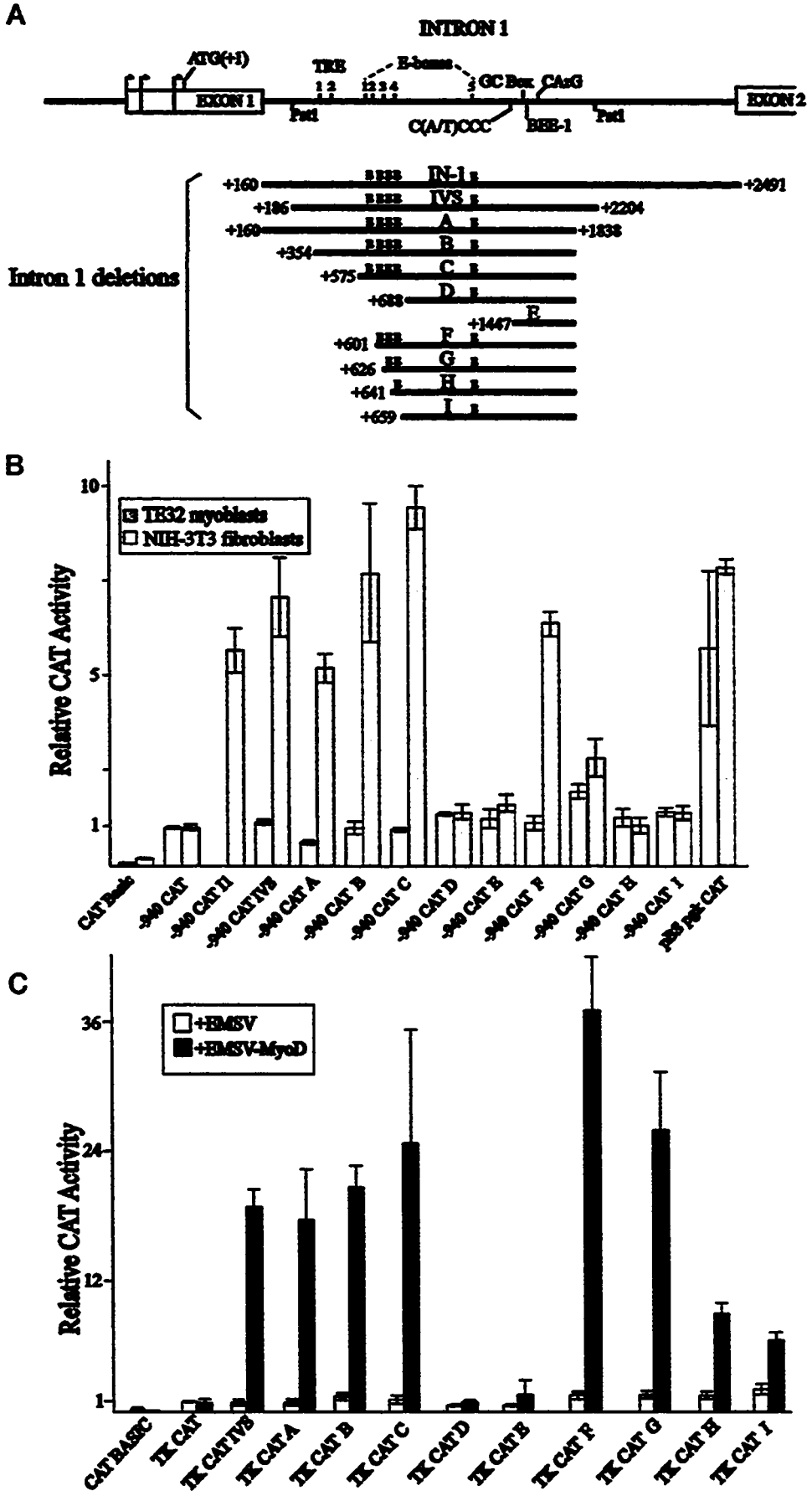

A
TK
CAT
IVS
SVI
I9R
B
+EMSV
+EMSV-MyoD
Relative CAT Activity
24
12
1
CAT BASIC
TK CAT
TK CAT IVS
TK CAT SVI
IVS TK CAT
SVI TK CAT
TK CAT I9R

Figure 3-9. Deletion mapping of DMPK first intron enhancer. (A), schematic map of DMPK first intron showing putative binding sites for various transcription factors. *IN*-1, entire 2.4-kb first intron; *IV*S, 2-kb first intron fragment; *A*–I, various first intron deletions; E, E-box. *Numbers* indicate 5' and 3' ends of first intron fragments relative to ATG initiation codon (see Fig. 3-3). (B), mapping of first intron sequences required for maximal enhancer activity in TE32 myoblasts. Constructs were prepared and transfected as outlined in Fig. 7 and under "Materials and Methods". The experiment was performed three times with similar results obtained in each. Shown is a representative experiment. (C), mapping of sequences required for full activity of the DMPK first intron enhancer in 10T1/2 cells co-transfected with MyoD. Constructs were prepared and transfected as described in the legend to Fig. 3-8 and under "Materials and Methods". The experiment was performed three times with similar results obtained in each. Shown is a representative experiment. Deletion of three of the five E-boxes in the DMPK first intron almost completely abolishes enhancer activity in both cell types.


A
INTRON 1
ATG(+1)
TRE
E-boxes
GC Box
CArG
EXON 1
EXON 2
Pst1
C(A/T)CCC
BBB-1
Pst1
Intron 1 deletions
IN-1
IVS
A
B
C
D
E
F
G
H
I
+160
+2491
+186
+2204
+1838
+354
+575
+683
+1447
+601
+626
+641
+659
B
TE32 myoblasts
NIH-3T3 fibroblasts
Relative CAT Activity
10
5
1
CAT Basic
-940 CAT
-940 CAT II
-940 CAT TVB
-940 CAT A
-940 CAT B
-940 CAT C
-940 CAT D
-940 CAT E
-940 CAT F
-940 CAT G
-940 CAT H
-940 CAT I
pBS pgK CAT
C
+EMSV
+EMSV-MyoD
Relative CAT Activity
36
24
12
1
CAT BASIC
TK CAT
TK CAT TVB
TK CAT A
TK CAT B
TK CAT C
TK CAT D
TK CAT E
TK CAT F
TK CAT G
TK CAT H
TK CAT I

size to the first intron and also contains a similar number of possible E-box elements, was included in the transfections as a control (figure 3-8A). Expression of CAT from constructs containing the first intron was activated up to 20-fold over the activity of TK CAT in the presence of exogenous MyoD (figure 3-8B). This level of *trans*-activation, in relation to the TK promoter, was consistent regardless of orientation and position of the first intron element. Furthermore, the presence of the ninth intron only weakly enhanced expression of CAT in the presence of MyoD (figure 3-8B). Therefore, the 2-kb first intron fragment acts as a MyoD-responsive enhancer element in myoblasts. However, it is unknown if MyoD interacts directly with first intron elements or has a *trans* effect through the activation of another transcription factor gene. We investigated this question by performing deletion analysis of the entire first intron, particularly of the first four conserved E-boxes. For these experiments, a 897 bp fragment of the DMPK promoter was used to drive the CAT gene, because it contains all possible transcription start sites. Deletion of 653 bp from the 3' end of the entire 2.4-kb first intron (IN-1) had no effect on CAT activity, indicating this region is dispensable for enhancer function (figure 3-9, *A* and B, compare 940 CAT IN-1 to 940 CAT A). Conversely, deletion from the 5' end appeared to relieve a slight inhibition (compare 940 CAT A, B, and C). Deletion of 113 bp, which includes the first four conserved E-boxes from the 5' end of 940 CAT C resulted in complete loss of enhancer activity, implicating these elements in enhancer function (compare 940 CAT C and 940 CAT D). Further deletions within the 113-bp region revealed that loss of 51 bp from the 5' end of 940 CAT C, which corresponds to deletion of two of the four E-boxes, severely compromised enhancer activity (compare 940 CAT C to 940 CAT G) and deletion of a further 15 bp completely abrogated

enhancer activity (compare 940 CAT C to 940 CAT H) (figure 3-9B). The enhancer deletion mutants failed to activate CAT expression in NIH 3T3 fibroblasts (figure 3-9B). These data indicate that a 51-bp region within the DMPK first intron containing two conserved E-box elements is required for enhancer function in TE32 myoblasts. To investigate the role of the E-boxes in the observed activation of CAT expression in 10T1/2 cells by MyoD, the same first intron deletion fragments were cloned downstream of the CAT gene driven by the thymidine kinase promoter. Maximal activity is observed with TKCAT F but is reduced dramatically with each E-box deletion (compare TK CAT F, G, and H), although some residual activity remains in deletions TK CAT H and I. Therefore, the first three E-boxes are required for maximal stimulation of CAT activity when MyoD is co-transfected with the first intron deletion constructs into 10T1/2 cells, implicating these elements as key mediators of the DMPK first intron enhancer function.

3.4 DISCUSSION

This is the first report describing regulatory elements controlling mRNA expression of the DMPK gene. Initially we identified transcription start sites within the 5' region of the DMPK gene and also confirmed one start site previously reported at nucleotide position 1394 (-773 relative to ATG translation initiation codon) (Mahadevan et al 1993). This site was first mapped using primer extension in a cell-free extract system that utilized a cloned fragment of DMPK 5'-untranslated region from -700 to -1550 relative to the ATG codon and therefore may have missed transcription start sites downstream from -700. Primer extension analysis of human TE32 myoblast and HFSF fibroblast RNA mapped two major transcriptional start sites at position -71 and -421 and also several minor sites. The relative importance of each of these start sites in various tissues is not

clear. The varied transcriptional start sites may be due to the lack of a canonical CCAAT and TATA box in the DMPK promoter region, resulting in multiple sites of transcription initiation often observed with promoters lacking these consensus sequences (Gudas et al 1992; Voss et al 1991; Ziegler et al 1991). However, most housekeeping and growth control gene promoters have their many initiation sites scattered within a 15–20-bp region (Sehgal et al 1988). Exceptions to this include the Ha-*ras* gene, which has multiple start sites located within a 90-bp region (Lu et al 1994), and the 3.7-kb mRNA for testicular inhibin/activin β-subunit, which initiates transcription from multiple sites over 150 nucleotides (Feng et al 1995). We believe that transcription initiation from the mapped start site locations (-71, -421, -773) in the DMPK 5' region are controlled by separate promoter elements, and we are currently investigating this possibility. Housekeeping style promoters have been found for such protein kinase genes as casein kinase II subunit β (Voss et al 1991), the murine *hck* gene (Ziegler et al 1991), choline kinase R (Uchida 1994), and the β-adrenergic receptor kinase (Itoh et al 1992). This study sought to identify *cis* elements that may activate expression of DMPK preferentially in myoblasts over fibroblasts. It is likely that distinct *cis* elements located elsewhere in the gene impact transcription of DMPK in other tissues. Increased levels of DMPK mRNA in myoblasts *versus* fibroblasts may be due either to an increase in DMPK transcription in myoblasts or to differential mRNA stability. In an effort to identify myoblast-specific *cis* elements, a series of CAT deletion mutants of DMPK 5' sequence were constructed and tested for CAT activity in TE32 myoblasts and NIH 3T3 fibroblasts (figure 3-4). However, only minor myoblast-specific expression of CAT (2–3-fold) was observed for deletion clones -1963 CAT, -537 CAT, and -403 CAT (figure 3-4), and this

activity alone cannot account for the increased levels of DMPK mRNA observed in myoblasts over fibroblasts. Two peaks of promoter activity mapped within the DMPK 5' region by transfection of CAT deletion constructs into NIH 3T3 cells (figure 3-4) was consistent with the mapped transcription start sites at position 2421 and 271. Of all the DMPK 5' region deletions, maximal promoter activity was observed with the 189-bp -232 CAT deletion mutant in all cell lines tested (figure 3-4). Low level DMPK mRNA expression was detected in many cell lines by RNase protection (data not shown) and Northern blot analysis (figure 3-1), suggesting a nearly ubiquitous low level presence of DMPK mRNA in several tissue types. We believe that transcription initiating from the -71 start site contributes to this basal level of DMPK mRNA. Promoter activity of the 189-bp -232 CAT was lost upon the deletion of 66 bp from this sequence (figure 3-4). Our data clearly demonstrate that the ubiquitous transcription factor Sp1 binds to an oligonucleotide probe containing a conserved GC box located within this deleted sequence. Furthermore, this binding is specific and purified Sp1 incubated with this oligonucleotide forms an identical complex (complex a, figure 3-5) to the slowest migrating complex observed with NIH 3T3 nuclear extracts. This evidence suggests that Sp1 binds to the GC box contained in this sequence, thus contributing to a ubiquitous basal level of transcription in several cell types. The promoter analysis data indicated that *cis* elements controlling the majority of DMPK transcription in myoblasts were most likely located elsewhere in the gene (figure 4). In support of this, transgenic mice expressing β-galactosidase from a 3.7-kb fragment of the DMPK promoter region exhibit mainly neural-specific expression (King et al 1996). Several muscle-specific genes have tissue-specific regulatory elements contained within their first introns (Christensen et al

1993; Feo et al 1995; Sternberg et al 1988; Yutzey et al 1989). Therefore we analyzed the DMPK first intron for conserved muscle-specific transcription factor consensus binding sites and found five E-boxes and a CArG element (figure 3-6) representing MyoD (Lassar et al 1989) and muscle actin promoter factor 1 and 2 (Ernst et al 1991) binding sites, respectively. We tested the ability of the DMPK first intron to *trans*-activate CAT gene expression in the context of a 679-bp fragment of its homologous promoter (figure 3-7A). A 4–6-fold orientation independent up-regulation was observed in TE32 myoblasts, but not in NIH 3T3 fibroblasts, demonstrating that the DMPK first intron contains a myoblast-specific enhancer element (figure 3-7B). The MyoD family of basic helix loop helix myogenic regulators bind the sequence CANNTG to activate muscle-specific gene transcription (Davis et al 1987; Lassar et al 1989). Existing evidence observed with P19 embryonal carcinoma cells and DM patient fibroblasts, both overexpressing a stably integrated MyoD gene, suggests that DMPK mRNA expression may be regulated either directly or indirectly by one of these transcription factors (Davis et al 1997; Otten & Tapscott 1995; Skerjanc et al 1994). We tested the responsiveness of the DMPK first intron to MyoD. This myogenic regulator could *trans*-activate expression of CAT in the presence of the DMPK first intron, but not the similarly sized ninth intron in 10T1/2 cells. Regardless of position or orientation around the TK promoter (figure 3-8) or the DMPK promoter (data not shown), CAT expression was *trans*-activated through the DMPK first intron by MyoD. Deletion analysis of the first intron revealed that a 51-bp sequence containing two conserved E-boxes is required for full enhancer function in TE32 myoblasts. Deletion of three of the first four E-boxes was required for near-complete abrogation of enhancer function in 10T1/2 cells co-transfected

with MyoD and CAT first intron deletions driven by the TK promoter. Since E-boxes are the only known *cis* elements present within this sequence, and given their complete conservation between mouse and human sequence (figure 3-6), it is very likely that factors binding these elements mediate enhancer function. It is apparent that the first three E-boxes each contribute in an additive fashion to enhancer activity as the sequential removal of each results in a decrease in activity in both TE32 and 10T1/2 cells co-transfected with MyoD. Mutating each of the four E-boxes within the 113-bp segment of first intron sequence will allow assessment of their relative importance for enhancer function. Interestingly, it has been shown that certain rhabdomyosarcoma cell lines, including TE32, are deficient in a MyoD co-factor that prevents them from fusing and forming multinucleate myotubes (Tapscott et al 1993). It is possible that in this cell line MyoD may be able to up-regulate a subset of genes not involved in the fusion process in the absence of this co-factor. An alternative explanation is that one of the other myogenic factors or another transcription factor may activate DMPK expression through the 51-bp element in the first intron. In any case, the first three E-boxes in the DMPK first intron are clearly required for maximal DMPK enhancer function and likely play an important role in expression of the gene in muscle.

In summary, DMPK transcription in myoblasts is at least partially regulated by a 51-bp MyoD-responsive element located in the first intron of the gene. This sequence cooperates with DMPK promoter elements, one of which binds Sp1, to upregulate transcription in myoblasts. These data, in conjunction with other studies showing MyoD responsiveness of the DMPK transcript (Davis et al 1997; Otten & Tapscott 1995;

Skerjanc et al 1994), implicate this myogenic factor in regulating transcription of this gene in muscle.

CHAPTER 4 EXPRESSION OF DMPK 3'UTR SENSITIZES MYOBLASTS TO APOPTOSIS

4.1 INTRODUCTION

DM is clinically divided into two groups. Adult DM, also known as classical DM, is characterized by myotonia, progressive weakness and wasting of select groups of muscles, cardiac conduction abnormalities, endocrine dysfunction, cataracts and premature balding (Harper 1989). Congenital (c)DM is characterized mainly by immaturity of skeletal muscle resulting in muscle hypotonia, severe respiratory distress, feeding difficulties, talipes, ptosis and an abundance of immature muscle fibres and muscle satellite cells. Importantly, myoblasts isolated from adult and cDM patients show a marked delay in the myogenic fusion process.

To date, the CTG expansion is the only DM mutation identified. There are no reports documenting premature stop codons, or large deletions within the DMPK gene suggesting that the CTG repeat does not cause loss of DMPK function. Two DMPK knockout mouse models report no DM specific features supporting the suggestion that DMPK protein function is not compromised in DM (Reddy et al 1996) (Jansen et al 1996). One exception exists. On closer inspection of one knockout model, it appears that loss of DMPK protein gives rise to cardiac conduction abnormalities similar to those observed in DM making it possible that loss of DMPK may play a role in cardiac features of the adult disease (Berul et al 2000). However, DMPK protein levels in patient heart samples assayed by western blot and immunofluorescence studies indicate only slight reductions of DMPK in this tissue (Maeda et al 1995).

Protein levels in skeletal muscle of DM patients have been reported as reduced (Fu et al 1993; Koga et al 1994), however, the antibody used in these studies recognizes a 54 KDa protein species which is not DMPK (Lam et al 2000). Other studies report slight (Maeda et al 1995) or no change (Dunne et al 1996b) in DMPK protein levels in patient tissues. Using an antibody reactive to proteins of 71 and 82 KDa that was verified using transgenic and knockout muscle extracts, our laboratory found slightly reduced DMPK levels in adult DM patient muscle samples and slightly elevated DMPK levels in cDM muscle protein extracts (Narang et al 2000). This suggests that in muscle, a loss of DMPK is not likely the cause of DM.

The location of the CTG repeat in the 3' UTR of the DMPK gene led some to believe that the CTG repeat may impact the expression of Six 5, a gene downstream of DMPK (Boucher et al 1995). Six 5 transcript levels in patient tissue have been reported as both decreased (Thornton et al 1997) (Klesert et al 1997) and increased (Eriksson et al 1999; Hamshere et al 1997). Genetic ablation of Six 5 in mice results in cataract formation in these mice suggesting a minor role for this gene in DM pathogenesis (Klesert et al 2000; Sarkar et al 2000).

Increased mRNA levels from congenital DM muscle and brain were reported (Sabourin et al 1993) and it was suggested that the CUG structure in the 3' UTR of the mutant mRNA may confer a gain of function to this molecule. Consistent with this notion, overexpression of the DMPK 3' UTR with wild type (Sabourin et al 1997) (Okoli et al 1998) or mutant (Amack et al 1999; Bhagwati et al 1999; Usuki et al 1997) numbers of CTG repeats inhibits myoblast differentiation suggesting that 3' UTR

elements can function in a dominant manner. Myoblasts from DM patients exhibit a similar delay in the myogenic fusion process.

Interestingly, it was reported that CUG repeats could form hairpin structures *in vitro* (Gacy et al 1995). Remarkably, mutant DMPK mRNA was shown by *in situ* hybridization to be trapped in the nuclei of patient cells suggesting that the CTG repeat was interfering with RNA processing and/or export (Taneja et al 1995). Further, the double stranded RNA activated protein kinase (PKR) was found to bind to CUG repeats in the mutant DMPK mRNA and become activated in a CUG length dependent manner *in vitro*. Therefore, it appears that the mutant DMPK message may become trapped in the nuclei of patient cells activating inappropriately ds RNA binding proteins, like PKR, which are capable of inducing apoptosis.

Apoptosis represents a normal, genetically controlled process for disposing of cells that are irreversibly damaged, no longer useful or pose a threat to the survival of an organism (Evan & Littlewood 1998). Although this process is poorly understood in muscle, it is known that a proportion of myoblasts induced to differentiate succumb to apoptosis (McClearn et al 1995; Sandri & Carraro 1999). In addition, a view currently emerging is that apoptosis of muscle nuclei is a normal part of muscle adaptation in response to atrophy (Allen et al 1997; Hikida et al 1997).

In muscle disease, apoptosis appears to be an important component of pathology in Duchenne's muscular dystrophy, SMA, merosin deficiency, and Limb girdle muscular dystrophy type IIA (Sandri & Carraro 1999). Myotubes cultured from Duchenne muscular dystrophy and merosin deficiency patients display increased levels of apoptosis compared to control myoblasts (Sandri et al 1998b; Vachon et al 1996).

Many muscle diseases (dystrophinopathies) result from disruption of the dystrophin complex which serves to connect extracellular matrix components with the muscle fibre sarcolemma (Cherin 1995). This complex is thought to protect the muscle fibre from detrimental effects associated with repeated contraction and relaxation of the muscle. It is evident that apoptosis is at least partially responsible for loss of muscle fibre during the course of disease progression of dystrophinopathies (Sandri & Carraro 1999). DM, which by the very nature of the mutation and the protein(s) affected is not a dystrophinopathy. However, like these diseases, DM patients experience fibre atrophy (Harper 1989) accompanied by apoptosis of myonuclei (Yamada et al 2000). Therefore, the mechanism leading to apoptosis during progression of DM most certainly results from a different mechanism than dystrophinopathy associated apoptosis.

Our aim is to understand the molecular mechanism of the apoptotic component associated with DM. To this end, we have constructed stable cell lines expressing the DMPK 3' UTR with normal (11) and mutant (99) CTG repeats in the mouse myogenic cell line C2C12 and assayed susceptibility of these cell lines to apoptosis in comparison to control cell lines. In addition, we have assayed activity of immunoprecipitated PKR following introduction of 11 and 99 CTG repeat DMPK 3' UTR containing constructs into C2C12 cells. We have also inhibited PKR activity in stable cell lines to address PKR's role in cell death induction. Furthermore, we have assayed apoptotic susceptibility of patient amniocytes and myoblasts in comparison to matched controls. Results indicate that expression of the DMPK 3' UTR and the presence of CTG repeat containing RNA renders cells susceptible to cell death and apoptosis via a PKR activation pathway. This data brings to light the fragile condition of DM cells and suggests a

framework for understanding a molecular mechanism possibly leading to DM symptoms revealing possible targets for treatment.

4.2 MATERIALS AND METHODS

4.2.1 TRANSGENE CONSTRUCTION

The transgene used in production of stable cell lines and transgenic mice was constructed by fusing sections of the DMPK promoter/enhancer with the GFP cDNA and the DMPK 3' UTR. A 1.5 Kb Eco RV/Sma I fragment from the DMPK gene 5' region encompassing the promoter was ligated into a blunted Sst II site in pBluescript (SK-) and designated pBS A. Next, a 2.4 Kb portion of the DMPK first intron including the DMPK muscle specific enhancer element was PCR amplified with primers containing Not I sites. The amplicon was digested and ligated as described above into dephosphorylated pBS A linearized with Not I to give pBS AB. A smaller piece of DMPK genomic sequence including the final stretch of the first intron plus a portion of the second exon was PCR amplified with primers containing Spe I sites. This fragment was cloned into linearized pBS AB digested with Spe I as described above to give pBS ABC. The GFP cDNA was excised from pEGFP-N1 (Clontech) using Bam HI and Not I (blunt). This fragment was cloned into pBS ABC digested with Bam HI and EcoRI (blunt) to form pBS ABCD. The DMPK 3' UTR containing 11 CTG repeats was PCR amplified using primers with Xho I and Xba I sites respectively and cloned into pCR 2.1 (Invitrogen). The 3' UTR fragment was excised from pCR 2.1 using Xho I and ligated into pBS ABCD linearized with Xho I to give pBS $ABCDE_{11}$. Finally, the 3' end of the DMPK gene containing 11 or 91 CTG repeats was isolated from pcDNA3-CAT-3' UTR (11 CTG repeats) or pcDMK 9-1 (99 or 91 CTG repeats) (Dr. Monica Narang) with Bam HI (blunt) and Hind III and ligated into

pBS ABCDE digested with EcoRV/Hind III to give the final contructs of pBS $ABCDEF_{11,\ 91\ or\ 99}$. Restriction enzyme and sequencing analysis were performed to ensure all clones were composed of the correct sequence.

4.2.2 CLONING OF UTR FRAGMENTS DOWNSTREAM OF CMV GFP

The CMV promoter was cloned upstream of GFP and the DMPK 3' UTR in pBS ABCDEF 11 and 99 by removing the DMPK promoter/enhancer element with an Sst I blunt/Bam HI partial digest and replacing it with a Nru I/Bam HI fragment containing the CMV promoter.

4.2.3 TRANSFECTION AND SELECTION OF STABLE CELL LINES

C2C12 myoblasts were transfected using the calcium phosphate method (Graham & van der Eb 1973) or the lipofectamine (Gibco-BRL) according to manufacturer's instructions. Briefly, 5 μg of CsCl purified plasmid DNA was added to an eppendorf tube containing 250 μl of 1X HBS pH 7.05-7.12 (25 mM Hepes, 140 mM NaCl, 5 mM KCl, 0.75 mM Na_2HPO_4 $2H_2O$, 6 mM dextrose) and mixed. To this, 12.5 μl of 2.5 M $CaCl_2$ was added dropwise, vortexed for 3-5 seconds and incubated at room temperature for 20 minutes. $CaPO_4$ precipitates were added to 2.5 X 10^5 cells in a 60 mm culture dish and incubated overnight. Cells were washed twice in 1X PBS and incubated for 3 minutes in 2 ml of 15 % glycerol in 1X PBS, washed once more in 1X PBS. Growth medium was added to the cells for 48 hours after which 750 μg/ml of Geneticin (Gibco-BRL) was added to the cells to initiate selection for stable integration. Selection in Genetecin was maintained for 9-11 days after which individual clones were isolated by limiting dilution in 96 well plates and assessed for GFP expression by fluorescence microscopy. Cells were diluted at a density of one cell per ml in the presence of 750

μg/ml Geneticin and 200 μl of cell suspension was added to each well. Individual clones were expanded, frozen and analyzed for expression of the transgene.

4.2.4 MYOBLAST DIFFERENTIATION VIABILITY ASSAYS

Stable cell lines expressing GFP with the DMPK 3' UTR with either 11 or 99 CTG repeats or the Pgk 3' UTR were plated in growth medium at a cell density of 5 X 10^5 cells in 35 mm culture dishes. The following day, growth medium was replaced with differentiation medium (D-MEM containing 2% horse serum). Twenty-four hours later, 5 μl of Hoechst 33342 (1 mg/ml) was added to the cells for 5 minutes and returned to the CO_2 incubator. The medium was removed, the cells washed once with 1X PBS and 500 μl of 1X PBS containing 2 μl of propidium iodide (PI) solution (1 mg/ml) was added to the cells. Two photographs were taken at 420 nm (UV, Hoechst) and 570 nm (red – PI) using a Zeiss Axiophot fluorescent microscope within two minutes of addition of PI. Percentages of cell death were determined by counting the number of cells stained red by PI divided by number of nuclei stained blue by Hoechst 33342 X 100. Cells were deemed apoptotic by identifying chromosomal condensation in nuclei of cells staining positive for both Hoechst 33342 and PI (Sun et al 1992).

Alternatively, $5X10^5$ cells were plated on day1. On day 2, growth medium was replaced with differentiation medium for 4 or 12 hours. Cells were washed twice in cold 1X PBS. Cells were then bathed in annexin V binding buffer (10 mM HEPES/NaOH [pH 7.4], 140 mM NaCl and 2.5 mM $CaCl_2$). Annexin V- phycoerthryin (10 μl – Molecular Probes) was added and cells were incubated in the dark at room temperature for fifteen minutes. DAPI (1μl of 1mg/ml) was added for 5 minutes and annexin V positive/DAPI negative cells were counted from images obtained using a Zeiss axiophot

fluorescent microscope and Northern eclipse imaging software. Seven fields for each cell line were counted.

4.2.5 STAUROSPORINE SENSITIVITY ASSAYS

Patient, normal cells and stable cell lines expressing GFP, the DMPK 3' UTR with either 11 or 99 CTG repeats or a Pgk control 3' UTR were plated in 96 well plates in triplicate from 2.5 X 10^4 to 1.0 X 10^5 cells per well in 200 µl of growth medium. The following day, growth medium was replaced with medium containing 1% fetal bovine serum with no drug, DMSO, or staurosporine (dissolved in DMSO) at concentrations ranging from 10 to 1280 nm staurosporine. After a four hour incubation period, medium was removed and replaced with fresh 1% FBS medium. Twenty-four hours later, 20 µl of WST-1 reagent (Boehringer-Mannheim) was added to the cells for two hours and then analyzed at 450 and 650 nm in a plate reader (Molecular Devices Spectra-Max 340) to determine metabolic activity.

4.2.6 ECDYSONE INDUCIBLE CELL LINE CONSTRUCTION

Ecdysone inducible cell lines were constructed by co-transfection of the hormone receptor plasmid (pVgRXR-Invitrogen) and pIND GFP11 or pIND GFP99 using lipofectamine (Gibco-BRL) according to manufacturer's instructions. Forty-eight hours post-transfection, zeocin (Invitrogen) (250 µg/ml) or bleocin (Calbiochem) (32 µg/ml) and Geneticin (Gibco-BRL) (750 µg/ml) were added to the cells and resistant clones were analyzed and expanded as described above.

4.2.7 CSCL RNA ISOLATION

In order to ensure liberation of CTG repeat containing mRNA from foci (Davis et al 1997), RNA was isolated using a CsCl gradient. Briefly, monolayers from two

confluent 100 mm culture dishes were harvested by scraping in 1X PBS and cells collected in eppendorf tubes. Cells were then disrupted in denaturing buffer (6M guanidinium isothyocyanate, Tris, EDTA, NaCl) by multiple passes through a syringe with a 231/2 guage needle. One gram of CsCl per ml of homogenate was dissolved in the lysate and then layered over a 5.7M cushion of CsCl. Samples were loaded into an SW41 rotor and centrifuged for 20 hrs at 30000 rpm in a Beckman TL-90 ultracentrifuge. Following centrifugation the supernatant was removed using a pasteur pipette, the pellet air dryed for 5 minutes and then resuspended in 50 µl of sterile, RNase free dH_2O. RNA was then analyzed by spectrophotometry to determine concentration.

4.2.8 RNASE PROTECTION ANALYSIS

To assess expression levels of inducible GFP-DMPK 3'UTR mRNA, total mRNA isolated from cells induced with muristerone for 0, 6, 24 or 48 hours was hybrized to a 396 bp probe from within the GFP gene and a 190 bp probe from within the mouse GAPDH cDNA overnight at 55°C. Probes were synthesized from plasmid DNA templates linearized downstream of the GFP and GAPDH inserts respectively utilizing the T7 promoter. Template DNA (5 µg) was digested as described (Plasmid minipreps and DNA digestion). Digested template DNA solution was brought to 50 µl with sterile RNase free water and extracted twice with an equal volume of phenol/chloroform (1:1). The DNA was precipitated by adding 0.1 volumes of 3M sodium acetate pH 5.2 and 2.5 volumes of cold 100% ethanol followed by mixing, a 10 minute incubation on ice and centrifugation at 10000 rpm for 10 minutes at 4°C. Template DNA was washed with cold 70% ethanol, air dried for 5 minutes and resuspended in 20 µl of sterile RNase free water. Probe labelling was performed in the presence of ^{32}P-UTP using an Ambion RNA probe

synthesis kit according to manufacturer's instructions. Briefly, about 250 ng (1 µl) of linearized template was mixed with 2µl of transcription buffer, 1 µl each of rCTP, rATP, rGTP (10 mM), 2 µl of rUTP (50 µm), 2 µl of T7 polymerase (5 U/µl) 1 µl of RNase inhibitor (RNA guard, 30 U- Pharmacia) , 2 µl of ^{32}P-UTP (40 µCi) and 8 µl of sterile RNase free dH_20. The probe synthesis reaction was incubated at 37°C for 30 minutes following mixing and a pulse spin to pellet the reaction constituents. Following probe synthesis, 1 µl of DNase I (2 U) was added to the tube and incubated at 37°C for 15 minutes. Yeast tRNA (2 µl of 10 mg/ml), 27 µl of sterile RNase free water and 50 µl of phenol/chloroform (1:1) was added to the synthesized RNA which was then vortexed for 20 seconds and spun at 14000 rpm for 3 minutes. Probe was precipitated by addition of 200 µl of 2.5 M ammonium acetate and 750 µl of 100% ethanol, incubated on ice for 20 minutes and spun for 20 minutes at 14000 rpm at 4°C. This was repeated once. Probe was washed by addition of 0.3 M sodium acetate in 75% ethanol, air dried for 5 minutes and resuspended in sterile RNase free dH_20. Probe was quantified by scintillation counting and resuspended at a concentration of 100000 CPM/µl.

RNase protection was performed using the RNAP kit from Ambion according to manufacturer's instructions. First, labelled probe and target RNA were co-precipitated. Equal volumes of GFP and GAPDH probes were mixed and 3 µl of this mixture was incubated with 5 µg of total RNA, 5 µl of 5M ammonium acetate, sterile RNase free dH_2O and 125 µl of 100% ethanol. This was mixed, incubated at –20°C for 15 minutes, spun at 14000 rpm for 15 minutes at 4°C, washed with 70% ethanol and air dried for 5 minutes. The pelleted probe/RNA mixture was resuspended in 20 µl of hybridization buffer (80% formamide, 10 mM sodium citrate pH 6.4, 300 mM sodium acetate pH 6.4,

1mM EDTA), heated at 90°C for 4 minutes and incubated at 55°C overnight. The following day, probe/RNA hybrids were digested with 100 µg/ml RNase A/T mixture in 200 µl of RNase digestion buffer (10 mM Tris-HCl pH 7.5, 300 mM NaCl, 5 mM EDTA) for 30 minutes at 37°C. Hybrids were precipitated by adding 300 µl of precipitation solution, mixing, incubating at –20°C for 15 minutes and centrifuged at 14000 rpm for 15 minutes at 4°C. Precipitated RNA/probe duplexes were air dried for 5 minutes, resuspended in 8 µl of gel loading buffer (95 % formamide, 0.025 % bromphenol blue, 0.025 % xylene cyanol, 18 mM EDTA, 0.025 % SDS) denatured at 90°C for 4 minutes and loaded on a 5% denaturing polyacrylamide gel. The gel was run for 1.5 hours at 250 volts, transferred to Whatman 3 CHR paper, covered with plastic wrap and exposed to X-ray film.

4.2.9 IMMUNOPRECIPITATION KINASE ASSAY

C2C12 myoblasts were transfected by the lipofectamine method as described with CMV GFP Pgk, CMV GFP_{11} or CMV GFP_{99} and cell lysates were prepared 48 hours later in kinase lysis buffer (20 mM Tris-HCl pH 7.5, 200 mM NaCl, 25 mM KCl, 0.5 % TritonX-100, 1 mM $MgCl_2$, 10 % glycerol). The cell lysates were spun for 5 minutes at 10000 rpm at 4°C and aliquots of the supernatant were transferred to a new tube. Antibody (1:100 antibody:lysate) to the desired antigen was added to the lysates and incubated overnight at 4°C while rotating. The following day, protein A/G-agarose beads (50 µl of a 50 % slurry) were added and incubated at 4°C on a rotator for 1 hour. Samples were spun for 5 minutes at 10000 rpm at 4°C and supernatants were discarded. Immunoprecipitates were incubated in kinase buffer (20 mM HEPES pH 7.5, 2 mM $MgCl_2$, 2 mM MnCl, 50 mM KCl plus 1.5 mM $NaVO_3$, 100 µm ATP, 0.1 mm DTT) and

10 μCi γ-^{32}P-ATP for 30 minutes at 30°C. Sample buffer (3X) was added and reaction products were boiled for 5 minutes, loaded on a 10 % SDS-polyacrylamide gel and electrophoresced as described. The gel was transferred to Whatman 3MM paper, covered with plastic wrap and subjected to autoradiography.

4.2.10 FLOW CYTOMMETRY AND CELL DEATH DETECTION WITH ANNEXIN V

C2C12 cells were treated with various concentrations of staurosporine for four hours and prepared for flow cytommetry the following day by trypsinization. Floating cells in the medium were combined with trypsinized cells, washed in cold 1X PBS and spun for 5 minutes at 1200 rpm. Washing was repeated and cells were resuspended in annexin V binding buffer (10 mM HEPES/NaOH [pH 7.4], 140 mM NaCl and 2.5 mM $CaCl_2$). Annexin V- phycoerthryin (10 μl – Molecular Probes) was added, the cells were mixed gently and incubated in the dark at room temperature for fifteen minutes. Cells were analyzed using the FL-3 photomultiplier tube of a Coulter flow cytommeter (Epics-XL) to determine levels of annexin V immunoreactivity.

4.3 RESULTS

We had previously established that overexpression of the DMPK 3'UTR in myoblasts resulted in a delay in terminal differentiation (Sabourin et al 1997). Also, work in our laboratory demonstrated that overexpression in mice of the entire DMPK gene, including the DMPK 3' UTR with 22 CTG repeats, resulted in many features of DM including central nuclei in muscle fibres, type I fibre atrophy, ringed fibres, mental abnormalities and retinal degeneration (Narang 2000). Therefore, we hypothesized that expression of the DMPK 3' UTR without the DMPK coding region might result in DM-like pathology

in transgenic mice. We designed two transgenes for oocyte injection utilizing endogenous DMPK regulatory elements, and the reporter gene GFP containing two versions of the 3'end of the DMPK gene including the 3' UTR, one with 11 CTG repeats (wild type) and one with 99 CTG repeats (mutant) (figure 4-1). Since trinucleotide repeats exhibit instability in bacteria, the transgene plasmids were analyzed by restriction digest and sequencing to ensure sequences were correct. Figure 4-2 A and B show that the 99 CTG repeat tract was completely in tact. These transgenes were initially tested for myoblast specific expression by transient transfection in C2C12 myoblasts and were capable of production of GFP in vitro (data not shown). Stable cell lines were then generated by co-transfection of the CTG 11, CTG 99 and control constructs (figure 4-1) with a plasmid carrying a neomycin resistance marker. To ensure that messenger RNA of the appropriate size was expressed from the transgenes; northern blot analysis was performed using RNA isolated from various neomycin resistant clones. As expected, SV40 poly A signal clones, which lack a 3' UTR, had the smallest message. Pgk 3' UTR clones exhibit a larger message than the SV40 poly A signal clones and the DMPK 3'UTR clones' mRNA was larger yet (see arrows, figure 4-3). Large transcripts are observed in the Pgk and DMPK 3' UTR clones but not the SV40 poly A clones (see arrowhead, figure 4-3). This is due to relatively weak polyadenylation signals in the DMPK and Pgk 3' UTR sequences compared to the SV40 poly A signal and results in read through transcription.

Initial analysis of neomycin resistant clones revealed evidence of cell death, particularly with the CTG 99 clones. Myoblast differentiation to multinucleate myotubes requires growth serum removal from the culture medium. During this process, many

Figure 4-1. DMPK promoter and 3' UTR – GFP constructs. The GFP cDNA was cloned downstream of a 1.5 Kb fragment of 5' UTR sequence of the DMPK gene corresponding to the promoter region and a 2.4 Kb fragment corresponding to the DMPK first intron. Conserved consensus translation initiation sites within the DMPK promoter region and first intron sequence were deleted. DMPK sequence corresponding to exons 13–15 and the 3'UTR with 11 CTG repeats (A), or 99 CTG repeats (B) were cloned downstream of the GFP cDNA. For control 3' UTR sequences, the Pgk 3' UTR or the SV40 poly A signal were cloned downstream of the GFP cDNA (C). Transcription initiation sites within the DMPK promoter sequence identified previously are indicated with arrows.

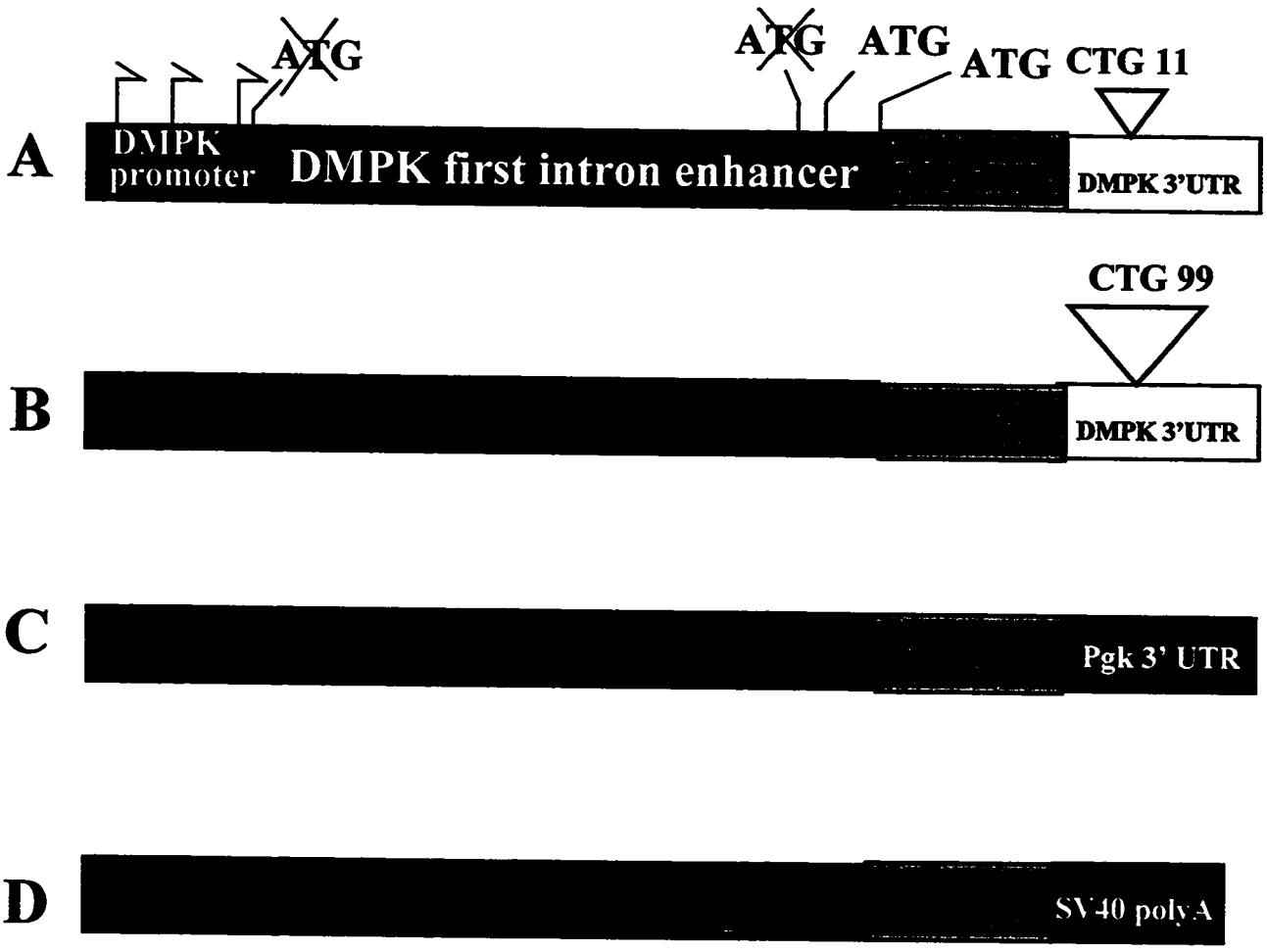
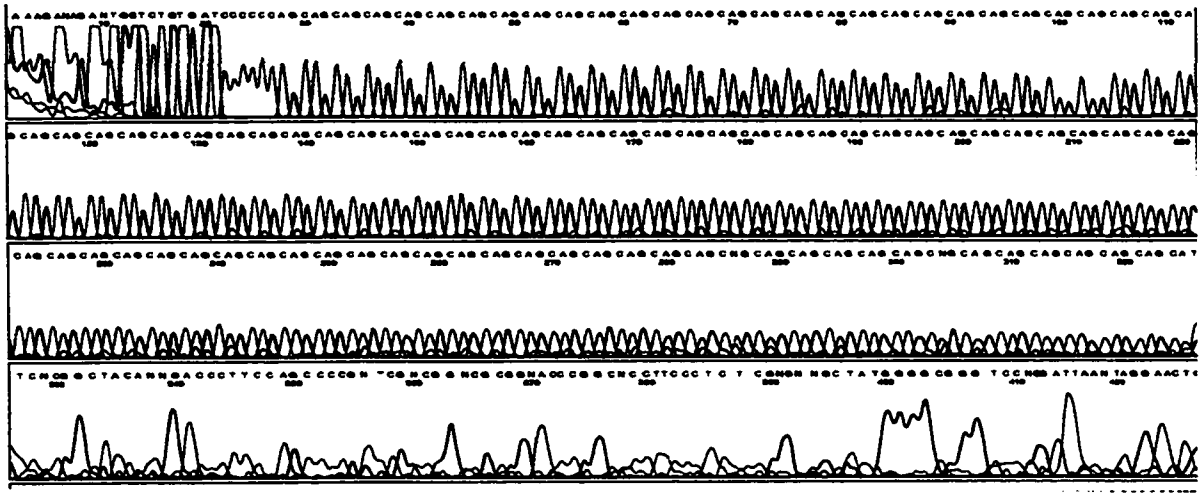

A
ATG
ATG
ATG
ATG
CTG 11
DMPK promoter
DMPK first intron enhancer
DMPK 3'UTR
CTG 99
B
DMPK 3'UTR
C
Pgk 3' UTR
D
SV40 polyA

Figure 4-2. Sequence analysis of repeat region of 99 CTG repeat transgene construct. The 99 CTG repeat transgene construct was sequenced on the top (A) and bottom (B) strands to ensure that the CTG repeat tract was uninterrupted. The sequence on both strands revealed 99 perfect CTG/CAG repeats.

A



B

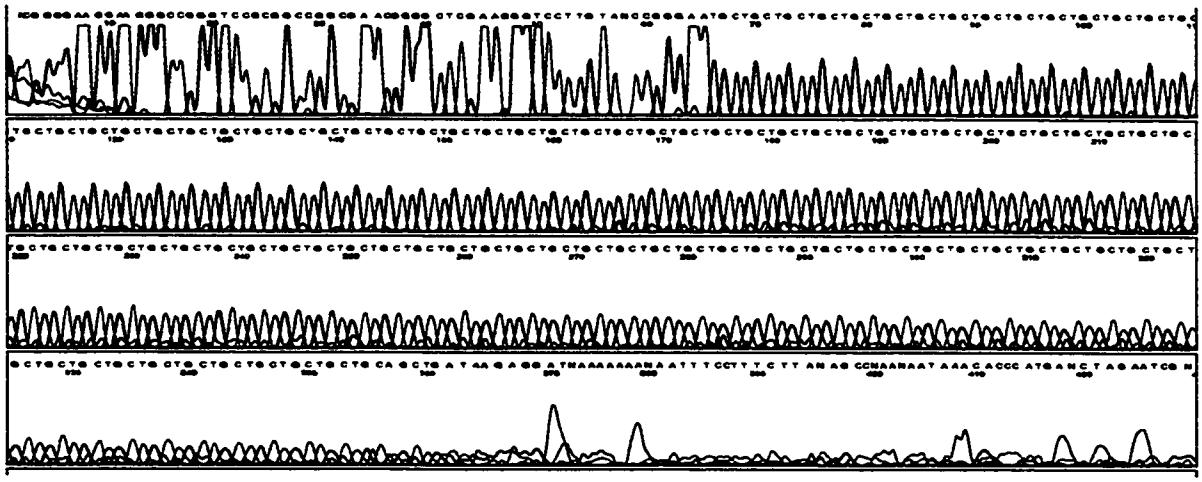
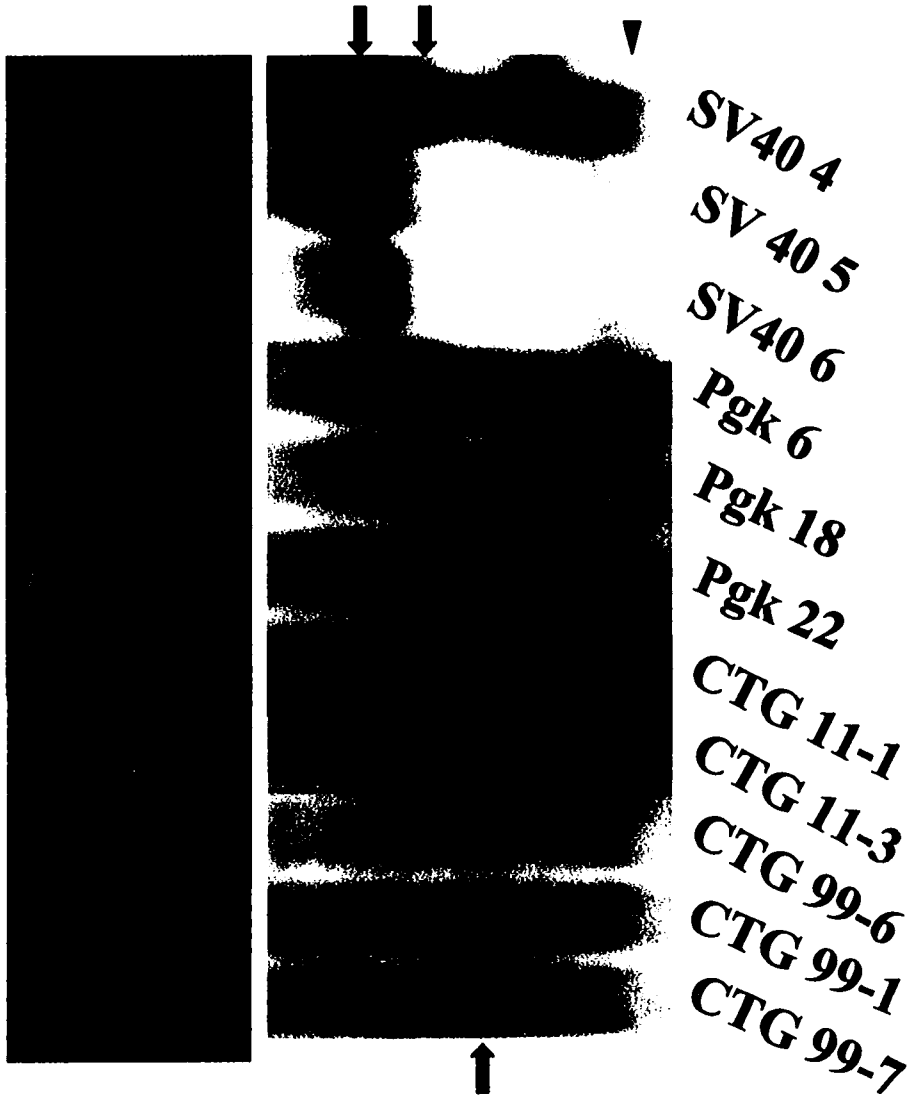


Figure 4-3. Northern Blot of control and DMPK 3'UTR expressing cell lines. To confirm expression of the GFP mRNA in all cell lines, 4 μg of total RNA was run on a formaldehyde agarose gel and transferred to uncharged nylon membrane. The northern blot was probed with the GFP cDNA and subjected to autoradiography. Three cell lines with an SV40 polyadenylation signal at the 3' end but no 3' UTR as well as three cell lines with a Pgk 3' UTR were included as controls. Two cell lines with 11 CTG repeats and three lines with 99 CTG repeats within the DMPK 3' UTR were also included on the blot. In all cases, mRNA molecules were of the correct size and GFP was part of the mRNA molecule. The lack of a large molecular weight mRNA in the cell lines with the SV40 poly A signal suggests that this species observed in the other cell lines is the result of inefficient polyadenylation. Position of the GFP mRNA from SV 40 poly A cell lines (black arrow) and Pgk 3'UTR cell lines (green arrow) are shown. In addition, the positions of the predominant mRNA species of DMPK 3' UTR CTG 11 and CTG 99 cell lines (blue arrow) and larger readthrough transcripts (black arrowhead) are shown.

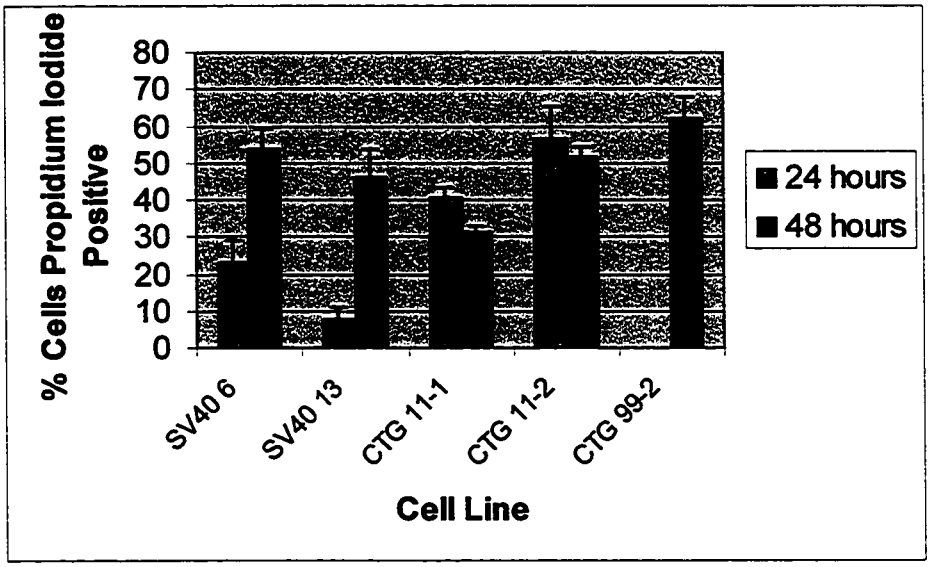

SV40 4
SV 40 5
SV40 6
Pgk 6
Pgk 18
Pgk 22
CTG 11-1
CTG 11-3
CTG 99-6
CTG 99-1
CTG 99-7

mononucleate cells fuse to form multinucleate myotube structures, however, some myoblasts not able to exit the cell cycle and fuse with other cells die by apoptosis. This is considered a normal feature of myogenesis (Sandri & Carraro 1999). We analyzed two CTG 11, one CTG 99 and two control clones with an SV40 poly A signal at the 3' end for apoptosis following growth factor withdrawal at 24 and 48 hours using differential dye uptake. Addition of the permeable dye Hoechst 33342 to cells results in blue staining of all nuclei still containing DNA (figure 4-4A). However, propidium iodide (PI), which also stains nucleic acids, cannot normally permeate the cell and only cells with compromised membrane integrity resulting from apoptosis or necrosis stain positive for this dye (figure 4-4A) (Sun et al 1992). After 24 hours in low serum medium, CTG 11 cell lines exhibited a greater amount of cell death when compared to control cell lines expressing GFP with an SV40 poly A signal (figure 4-4B). By 48 hours post serum withdrawal, the percentage of PI positive cells had equilibrated between the CTG 11 cell lines compared with the control cell lines. This data indicates that expression of the DMPK 3' UTR results in elevated differentiation associated cell death in myoblasts. Cells exhibiting chromatin condensation and nuclear shrinkage were prevalent suggesting that the observed cell death is apoptotic (figure 4-4A). After 48 hours, percentages of cells from control and CTG 11 and 99 cell lines undergoing cell death were similar indicating that the increased cell death at 24 hours is transient. To confirm if the observed cell death was apoptotic, we employed annexin V staining in combination with DAPI, a non-permeable dye that stains chromatin. Cells positive for annexin V but negative for DAPI are indicative of an early stage of apoptosis (Vermes et al 1995). As a positive control for the assay, the control cell line Pgk 25 was treated

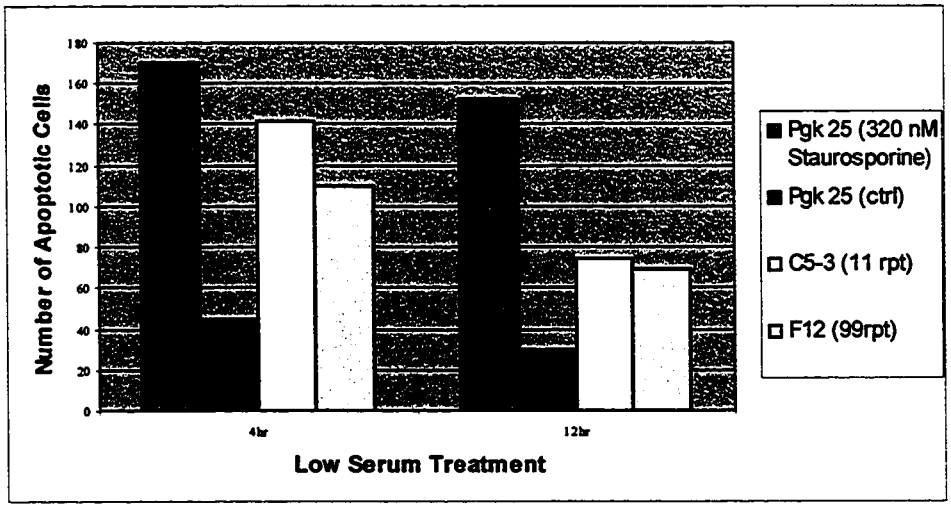
Figure 4-4. Differentiation induced cell death is higher in DMPK 3'UTR cell lines. Various control and DMPK 3'UTR cell lines were subjected to serum withdrawal and then assayed for viability using the cell permeable nuclear stain Hoechst 33342 and the cell impermeable nuclear stain propidium iodide. Cells containing nuclei positive for both dyes have compromised cell membranes and are undergoing cell death. (A) Cells stained with Hoechst 33342 (blue) and propidium iodide (red). (B) The two CTG 11 cell lines have increased cell death compared to the control cell lines. (C) Annexin V in combination with DAPI was used to identify early apoptotic cells following serum withdrawal. Staurosporine treatment (320 nm) was used as a positive control for apoptosis. At 4 and 12 hours following serum withdrawal, DMPK 3' UTR cell lines are more susceptible to apoptosis than control cells.

A

B


% Cells Propidium Iodide Positive
80
70
60
50
40
30
20
10
0
SV40 6
SV40 13
CTG 11-1
CTG 11-2
CTG 99-2
Cell Line
24 hours
48 hours

C


Number of Apoptotic Cells
180
160
140
120
100
80
60
40
20
0
4hr
12hr
Low Serum Treatment
Pgk 25 (320 nM Staurosporine)
Pgk 25 (ctrl)
C5-3 (11 rpt)
F12 (99rpt)

with 320 nm staurosporine. At 4 hours following serum withdrawal, DMPK 3' UTR cell lines with 11 and 99 CTG repeats show elevated levels of apoptotic cells compared with the control cell line Pgk 25 (figure 4-4C). Although there is a less dramatic difference, this pattern holds up at 12 hours following serum withdrawal.

To further define apoptotic sensitivity of CTG 11 and CTG 99 cell lines, we determined whether cell lines expressing the DMPK 3' UTR were more susceptible than controls to staurosporine, an inhibitor of protein kinase C. To establish that staurosporine could induce apoptosis in the parental C2C12 cell line, an assay was developed utilizing annexin V-PE staining and flow cytommetry. Annexin V is a membrane phospholipid normally present on the interior surface of the cell membrane, however, during early events of apoptosis, annexin V is flipped to the exterior of the cell membrane without compromising membrane integrity (Vermes et al 1995). As staurosporine concentration increases, an increasing number of cells stain positive for annexin V indicating that staurosporine kills C2C12 myoblasts in a dose dependent manner (figure 4-5). Staurosporine sensitivity was then investigated in CTG 11, 99 and control cell lines. At 80 and 320 nm, the Pgk control cell lines were largely unaffected by the drug exhibiting survival rates at or near 100%. However, at 1280 nm, the Pgk cell lines succumb to the effects of staurosporine and % survival ranges from about 5-50% (figure 4-6A). Significantly, CTG 11 and 99 cell lines are susceptible to staurosporine at much lower concentrations. At 20 nm, like the Pgk 3' UTR control lines, CTG 11 cell lines are almost completely resistant to the effects of staurosporine while CTG 99 cell lines exhibit survival rates between 65 and 100% (figure 4-6, A-C). At 80 nm staurosporine, % survival for CTG 11 cell lines ranged from 40 to 75% (figure 4-6B) while CTG 99 cell

Figure 4-5. Parental C2C12 myoblasts exhibit apoptotic staurosporine sensitivity. C2C12 myoblasts were treated with various concentrations of staurosporine, incubated with Annexin V –phycoerythrin and subjected to flow cytometry analysis. These cells are sensitive to staurosporine and die in a dose dependent manner by an apoptotic process as measured by Annexin V –phycoerythrin fluorescence.

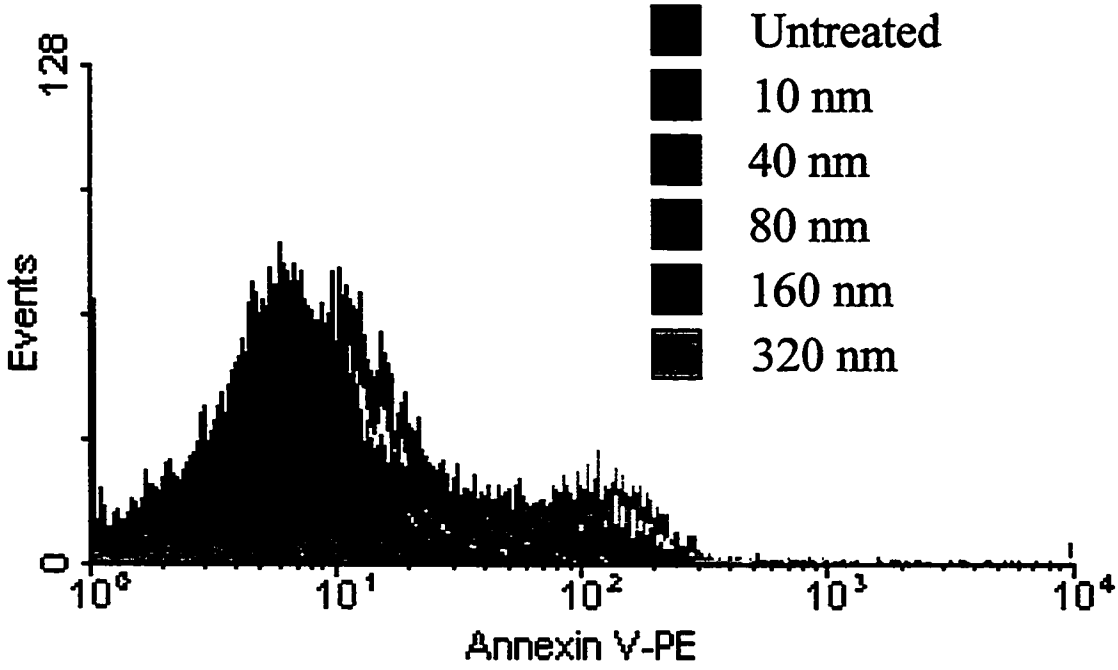
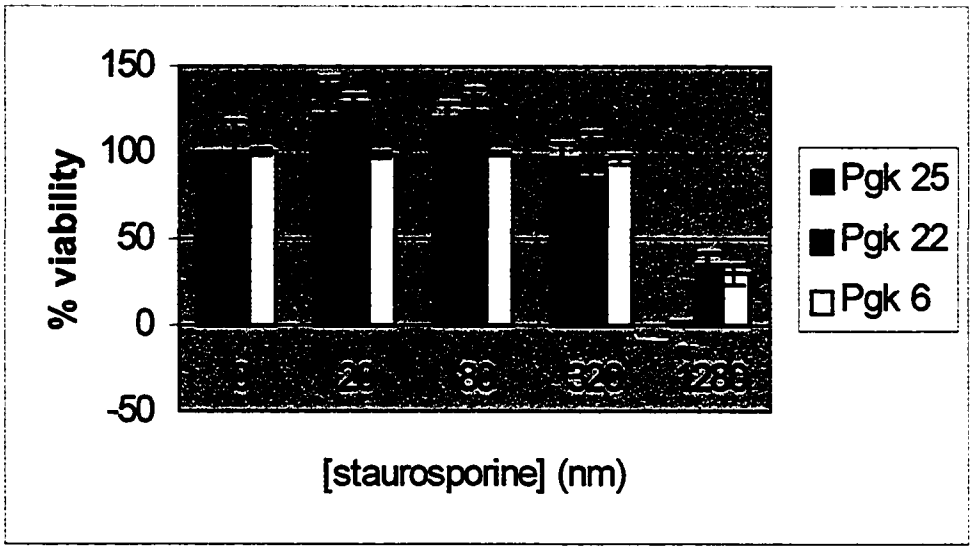
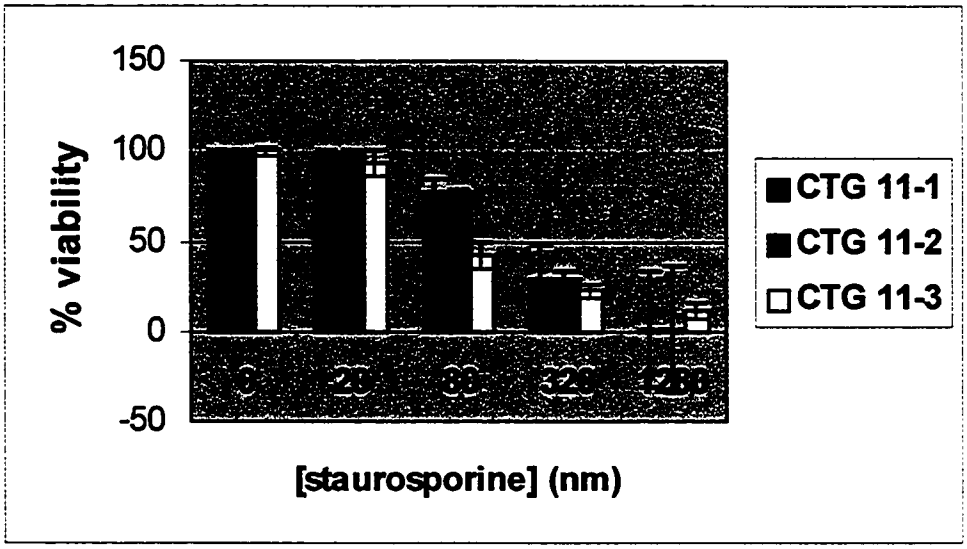

Untreated
10 nm
40 nm
80 nm
160 nm
320 nm
128
0
Events
10^0
10^1
10^2
10^3
10^4
Annexin V-PE

Figure 4-6. Stable cell lines expressing the DMPK 3' UTR are more sensitive to staurosporine apoptosis than control cell lines. Three different clones of control 3' UTR, 11 CTG repeat, or 99 CTG repeat DMPK 3' UTR stable cell lines were treated with a range of staurosporine concentrations and assayed for metabolic activity using WST-1 reagent. (A) Pgk 3' UTR cell lines had the highest threshold to staurosporine induced apoptosis. (B) The 11 CTG repeat cell lines displayed a low threshold to staurosporine induced apoptosis while the 99 CTG repeat DMPK 3' UTR cell lines exhibited the lowest threshold to staurosporine induced apoptosis (C).

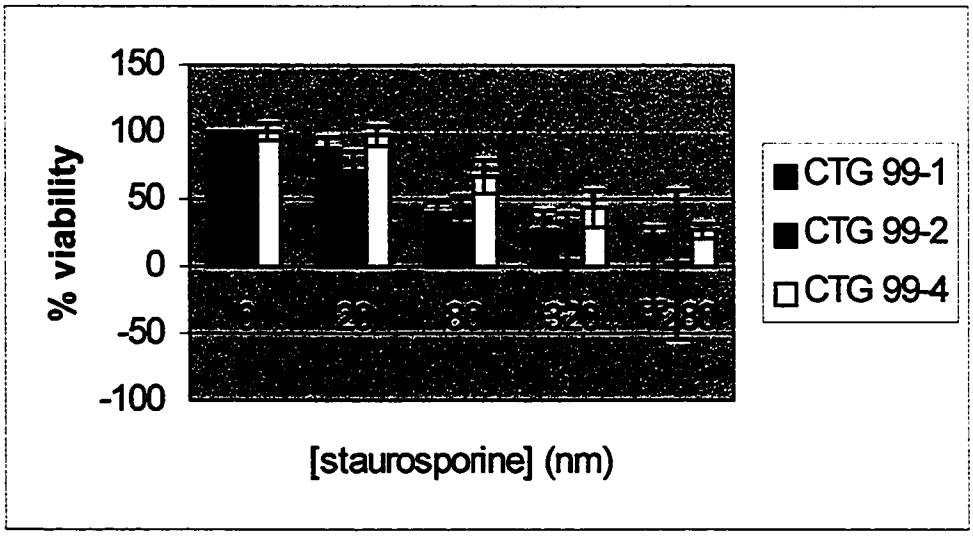
A


150
100
50
0
-50
% viability
Pgk 25
Pgk 22
Pgk 6
[staurosporine] (nm)

B


150
100
50
0
-50
% viability
CTG 11-1
CTG 11-2
CTG 11-3
[staurosporine] (nm)

C


150
100
50
0
-50
-100
% viability
CTG 99-1
CTG 99-2
CTG 99-4
[staurosporine] (nm)

line survival ranged from about 30 to 65% (figure 4-6C). At 320 nm staurosporine, a concentration that has little effect on the control cell lines, CTG 11 cell lines had survival rates around 25% (figure 4-6B) while CTG 99 cell lines had survival rates from about 5% to 40% (figure 4-6C). This data indicates that expression of the DMPK 3' UTR somehow alters intracellular signaling making cells more sensitive to changes in PKC kinase activity.

In order to establish if expression level of the 3' UTR was related to staurosporine sensitivity, northern blot (figure 4-7) and slot blot (figure 4-8) analysis was performed on the cell lines used in the staurosporine sensitivity experiments. Figure 4-7A indicates that the DMPK 3' UTR is expressed in all DMPK 3' UTR cell lines with the possible exception of CTG 99-4 in which expression is almost undetectable by northern blot (figure 4-7A). Figure 4-7B illustrates that GFP is part of the transgene mRNA molecule produced in DMPK 3' UTR and control cell lines and that mRNA levels are comparable between control and DMPK 3' UTR clones. To ensure that all clones had retained their myoblast identity, the northern blot was probed with MyoD (figure 4-7C). As a control for equal mRNA loading, the northern blot was probed with GAPDH. This probing revealed that on this blot, CTG 99 clone 99-4 mRNA was not in tact and therefore expression levels for this clone remain unknown. Additionally, CTG 99 cell lines are generally expressed at lower levels than the CTG 11 cell lines. In order to quantitate this observation, slot blot analysis was performed on the same mRNA samples. Figure 4-8 A, B and C show analysis of RNA isolated from Pgk, CTG 11 and CTG 99 cell lines. Yeast RNA and 10X SSC containing no RNA were blotted to the membrane and represent negative controls. The blot shown in figures 4-8 A, B and C was probed with the DMPK

Figure 4-7. Northern Blot RNA expression analysis of GFP-3'UTR transgenes in C2C12 myoblast stable cell lines. Total RNA (4μg) was isolated from four cell lines expressing 11 CTG repeats in the DMPK 3' UTR (CTG 11), four cell lines expressing 99 CTG repeats (CTG 99) and two cell lines expressing the phosphoglycerate kinase (Pgk) 3 ' UTR all linked to the reporter GFP. RNA was subjected to Northern Blot analysis. The northern blot was probed with (A) the 3' portion of the DMPK 3' UTR, (B) the coding region of the GFP gene, (C) the mouse MyoD coding region and (D) human GAPDH. Generally, transgene expression in GFP 99 cell lines is lower than in GFP 11 and control cell lines. Also, the 3' UTR is part of the expressed mRNA in both CTG 11 and CTG 99 cell lines

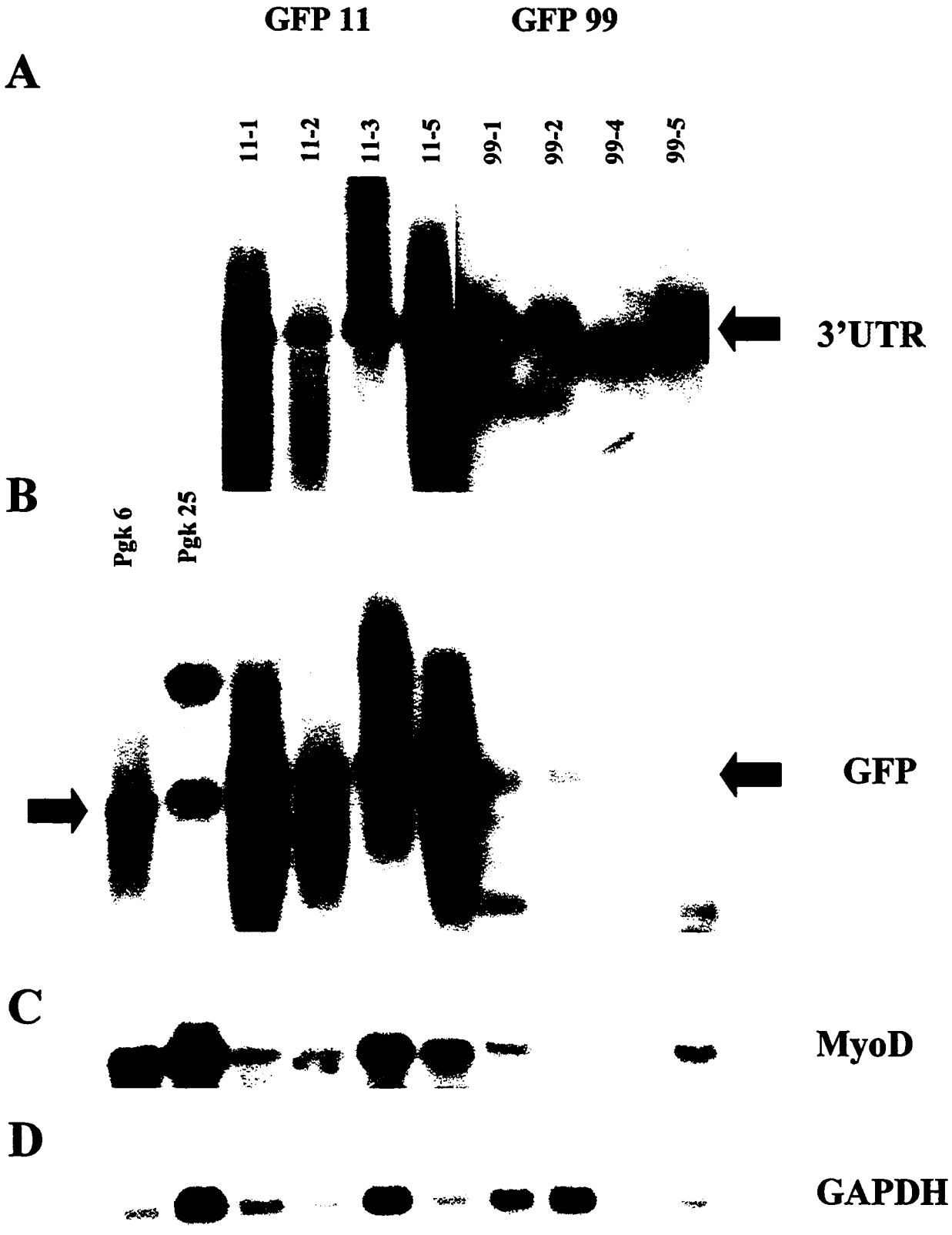
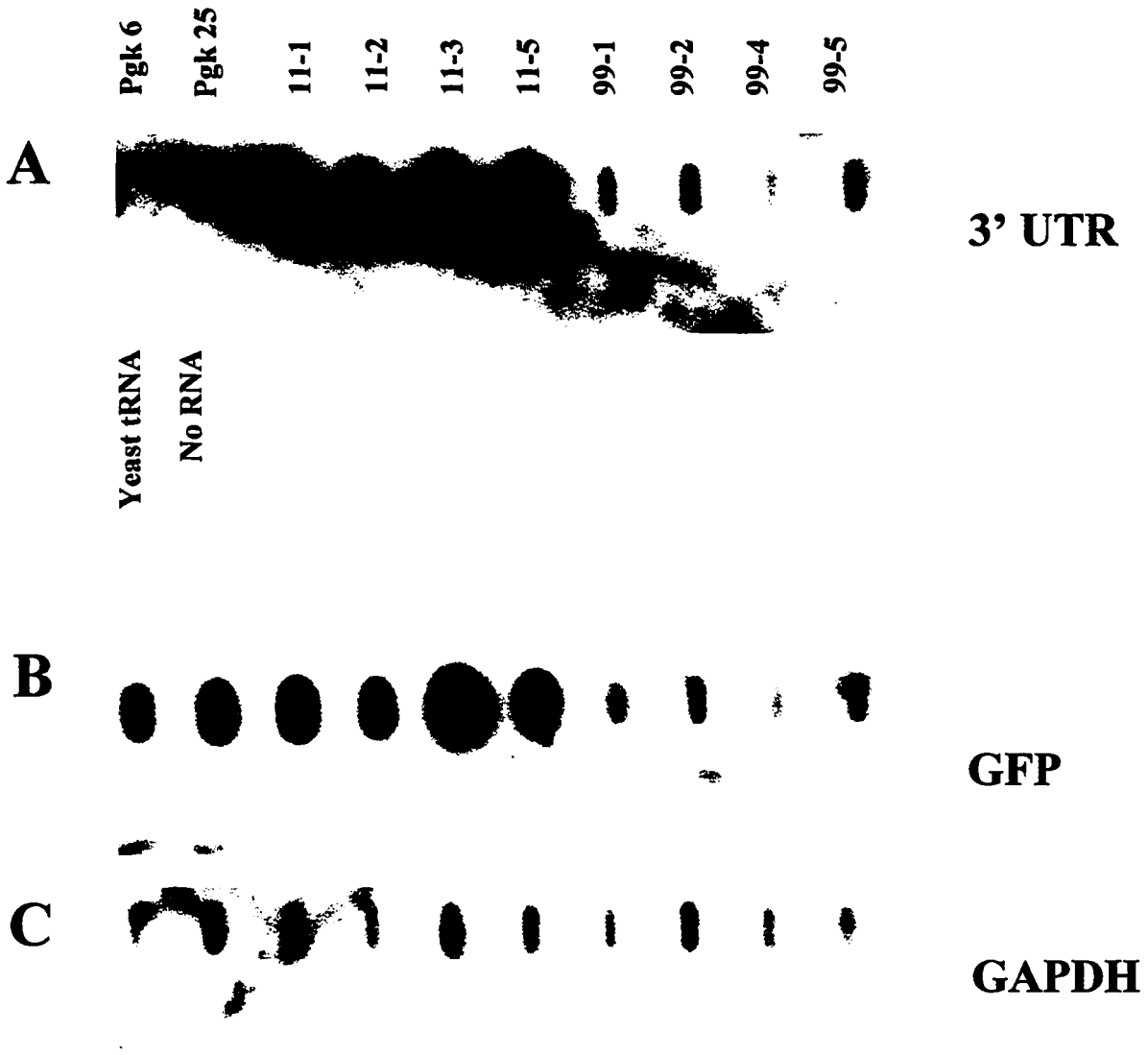
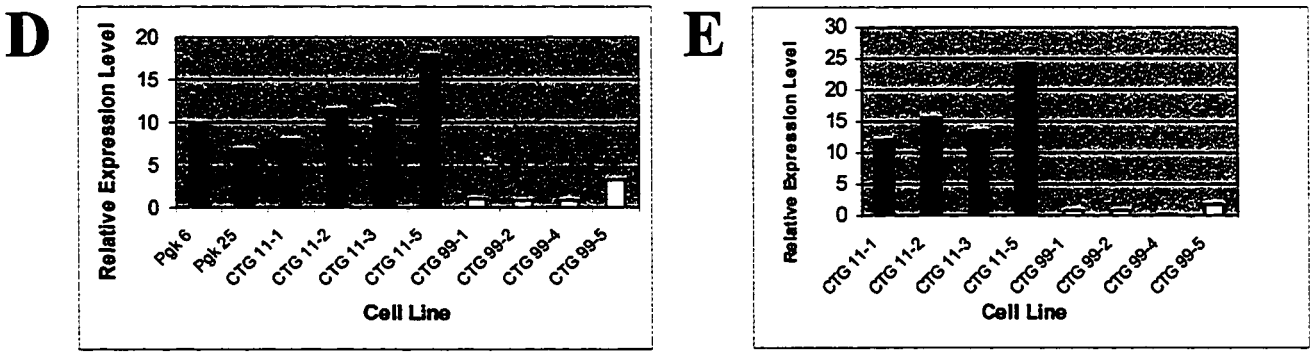

GFP 11
GFP 99
A
11-1
11-2
11-3
11-5
99-1
99-2
99-4
99-5
3'UTR
B
Pgk 6
Pgk 25
GFP
C
MyoD
D
GAPDH

Figure 4-8 Slot blot RNA expression analysis of GFP-3'UTR transgenes in C2C12 myoblast stable cell lines. . Total RNA (4μg) was isolated from four cell lines expressing 11 CTG repeats in the DMPK 3' UTR (CTG 11), four cell lines expressing 99 CTG repeats (CTG 99) and two cell lines expressing the phosphoglycerate kinase (Pgk) 3 ' UTR all linked to the reporter GFP. RNA was subjected to slot blot analysis. Yeast RNA and a sample containing no RNA served as negative controls. The slot blot was probed with (A) the 3' portion of the DMPK 3' UTR, (B) the coding region of the GFP gene, and (C) human GAPDH. The blots were scanned by laser densitometry, background was subtracted and GFP and 3' UTR RNA expression levels were normalized to GAPDH RNA expression levels. (D) Relative RNA expression levels of GFP for all cell lines and (E) relative RNA expression levels of the DMPK 3' UTR for CTG 11 and CTG 99 cell lines are shown. Expression of the RNA for the GFP reporter is substantially lower in the CTG 99 cell lines compared with the CTG 11 and control cell lines. Also, the amount of DMPK 3' UTR RNA is substantially reduced in CTG 99 compared to CTG 11 cell lines.


A
Pgk 6
Pgk 25
11-1
11-2
11-3
11-5
99-1
99-2
99-4
99-5
3' UTR
Yeast tRNA
No RNA
B
GFP
C
GAPDH


D
Relative Expression Level
20
15
10
5
0
Pgk 6
Pgk 25
CTG 11-1
CTG 11-2
CTG 11-3
CTG 11-5
CTG 99-1
CTG 99-2
CTG 99-4
CTG 99-5
Cell Line
E
Relative Expression Level
30
25
20
15
10
5
0
CTG 11-1
CTG 11-2
CTG 11-3
CTG 11-5
CTG 99-1
CTG 99-2
CTG 99-4
CTG 99-5
Cell Line

3' UTR, the GFP cDNA and the GAPDH cDNA respectively and scanned using laser densitometry. Relative GFP and DMPK 3' UTR mRNA levels were then quantified (figure 4-8 C and D). Clearly, relative GFP and DMPK 3'UTR mRNA levels are correlative (figures 4-8 D and E). In addition, it is evident that CTG 99 cell lines express considerably less GFP and DMPK 3' UTR mRNA than the CTG 11 cell lines (figures 4-8 D and E). Further, the Pgk clones, on average, express at similar albeit slightly lower levels of GFP mRNA than the CTG 11 clones but considerably more than the CTG 99 clones (figure 4-8 D). Relative expression levels of the DMPK 3' UTR in CTG 11 and CTG 99 clones are generally constant between clones with the exception of CTG 11-5 which is expressed at a higher level (figure 4-8E). In accordance, staurosporine sensitivities between clones of the same cell type are relatively constant (figure 4-6B, C). Exceptions include CTG 11-3 which is more sensitive at 80 nm staurosporine (figure 4-6B) and CTG 99-4 which is more resistant at 20, 80, 320 and 640 nm staurosporine (figure 4-6C). Establishing the expression level of CTG 99-4 and the staurosporine sensitivity of CTG 11-5 would be of interest.

Despite transgene mRNA expression levels in CTG 99 clones being ten fold less than in CTG 11 clones (figure 4-8E), similar sensitivities to staurosporine mediated apoptosis are observed between the two cell types (figure 4-6, B and C).

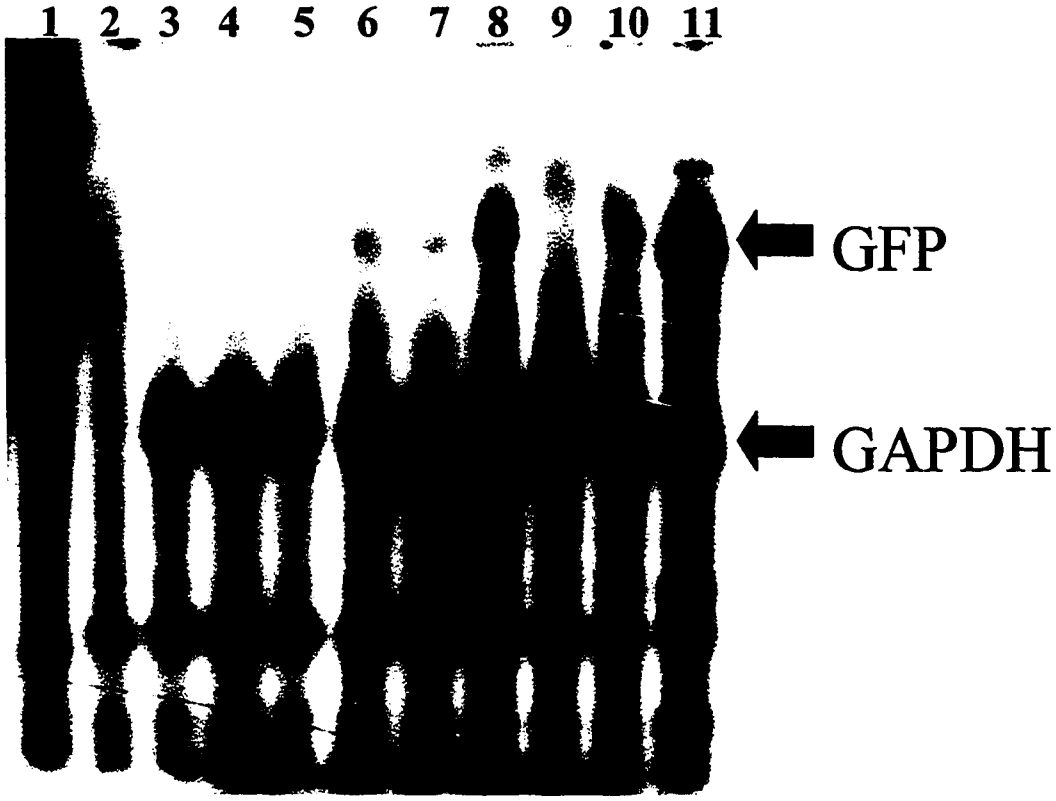
To reinforce the suggestion that expression of the DMPK 3' UTR can induce cell death, stable ecdysone inducible cell lines were generated in the mouse myoblast line C2C12. The GFP cDNA linked to the DMPK 3' UTR with 11 or 99 CTG repeats were cloned into the vector pIND (figure 4-9 A) and co-transfected with the hormone receptor

Figure 4-9. Inducible expression of DMPK 3' UTR containing 11 and 99 CTG repeats. (A) The DMPK 3' UTR containing 11 or 99 CTG repeats was cloned downstream of the heat shock minimal promoter (HSP) and multimeric ecdysone/glucocorticoid binding sites in the inducible vector pIND. Following co-transfection of these clones with the hormone receptor expression vector (pVgRXR), stable cell lines were selected on the basis of GFP expression. (B) RNase protection analysis of inducible CTG 11 and CTG 99 stable cell lines. Stable CTG 11 and CTG 99 inducible cell lines 11-14 and 99-10 were induced with muristerone for 0, 6, 24 or 48 hours. RNA was purified for each time point and 5 μg was analyzed by RNAP using a portion of GFP and murine GAPDH cDNAs as probes. The probe mixture in the presence of yeast RNA but no RNase was run in lane 1. Lane 2 is identical to lane 1 except that RNase was included. In lanes 3-6, RNA from clone 11-14 induced for 0, 6, 24 and 48 hours was hybridized to both probes in the presence of RNase. In lanes 7-10, RNA from clone 99-10 induced for 0, 6, 24 and 48 hours was hybridized to both probes in the presence of RNase. In lane 11, RNA isolated from stable cell line Pgk 6 known to express GFP was hybridized with both probes in the presence of RNase and served as a positive control. (C) Protected fragments corresponding to GFP and GAPDH mRNA molecules respectively were further analyzed by densitometry. GFP mRNA levels were normalized to GAPDH mRNA levels. Inducible clone 99-10 expressed more GFP mRNA than clone 11-14 by 48 hours post induction while both of these clones expressed considerably less GFP mRNA than the control clone.

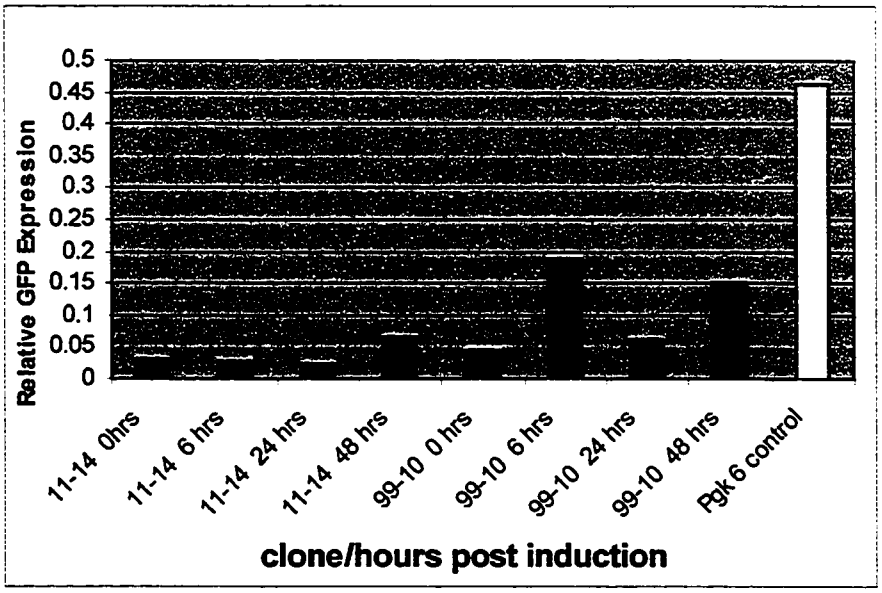
A


CTG 11, 99
DMPK 3' UTR
BgH poly A

B

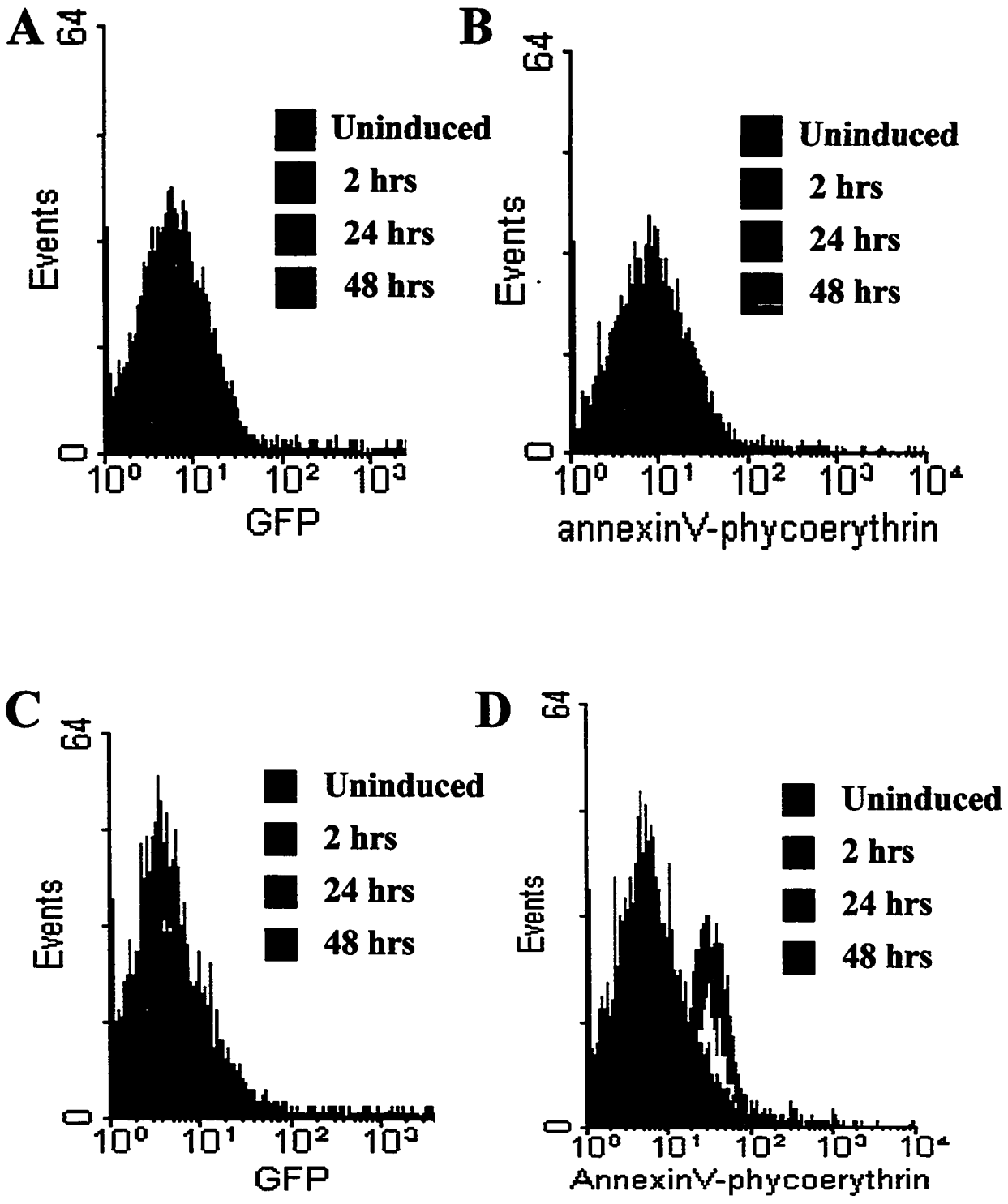

1 2 3 4 5 6 7 8 9 10 11
GFP
GAPDH

C


Relative GFP Expression
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
11-14 0hrs
11-14 6 hrs
11-14 24 hrs
11-14 48 hrs
99-10 0 hrs
99-10 6 hrs
99-10 24 hrs
99-10 48 hrs
Pgk 6 control
clone/hours post induction

expression construct. Stable clones were selected in the presence of genetecin and zeocin. Although attempts were made to obtain inducible clones expressing the Pgk 3' UTR or no UTR, we were unsuccessful in obtaining such cell lines. Regardless, one clone each containing 11 (11-14) and 99 (99-10) CTG repeats respectively were analyzed for inducible GFP mRNA expression by RNase protection (figure 4-9 B). Inducible expression of 11-14 was quite poor, however, after 48 hours some expression of GFP mRNA was observed (figure 4-9, B [lane 6]and C). Clone 99-10 showed two peaks of induction, one at 6 hours following addition of the ecdysone analog muristerone and one at 48 hours (figure 4-9, B [lanes 8 and 10] and C). In order to quantitate the inducible expression, bands on the RNase protection gel corresponding to GFP and GAPDH were scanned by laser densitometry and expression of GFP mRNA was normalized to GAPDH mRNA levels (figure 4-9 C). By 48 hours post-induction, clone 99-10 expressed more GFP mRNA than clone 11-14. Next we investigated induction of GFP protein and the occurrence of cell death following induction of expression of these clones by the addition of muristerone. Clone 11-14 showed a modest induction of protein by 48 hours post-induction but showed only a marginal induction of cell death as measured by annexin V-phycoerythrin flow cytometry analysis (figure 4-10, A and B). In contrast, clone 99-10 showed less induction of GFP protein by 48 hours than did clone 11-14 but a considerable induction of cell death was observed (figure 4-10, C and D). This data indicates that induction of a mRNA molecule containing the DMPK 3' UTR with 99 CTG repeats can induce cell death whereas induction of a similar molecule with only 11 CTG repeats cannot. Although it is apparent that CTG repeats within the DMPK 3' UTR are capable of inducing inappropriate cell death, the mechanism of cell death is unclear.

Figure 4-10. Apoptosis is triggered following muristerone treatment of inducible clone 99-10. (A) Addition of muristerone to 11 CTG repeat clone 11-14 results in a modest induction of GFP protein and a very slight induction of apoptosis (B) by 48 hours. C) Addition of muristerone to 99 CTG repeat clone 99-10 results in a similar modest induction of GFP protein but a much more pronounced induction of apoptosis (D) at 48 hours. Induction of apoptosis is more prevalent in the DMPK 3' UTR cell line expressing 99 CTG repeats.


A
64
Events
0
Uninduced
2 hrs
24 hrs
48 hrs
10⁰ 10¹ 10² 10³
GFP
B
64
Events
0
Uninduced
2 hrs
24 hrs
48 hrs
10⁰ 10¹ 10² 10³ 10⁴
annexinV-phycoerythrin
C
64
Events
0
Uninduced
2 hrs
24 hrs
48 hrs
10⁰ 10¹ 10² 10³
GFP
D
64
Events
0
Uninduced
2 hrs
24 hrs
48 hrs
10⁰ 10¹ 10² 10³ 10⁴
AnnexinV-phycoerythrin

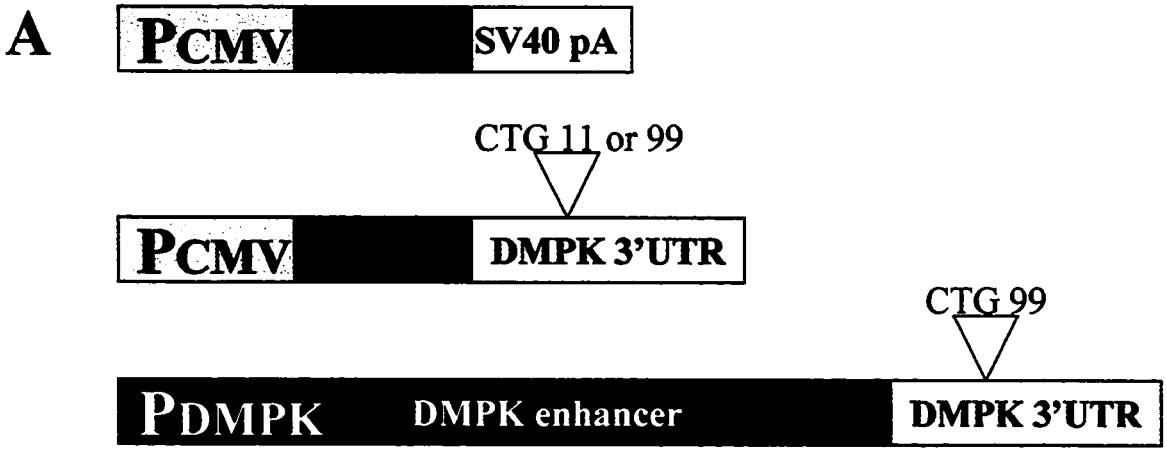
It was recently shown that double stranded RNA activated protein kinase (PKR) could bind CTG repeats within the DMPK 3' UTR and become activated *in vitro*. Therefore we investigated the possibility of PKR becoming activated following transient transfection into C2C12 myoblasts of constructs with no 3' UTR (SV 40 poly A) or the DMPK 3' UTR containing 11 or 99 CTG repeats (figure 4-11A). Forty-eight hours post-transfection, PKR was immunoprecipitated and its kinase activity assayed. When no 3' UTR was present, no detectable kinase activity was detected (lane 1, figure 4-11B). However, when a construct containing 11 CTG repeats was transfected, there was a noticeable induction of

PKR activity (lane 2, figure 4-11B). When a construct containing 99 CTG repeats was transfected, PKR kinase activity increased further (lane 3, figure 4-11B). Finally, when the DMPK promoter/enhancer is used in place of the CMV promoter in the 99 CTG repeat construct, the increase in PKR activity due to the increased number of CTG repeats in the transfected construct remains unchanged (lane 4, figure 4-11B). This assay was repeated several times with each repetition giving very similar results. Therefore, CTG repeats expressed in a mRNA molecule in C2C12 myoblasts can induce PKR activity in a CTG tract length dependent manner.

Next we attempted to link PKR activity with increased sensitivity of DMPK 3' UTR expressing cell lines to staurosporine mediated apoptosis. To accomplish this we transiently transfected a dominant negative (dn) form of PKR into stable cell lines Pgk 22, CTG 11-3 and CTG 99-2. As a negative control, the parental vector pcDNA3 was transfected. Transfection of dn PKR had no effect on the survival of the control cell line

Figure 4-11. Double stranded RNA activated protein kinase (PKR) becomes activated upon transfection of DMPK 3' UTR sequences. (A) Constructs transiently transfected into C2C12 myoblasts are shown. (B) 48 hours following transfection of DMPK 3' UTR constructs and a no UTR control construct into C2C12 myoblasts, PKR was immunoprecipitated and assayed for kinase activity. Lane 1: PKR immunoprecipitated from cells transfected with a CMV driven GFP construct. Lane 2 and 3 are identical to lane 1 except that the transfected construct has the DMPK 3' UTR containing 11 and 99 CTG repeats respectively at the 3' end (see (A)). Lane 4 is identical to lane 3 except that the DMPK promoter/enhancer was used to drive the construct in place of the CMV promoter. PKR activity increases depending on the number of CTG repeats in the transfected construct but is silent in control transfected extracts.

A


PCMV
SV40 pA
CTG 11 or 99
PCMV
DMPK 3'UTR
CTG 99
PDMPK
DMPK enhancer
DMPK 3'UTR

B

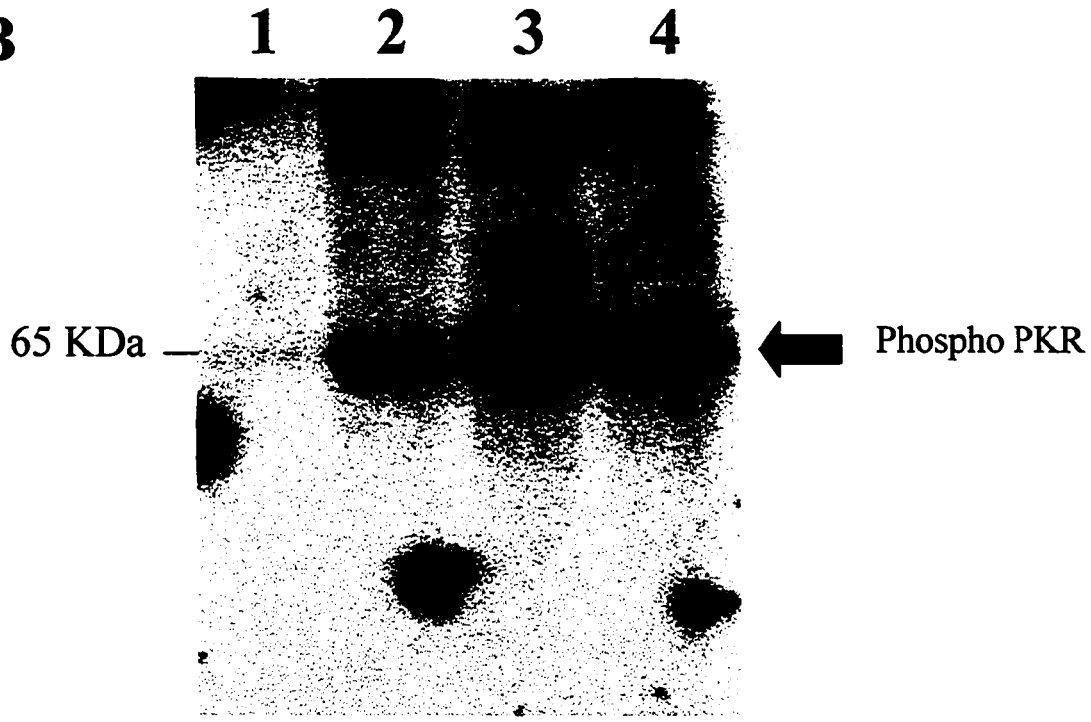
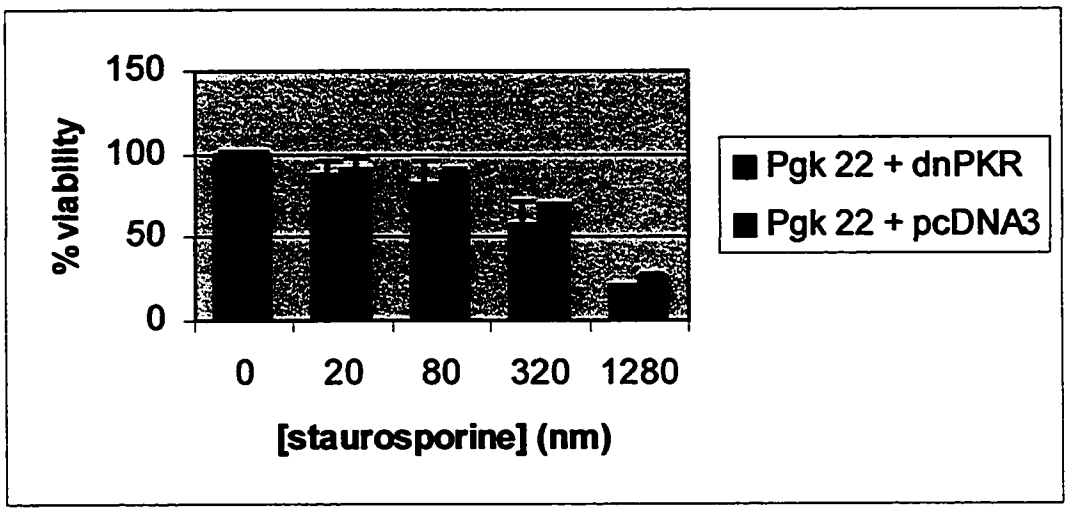
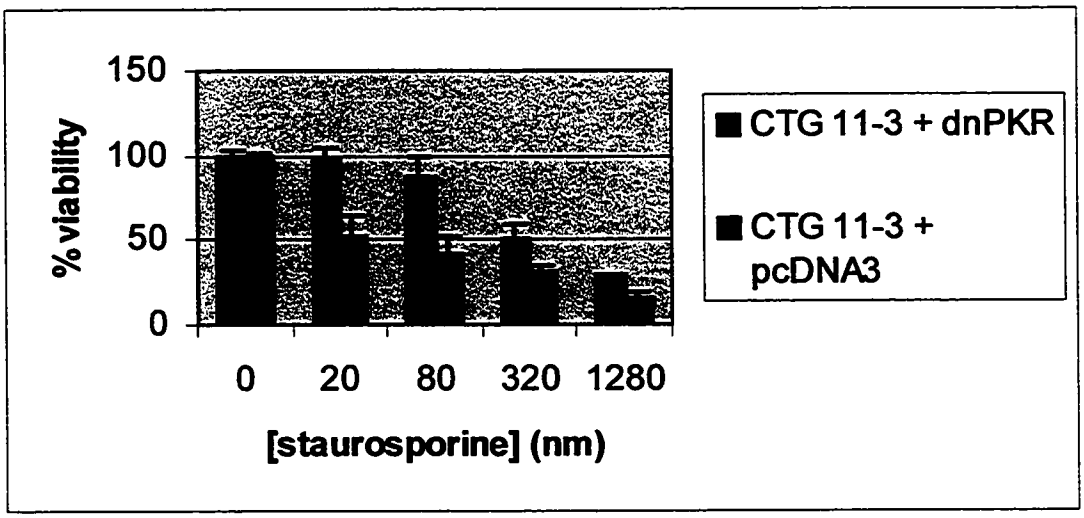

1
2
3
4
65 KDa
Phospho PKR

Figure 4-12. Transfection of dominant negative PKR increases survival in a CTG 11 cell line. Dominant negative PKR (dnPKR) or control vector (pcDNA3) were transfected into a CTG 11, CTG 99 or Pgk control clone and assayed for staurosporine sensitivity 48 hours later. (A) Transfection of dnPKR into control clone Pgk 22 had no effect on cell survival. (B) DnPKR delays onset of cell death in CTG 11-3 cell line. (C) Transfection of dnPKR has no impact on survival of CTG 99-2 cell line. Inhibiting PKR activity can increase survival of a CTG 11 cell line but not a CTG 99 or control cell line.

A


150
100
50
0
% viability
0
20
80
320
1280
[staurosporine] (nm)
Pgk 22 + dnPKR
Pgk 22 + pcDNA3

B


150
100
50
0
% viability
0
20
80
320
1280
[staurosporine] (nm)
CTG 11-3 + dnPKR
CTG 11-3 + pcDNA3

C

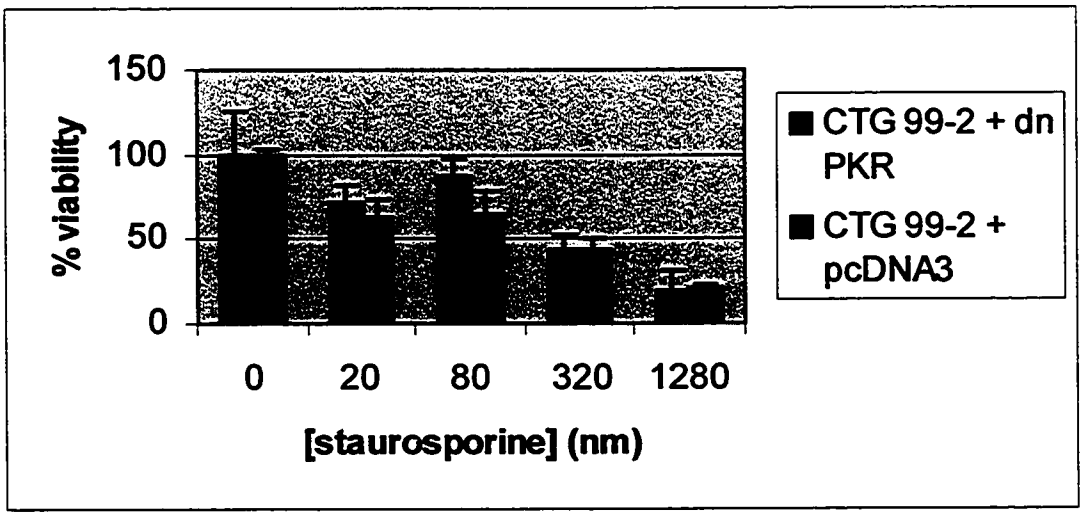
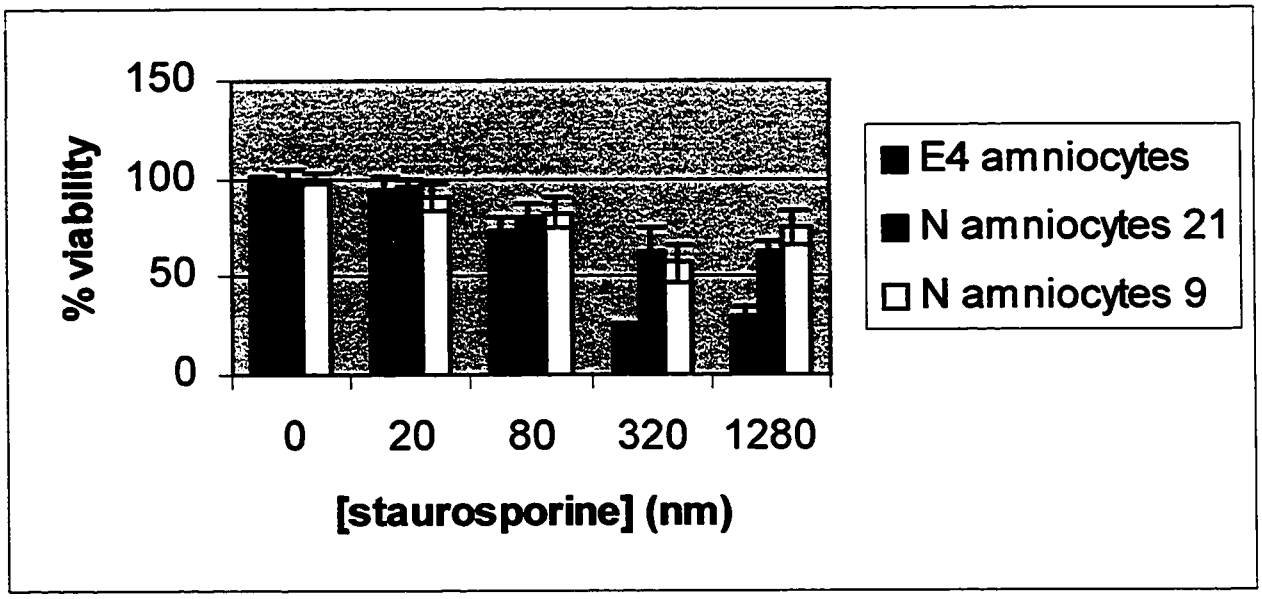
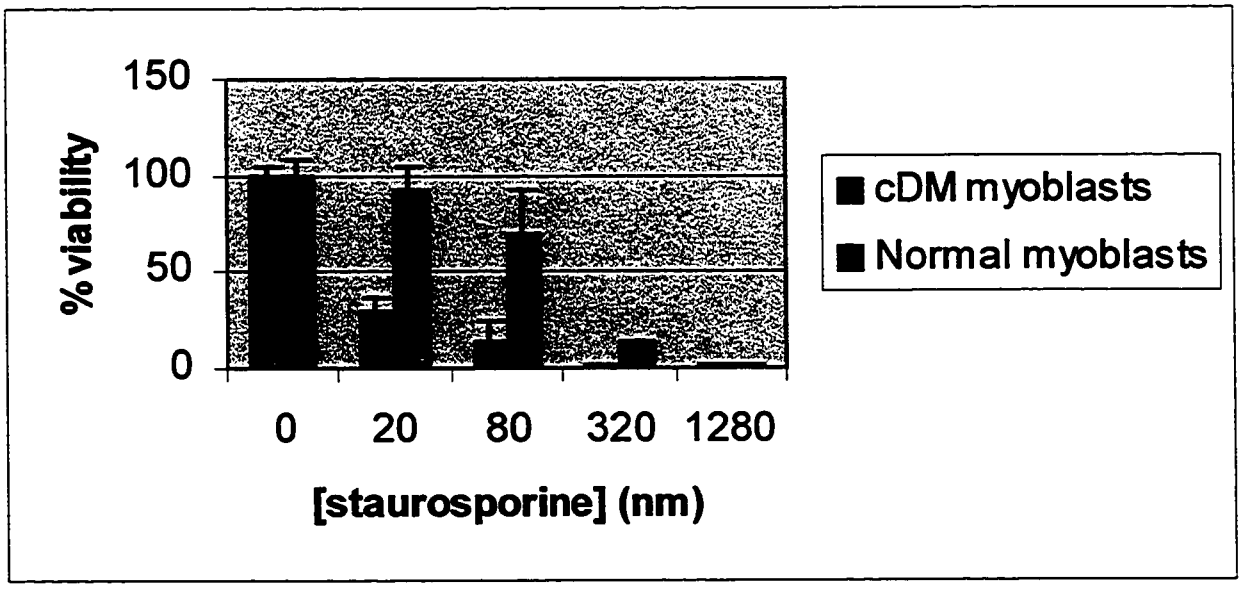

150
100
50
0
% viability
0
20
80
320
1280
[staurosporine] (nm)
CTG 99-2 + dn PKR
CTG 99-2 + pcDNA3

Figure 4-13. Sensitivity of DMPK patient cells to staurosporine induced cell death. (A) Amniocytes from a patient with the most severe DM mutation (E4) and normal control amniocytes were subjected to a staurosporine sensitivity assay. E4 amniocytes were more sensitive to staurosporine induced cell death than control amniocytes. (B) Myoblasts from an E4 individual and age-matched myoblasts from a normal control were analyzed in the staurosporine sensitivity assay. E4 myoblasts were more sensitive to staurosporine induced cell death than control myoblasts. Cells from DM patients are more sensitive to staurosporine mediated cell death than normal control cells.

A


150
100
50
0
% viability
0
20
80
320
1280
[staurosporine] (nm)
E4 amniocytes
N amniocytes 21
N amniocytes 9

B


150
100
50
0
% viability
0
20
80
320
1280
[staurosporine] (nm)
cDM myoblasts
Normal myoblasts

Pgk 22 and very little effect on cell line CTG 99-2, however, CTG 11-3 displayed significantly greater survival at 20, 80, 320 and 1280 nm staurosporine when dn PKR was transfected. It is not clear why dn PKR did not increase survival of CTG 99-2, however, inhibition of PKR activity via transfection of dn PKR could rescue CTG 11-3 staurosporine sensitivity (figure 4-12).

Finally, we tested whether cell lines obtained from myotonic dystrophy patients were more susceptible to staurosporine induced cell death than control cell lines. Compared to two control amniocyte cell lines, an amniocyte cell line obtained from a DM patient with a severe mutation (E4) of up to several thousand CTG repeats were significantly more sensitive to staurosporine apoptosis at 320 and 1280 nm concentrations (figure 4-13A). Because amniocytes are a non-muscle cell line, it was of interest to investigate staurosporine sensitivity in DM myoblasts because this cell type expresses more DMPK mRNA than amniocytes. Significantly, at 20, 80 and 320 nm concentrations, DM myoblasts with an E4 mutation were much more sensitive to staurosporine than myoblasts obtained from an age matched control (figure 4-13B). Interestingly, myoblasts are more sensitive to staurosporine than amniocytes (compare normal cells in figure 4-13A and B). Despite this, the E4 DM myoblasts were affected more severely than E4 DM amniocytes relative to their respective controls (figure 4-13A, B). In accordance with data obtained from the stable cell lines (figure 4-6), altered intracellular signaling within patient cells expressing CTG repeats results in increased sensitivity to the effects of staurosporine.

4.4 DISCUSSION

Expression of the DMPK 3' UTR with 11 or 99 CTG repeats in C2C12 myoblasts presents the opportunity to examine effects of limited CTG repeat expansion on processes occurring during myoblast differentiation *in vitro*. One such process is the loss of a portion of myoblasts to apoptosis during the first 24 hours following growth factor withdrawal (Mampuru et al 1996). Clearly, C2C12 myoblasts expressing the DMPK 3' UTR with 11 or 99 CTG repeats were more susceptible to growth factor withdrawal associated cell death at 24 hours than control cells expressing a Pgk 3' UTR. In these experiments, CTG 11 clones displayed greater levels of cell death compared with CTG 99 clones as measured by propidium iodide uptake (figure 4-4B). Furthermore, this cell death is apoptotic. At 4 and 12 hours following serum withdrawal, DMPK 3' UTR clones with 11 and 99 CTG repeats displayed more annexin V positive/DAPI negative, or early apoptotic cells than the control cell line Pgk 25 (figure 4-4C). Levels of transgene mRNA encoding the DMPK 3' UTR in CTG 11 clones were significantly higher than in CTG 99 clones (figure 4-3). Higher expression levels may compensate for fewer CTG repeats possibly accounting for greater apoptosis levels in cell lines with 11 CTG repeats than cell lines with 99 CTG repeats.

Stable cell lines were tested for resistance to staurosporine induced apoptosis. This drug, which was originally thought to inhibit protein kinase C specifically, inhibits other serine/threonine kinases like cAMP-dependent protein kinase (PKA), phosphorylase kinase, and S6 kinase just as effectively (O'Brian & Ward 1990). In addition, staurosporine can also inhibit some tyrosine kinases like EGFR, IGFR and pp60 v-src (O'Brian & Ward 1990). Regardless, the DMPK 3' UTR expressing cell lines were

more sensitive to staurosporine induced apoptosis than control cell lines expressing the Pgk 3' UTR (figure 4-6A, B, C). These results are in good agreement with increased differentiation associated cell death observed in stable cell lines suggesting that expression of the DMPK 3' UTR lowers the apoptotic threshold in C2C12 cells.

Of significance, mRNA levels of the GFP-DMPK 3' UTR transgene were reduced ten fold in CTG 99 cell lines compared with CTG 11 cell lines used in the staurosporine assays (figure 4-8D, E). Despite this difference in expression, the susceptibility of the two types of cell lines to staurosporine mediated cell death were very similar (figure 4-6B, C). It is possible that the mechanism leading to increased staurosporine susceptibility depends on total number of CTG repeats present within the cell. In other words, more mRNA molecules with fewer CTG repeats would be equivalent to fewer mRNA molecules with more CTG repeats.

Lower levels of the GFP-DMPK 3' UTR transgene mRNA in clones with 99 CTG repeats compared with clones harbouring 11 CTG repeats was a general observation seen in many of the stable cell line clones. This difference in expression levels between the two types of cell lines may be due to death of higher expressing CTG 99 cells within clonal populations during the selection and cloning process. Ultimately, this would result in selection of lower expressing CTG 99 clones. Presumably this selection pressure on CTG 11 clones would be weaker because of fewer CTG repeats allowing selection of lines expressing higher levels of transgene RNA.

One way to avoid selection problems during cloning of cell lines is to use an inducible expression system (figure 4-9A). One CTG 11 and one CTG 99 inducible clone was analyzed for mRNA expression at various time points following induction with

the ecdysone analog muristerone (figure 4-9B). By 48 hours post-induction, transgene mRNA levels of the CTG 11 clone 11-14 were roughly a third of levels of the CTG 99 clone 99-10 (figure 4-9B, C). However, despite exhibiting low protein levels at 48 hours post induction, clone 11-14 displayed a greater induction of GFP protein over the time course than did clone 99-10, which expressed three fold higher levels of transgene mRNA (figure 4-10A, C). Reduced protein levels in clone 99-10 may be explained by nuclear retention of the transgene transcript due to the presence of an expanded repeat (Taneja et al 1995) (Davis et al 1997), however, this is normally not observed under 150 CTG repeats in patient fibroblasts (Hamshere et al 1997). It is possible that an inducible system may overwhelm nuclear mRNA export capacity in C2C12 cells and, in combination with an expanded CTG repeat may result in nuclear retention of transcript and lower protein levels for clone 99-10. Examination of the cell lines by annexin V staining and flow cytommetry following 48 hours of induction revealed a higher rate of cell death in clone 99-10 over 11-14 (figure 4-10B, D). Thus for the first time, expression of the transgene mRNA in a 99 repeat cell line was higher than an 11 repeat line and cell death was in turn much more dramatic for the 99 CTG repeat clone 99-10. This data is entirely consistent with an increased susceptibility to cell death with expression of the DMPK 3' UTR observed with the first set of stable cell lines. In addition, by employing an alternative approach, this data solidifies the hypothesis that total number of CTG repeats is crucial for cell death induction; whether achieved by high level expression of a low number of CTG repeats or by lower level expression of a longer CTG repeat tract. In this case, induced transgene mRNA levels in clone 11-14 were too low to achieve a marked amount of cell death.

Data presented thus far suggest that expression of the DMPK 3' UTR increases susceptibility of C2C12 myoblasts to cell death. Recently it was demonstrated that C2C12 stable cell lines expressing the DMPK cDNA with a mutant (46) number of CTG repeats in the 3' UTR were more susceptible to reactive oxygen species induced cell death than control clones with only 5 CTG repeats in the 3' UTR (Usuki & Ishiura 1998). Although we do not see a difference between cell lines expressing 11 and 99 CTG repeats in the staurosporine assay, in the inducible system we do observe increased cell death of the 99 CTG repeat cell line compared with the 11 CTG repeat cell line. Therefore, it is likely that given equivalent expression levels, cells expressing more CTG repeats will display increased apoptosis susceptibility. Therefore, this observation by Usuki *et. al* is consistent with our results.

The double stranded RNA binding protein kinase (PKR) has been shown to bind CTG repeats and become activated in a repeat number dependent manner *in vitro* (Tian et al 2000). Upon transfection of DMPK 3' UTR constructs, PKR became activated in a repeat dependent manner (figure 4-11B) indicating that within C2C12 myoblasts, CTG repeats are bound by PKR resulting in its activation. In agreement with cell death induction by the DMPK 3' UTR, 11 CTG repeats was sufficient to activate this kinase, however, 99 CTG repeats were more effective (figure 11B). Significantly, the RNA binding domain of PKR requires 11-13 bp of ds RNA for binding, therefore, 11 CTG repeats would be sufficient for PKR binding having 16 ds RNA bp (St Johnston et al 1992). PKR is known to have many effects on cell growth and differentiation, however, its most notable function is inhibition of translation achieved through phosphorylation of translation factor eIF2α resulting in translational repression (Williams 1999). This may

explain that despite higher transgene mRNA levels, inducible clone 99-10 expressed less transgene protein than clone 11-14 which expressed approximately one third the amount of transgene mRNA (figure 4-9B, C). In addition to translation inhibition, following treatment of cells with double stranded (ds) RNA, TNFα or lipopolysacharide (LPS), activated PKR can induce apoptosis (Der et al 1997). PKR mediated apoptosis can result from translational repression, presumably from a shortfall of apoptosis inhibitory proteins or through various other signaling events mediated by PKR resulting in caspase 8 activation via Fas associated death domain protein (FADD) (Gil & Esteban 2000).

A role for PKR in staurosporine mediated cell death in DMPK 3' UTR expressing cell lines was substantiated by a partial rescue of this phenotype when PKR activity was inhibited by transfection of a dominant negative (dn) form of PKR (figure 4-12B). Transfection of a plasmid encoding dn PKR resulted in greater resistance to staurosporine mediated cell death compared to cells transfected with empty vector (pcDNA3). No increased resistance to the drug was observed in control cell lines (Pgk 22) or in the CTG 99 cell line (figure 4-12A, C). The failure of transfected dn PKR to protect the CTG 99 cell line from the effects of staurosporine may have been due to a resistance of this cell line to transfection resulting in dn PKR protein levels too low to have an effect. It is also possible that the dn PKR is expressed adequately in the CTG 99 cell line but has no effect. Interestingly, PKR activity is unaffected by staurosporine at the concentrations used in the assay employed in this study (Martin de la Vega et al 1999) suggesting that staurosporine inhibits protective signaling pathways. Consistent with this notion, in response to IL-3 signaling in the IL-3 dependent myeloid cell line NSF/N1.H7, mitochondrial PKC, a kinase inhibited by staurosporine, phosphorylates Bcl-2 on serine

70, conferring potent anti-apoptotic activity to this molecule (Deng et al 2000). In addition, IL-3 withdrawal from this cell line results in PKR activation and increased rates of cell death (Ito et al 1994). Therefore, loss of the protective effect of PKC phosphorylation of Bcl-2 coupled with induction of PKR following IL-3 withdrawal results in increased cell death. If similar signaling pathways exist in myoblasts, it seems reasonable that further activation of PKR due to expression of the DMPK 3' UTR in the C2C12 stable cell lines might be expected to exacerbate cell death. By the same token, reduction of PKR activity by expression of dnPKR might improve survival of these cell lines. This is what we observed, suggesting a molecular explanation for increased apoptotic sensitivity of DMPK 3' UTR stable cell lines to staurosporine.

Although DMPK 3' UTR expression results in compromised survival of a mouse myoblast cell line, it was not known if survival of cells derived from DM patients were similarly affected. In agreement with data observed in C2C12 stable cell lines, amniocytes derived from a severely affected (E4) DM patient were more sensitive to staurosporine than amniocytes derived from a normal individual (figure 4-13A). This was also the case for DM derived myoblasts compared with myoblasts from a normal age matched control (figure 4-13B). The susceptibility of the DM myoblasts compared to normal myoblasts was more severe than the DM amniocytes compared with normal amniocytes (figure 4-13A, B). This difference is likely due to higher levels of DMPK mRNA expression in myoblasts compared with amniocytes, thus more CTG repeats would be present in the myoblasts resulting in greater cell death susceptibility.

Expression of the DMPK 3' UTR was previously shown to inhibit differentiation of C2C12 cells *in vitro* (Sabourin et al 1997) (Amack et al 1999) (Bhagwati et al 1999).

However, the possibility that apoptosis, a process intimately associated with differentiation, could play a role in this effect of the DMPK 3' UTR had not been addressed. One study has demonstrated that expression of a DMPK cDNA with 46 CTG repeats in the DMPK 3' UTR was more susceptible to reactive oxygen species (ROS) mediated cell death than a similar cDNA with only 5 CTG repeats (Usuki & Ishiura 1998). We now demonstrate that expression of just the DMPK 3' UTR is sufficient to confer increased apoptotic sensitivity to C2C12 myoblasts perhaps affecting their differentiation potential. Interestingly, inappropriate activation of PKR may be pivotal to this phenotype. PKR activity is regulated during (Kronfeld-Kinar et al 1999) and required for differentiation of C2C12 myoblasts (Salzberg et al 2000). However, over-activation of this enzyme may lead to alteration of the balance between differentiation and apoptosis signals resulting in deleterious effects on differentiation.

Other 3' UTR elements are capable of activating PKR. One example, the α-tropomyosin 3' UTR, known to act as a tumour suppressor element (Rastinejad et al 1993), can bind and activate PKR *in vitro* (Davis & Watson 1996) perhaps explaining its anti-proliferative action. In addition, the TNF α 3' UTR contains an element recognized by and able to activate PKR *in vitro* resulting in specific splicing regulation of this transcript (Osman et al 1999). Intriguingly, the nuclear sequestration of mutant DMPK mRNA (Taneja et al 1995) may result in inappropriate activation of nuclear PKR (20% of PKR is nuclear (Jeffrey et al 1995; Jimenez-Garcia et al 1993; Osman et al 1999)) and therefore may be a factor in disregulation of splicing of certain messages in myotonic dystrophy cells (Philips et al 1998). Mutant DMPK mRNA is able to escape the nucleus of a DM cell (Taneja et al 1995) perhaps leading to cytoplasmic PKR activation.

Therefore, PKR activation in the nucleus as well as in the cytoplasm may contribute to DM pathology including inappropriate cell death and delayed differentiation of muscle. Taken together, the data presented highlights a possible molecular mechanism by which an expanded CTG repeat may lead to defects of differentiation of myoblasts and loss of muscle mass in DM.

CHAPTER 5 ANALYSIS OF EXPRESSION OF THE DMPK 3' UTR WITH 11 OR 91 CTG REPEATS IN TRANSGENIC MICE

5.1 INTRODUCTION

The mutation causing Myotonic dystrophy is an expanded CTG repeat within the 3' UTR of the DMPK gene. The emerging consensus suggests that the 3' UTR, alone, may play a major role in disease pathogenesis. Therefore, we proposed to generate a transgenic mouse model expressing a mutant DMPK 3' UTR in association with the coding region of a reporter gene (GFP) to investigate the role of the 3' UTR in mediating features of DM pathology.

Muscle features of adult DM include myotonia and progressive muscular weakness and wasting. In contrast, congenital DM infants are hypotonic, or lacking developed skeletal muscle, have an abundance of muscle satellite cells and experience difficulties suckling due to paucity in development of facial muscles (Harper 1989).

Following identification of the CTG expansion mutation, DMPK mRNA expression level studies were performed in an effort to understand DM molecular pathogenesis. DMPK mRNA levels from DM tissues were reported to be reduced (Carango et al 1993; Fu et al 1993; Hofmann-Radvanyi et al 1993) and also increased (Sabourin et al 1993). The differences observed in these studies were attibuted to differences in tissue sampling and RNA isolation methodologies employed (Wieringa 1994). Further studies reported both decreases of mutant and normal polyadenylated DMPK mRNA (Wang et al 1995) while another found unchanged levels of unprocessed mutant and normal DMPK mRNA from patients but reduced levels of the mutant processed but not the normal processed transcript (Krahe et al 1995).

RNA *in situ* hybridization studies on patient fibroblasts and myoblasts demonstrated that the DMPK mutant mRNA was trapped in "foci" within the nucleus (Taneja et al 1995). Further studies showed that the mutant DMPK mRNA was resistant to biochemical retrieval from these structures providing an explanation for the variable yields of isolated mRNA from patient tissues (Davis et al 1997).

The apparently normal levels and localization of DMPK in tissues of even the most severely affected patients suggests that lack of functioning DMPK protein is not likely the cause of DM. Elevated expression of DMPK mRNA was reported in brain and skeletal muscle of cDM patients (Sabourin et al 1993) suggesting that overexpression of DMPK may play a role in DM pathology. To test this hypothesis, the DMPK cDNA, including both untranslated regions, was overexpressed in C2C12 mouse myoblasts. Differentiation of these cells was inhibited and a portion of the 3' UTR was found to be the minimum sequence within the cDNA responsible for this phenotype (Sabourin et al 1997). Subsequently, a number of studies confirmed this result but implicate the CTG repeats in the observed perturbation of myoblast differentiation (Amack et al 1999; Bhagwati et al 1999; Okoli et al 1998; Usuki & Ishiura 1998).

Two transgenic mouse models have been generated with the entire 15 Kb genomic fragment including both 5' and 3' UTR sequences. One study found a cardiomyopathy that was dependent upon transgene expression level (Jansen et al 1996) while a model generated in our laboratory showed more prominent features of DM (Narang 2000). These features included centrally located nuclei in muscle fibres, type I fibre atrophy, delayed differentiation of cultured muscle satellite cells and the presence of ringed fibres (Narang 2000).

Recent investigations have probed possible new dominant mechanisms of DM. Proteins binding the CUG repeat in the mutant mRNA are candidates for factors sequestered by this sequence as it expands to huge lengths in severe cases of DM. A protein termed CUG-BP was initially thought to bind in this manner (Roberts et al 1997; Timchenko et al 1996) but has subsequently been shown to bind only to the base of the CUG hairpin and does not exhibit preferential nuclear localization in DM cells (Michalowski et al 1999). Recently, a dsRNA binding protein termed muscleblind has been shown to bind CUG repeats in mutant DMPK mRNA in a repeat dependent manner and represents a good candidate CUG-sequestered factor in DM (Miller et al 2000). Another factor shown to bind the DMPK CUG repeats present in RNA in a repeat length dependent fashion is the double stranded RNA activated kinase (PKR). Binding of PKR to CUG repeats *in vitro* results in repeat length dependent kinase activation (Tian et al 2000) implicating hyperactivation of this enzyme in DM pathogenesis.

Our laboratory is focused on understanding the mechanism of mutation in DM. We hypothesized that the expression of a reporter construct containing the DMPK 3'UTR fused to unrelated coding sequence would result in DM pathology in a mouse model. To test this possibility, we designed two transgenic mouse strains utilizing endogenous DMPK gene regulatory sequences with the DMPK 3'UTR with either 11 or 91 CTG repeats linked to the reporter gene, green fluorescent protein (GFP). We demonstrate that the DMPK gene regulatory units employed confer proper spatial and temporal expression of the reporter gene and that expression of the DMPK 3' UTR *in vivo* is capable of replicating a subset of features of myotonic dystrophy.

5.2 MATERIALS AND METHODS

5.2.1 DNA ISOLATION FROM MOUSE TAILS AND SOUTHERN BLOTTING

Mouse litters were weaned at three weeks of age. Pups were ear tagged, tail clipped and DNA was isolated from mouse tails using the DNeasy™ DNA isolation kit (Qiagen) according to manufacturer's instructions. To a 0.5 cm length of mouse tail, 180 µl of buffer ATL and 20 µl of proteinase K (20 mg/ml) was added. Tails were incubated overnight at 55°C with occasional mixing, followed by vortexing for 15 seconds and addition of the buffer AL/ethanol mixture and further vortexing. The tail mixtures were then added to a Qiaquick column and spun for 1 minute at 8000 rpm. The column was washed once each with wash buffers AW1 and AW2 followed by centrifugation for 1 minute at 8000 rpm for the first wash and 14000 rpm for 3 minutes for the second wash. DNA was eluted twice from the column by addition of 30 µl of elution buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 7.5) followed by centrifugation for 1 minute at 8000 rpm. DNA (16 µl) was digested with BamHI (1 µl BamHI, 1 µl Rnase A [10 mg/ml], 2 µl of React 3 buffer [Gibco-BRL]) overnight, mixed with 2 µl of gel loading buffer (0.1 % bromphenol blue, 0.1 % xylene cyanol, 50 % glycerol, 10 mM Tris-HCl pH 7.5), and loaded on a 1% agarose gel. Following electrophoresis in 1X TAE (0.04 M Tris acetate, 1mM EDTA), the gel was soaked in 200 ml of 1X TAE buffer containing 1 µg/ml ethidium bromide for 10 minutes with shaking. To visualize the digested DNA, the stained gel was photographed using a Polaroid MP-4 land camera and Polaroid polapan 667 film. The gel was destained in 200 ml of 1X TAE for ten minutes with shaking, rinsed with dH_2O and then denatured in 0.4 N NaOH with shaking for 30 minutes. The DNA was transferred to positively charged nylon membrane (Pall-Biodine) by downward

capilliary flow blotting (Current Protocols in Molecular Biology, pp.2.9.1-2.9.10). About 6 cm of paper towel is placed on the lab bench. Six pieces of superabsorbant gel blot paper (Schleicher and Schuell) cut to the size of the gel were placed on the paper towels. Four pieces of dry and one piece of Whatman 3 CHR paper pre-soaked in transfer buffer (0.4 N NaOH) and cut to the size of the gel were placed on top of the absorbant sheets. The nylon membrane pre-soaked in dH_2O for five minutes was placed on the fifth sheet of Whatman paper followed by the gel and three more sheets of Whatman 3 CHR paper soaked in transfer buffer. A wick was then pre-soaked in transfer buffer, one end placed in a reservoir containing transfer buffer and the other placed on top of the final piece of Whatman 3 CHR paper. The gel tray was placed on top of the stack and served as a weight. Transfer was complete in one hour after which the membrane was washed in 2X SSC for five minutes with shaking and air dried for fifteen minutes on a sheet of Whatman 3 CHR paper. The DNA was immobilized to the membrane by baking at 80°C for thirty minutes.

5.2.2 RNA ISOLATION FROM ANIMAL TISSUES

RNA was isolated using trizol reagent (Gibco-BRL) according to manufacturer's instructions. Briefly, 1ml of Trizol was added to 100mg of animal tissue and ground using a homogenizer. Samples were incubated at 15 – 30°C for 5 minutes after which 200 µl of chlolroform was added, shaken vigourously for 15 seconds and spun at 12000 xg for 15 minutes at 4°C. The aqueous phase was removed and the RNA precipitated by mixing with 500 µl of isopropanol per ml of Trizol reagent. The mixture was incubated for 10 minutes at room temperature and centrifuged as described above. The RNA was

washed with 70 % ethanol, resuspended in 50 µl of sterile RNase free distilled water and analyzed by spectrophotometry to determine the concentration.

5.2.3 PROTEIN LYSATES AND WESTERN BLOTTING

Western blotting was performed as described in (Sambrook 1989) (pp. 18.47-18.59). Briefly, protein lysates from tissues were obtained by homogenization on ice with a polytron in homogenization buffer (10mM Tris pH 8.0, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM PMSF). Samples were spun for 5 minutes at 500 rpm, made up to 2% SDS and boiled for 20 minutes. Lysates from cultured cells were prepared in RIPA buffer (150 mM NaCl, 1% NP40, 0.5 % deoxycholate, 0.1 % SDS, 50 mM Tris HCl pH 7.5) plus protease inhibitors (PMSF [100 µg/ml], aprotonin [2 µg/ml], pepstatin A [1 µg/ml], antipain [2 µg/ml]). Protein concentrations were determined using a Pierce micro BCA kit according to manufacturer's instructions. Individual protein lysates (30-50 µg) were mixed with 3X loading buffer (187.5 mM Tris-HCl pH 6.8, 6 % SDS, 30 % glycerol, 0.03 % bromphenol blue) and boiled for 5 minutes. Samples were loaded on a 10-12 % SDS-polyacrylamide gel made with lower buffer (1.5 M Tris pH 8.8, 0.1 % SDS) and stacking gel made with upper buffer (1.0 M Tris pH 6.8, 0.1 % SDS) and polymerized with 10 % ammonium persulfate and TEMED. Gels were run at about 115 volts for 1 hour in electrode buffer (25 mM Tris, 250 mM glycine pH 8.3, 0.1 % SDS). On completion of electrophoresis, the stacking gel was removed and proteins were transferred to PVDF membrane by semi-dry electroblotting. Three pieces of Whatman 3MM paper cut to the size of the gel were soaked in transfer buffer (Tris, glycine, 0.375 % SDS, 20 % methanol), placed on the electroblotter surface (Hoefer Semi-Phor-Amersham-Pharmacia Biotech). Air bubbles were removed by rolling a pipette over the

pieces of Whatman paper. The PVDF membrane was soaked in methanol for 20 seconds and then put in transfer buffer prior to placement over the Whatman paper. The gel, which was pre-soaked in transfer buffer, was placed on top of the membrane. Next, three more sheets of Whatman paper pre-soaked in transfer buffer were put over the gel, the cover placed on the electroblotter apparatus and power applied (15 V) for 40 minutes. The membrane was soaked in Ponceau S stain diluted 1:10 in 1X PBS for 5 minutes and washed gently in several changes of dH_2O to visualize transferred protein. Ponceau S was completely removed from the membrane with several changes of dH_2O over 5 minutes. The membrane was then incubated overnight at 4°C in blocking buffer (5 % skim milk in 1X PBS). All subseqent treatments were performed with shaking. The following day the membrane was washed 2X 10 minutes in wash buffer (1X PBS/0.05 % NP40) and incubated for 1 hour in primary antibody diluted in wash buffer containing 1% skim milk powder. The membrane was washed 4X 15 minutes in wash buffer and incubated with secondary antibody diluted in wash buffer containing 1% skim milk for 1 hour. Following this incubation, the membrane was treated 4X 15 minutes in wash buffer. The immunoreactive bands were visualized by treating the membrane with chemiluminescent reagents (ECL kit, Amersham) according to manufacturer's instructions.

5.2.4 ^{33}P *IN SITU* HYBRIDIZATION

Riboprobes encoding portions of the GFP and myogenin genes were generated as described above (See RNase protection) using ^{33}P-UTP in place of ^{32}P-UTP. Mouse embryos were obtained from pregnant CD-1 mice. (ordered timed pregnancies, Charles River). The day the vaginal plug appeared was designated E0. Pregnant mice were

euthanized by CO_2 and embryos removed at various times between E9.5 to E20. Mouse embryos were dissected free of extra embryonic membranes in cold 1X PBS and transferred into 4%PFA/0.2% gluteraldehyde in PBS and stored at 4°C overnight. Procedures for mRNA detection from this point forward were performed under RNase-free conditions using RNase-free solutions, vials and containers. E9.5 to E12 embryos were placed in formalin overnight and embryos older than E12 were preserved for an additional 2-3 days. Embryos were processed in PBS for 30 min at 4°C, 0.85% saline solution for 30 min at 4°C, (subsequent steps were performed at 25°C) 50% ETOH in 0.425% saline 2X for 15 min, 70% ETOH for 30 min, 85% ETOH for 30 min, 95% ETOH for 30 min, 100% ETOH 2X for 30 min, xylene 2X for 30 min, and a 1:1 solution of xylene:melted paraplast at 60°C for 45 min. The embryos were transferred through paraplast 3X for 20 min each at 60°C, embedded in paraffin wax, oriented in plastic moulds, preheated to 60°C, cooled and stored at 4°C. Mouse embryo sections (6 μm) were obtained using a microtome (Zeiss) and floated on a bath of distilled water at 50°C until creases dissipated. The sections were collected on silane coated slides (SIGMA), dried at 37°C overnight and stored desiccated at 4°C.

Embryo sections were deparaffinized in xylene 2X for10 min and rehydrated through a series of ethanols, 2X 100% for 1 min each, and 1X 95/85/70/50/30% for 1 min each. Slides were rinsed in 0.9% saline for 5 min and then in PBS for 5 min. The sections were postfixed in freshly prepared and filtered 4% PFA in PBS for 20 min. The slides were washed twice in PBS for 5 min each and treated with proteinase K (Gibco/BRL, 20 μg/ml in 50mM tris-HCl pH 7.2 and 5mM EDTA pH 7.2) for 8 min. Following this, the slides were washed in PBS for 5 min and postfixed in 4% PFA in PBS

for another 20 min. Slides were dipped in distilled water and acetylated in 0.1 M triethanolamine containing 0.25% acetic anhydride in 0.9% NaCl for 10 min. Subsequently, the slides were rinsed in PBS and 0.9% saline for 5 minute each. The sections were dehydrated through a series of 30/50/70/85/95 and 100% ethanols for 1 minute each and the slides were air dried for 1 hour at room temperature. Hybridization solution (200 µl of 50% formamide, 0.3 M NaCl, 20 mM tris-HCl pH 7.4, 5 mM EDTA pH 7.4, 10 mM $NaPO_4$ pH 8.0, 1X Denhardt's, 10% Dextran sulfate) was added to each slide, a coverslip was then applied and embryo sections were incubated in a humidified chamber at 58°C overnight. Each probe contained 2 x 10^7 cpm/ ml of hybridization solution.

The following day the coverslips were gently removed in 5X SSC/10 mM DTT at 50°C. The slides were then washed in 50% formamide, 2X SSC, 100 mM DTT at 60°C for 30 minutes, rinsed briefly in NTE buffer (0.5 M NaCl, 10 mM tris-HCl pH 8.0, 5 mM EDTA pH 8.0) and then in NTE buffer containing RNase A (20 µg/ml Boehringer Mannheim) for 40 min at 37°C. Slides were washed in 2X SSC and 0.1X SSC for 15 minutes each at room temperature. They were dehydrated through a series of ethanols: 30/60/80/95% containing 0.3 M ammonium acetate and 2X 100% ethanol for approximately 1 minute each and then air dried.

Next, the slides were processed. Slides were dipped into photographic emulsion prewarmed to 42°C (Kodak NTB-2 autoradiographic emulsion) in a darkroom. The slides were air dried for 2 hours and placed horizontally in a light-tight box containing desiccant. The box of slides was exposed for 3 weeks at 4°C before development. Prior to development, the box of slides were removed from the 4°C and warmed to room

temperature. In the dark, slides were transferred through a 1:1 dilution of Kodak D19 developer and water for 5 min, distilled water for 10 seconds, Kodak fixer for 5 min and distilled water for 5 min, all at 16°C. Slides were stained with a 1:50 dilution of 0.02% toluidine blue for 5 min, dehydrated in a series of ethanols: 50/75/95/100%, and immersed in xylene for several minutes prior to mounting the sections with Permount (SIGMA). Images were analyzed using both light and dark field optics with a Zeiss Axiophot microscope connected to a Sony PowerHAND video camera, using Northern Eclipse 4.0 software. An Olympus DF Planaro dissecting microscope was used to capture the whole embryo section under darkfield illumination and photographs were captured with an Olympus SC35 Type 12 camera.

5.2.5 MUSCLE HISTOLOGY

Muscle specimens were obtained from transgenic and wild type mice as described above and immersed in 10% neutral buffered formalin (Sigma) for 1-2 weeks. Muscle tissue was then processed and immersed in paraffin wax and sectioned using a microtome. Sections were deparaffinized and rehydrated by treatment in xylene for 2X 10 minutes, 3 minutes in 100%, 95%, 2X 3 minutes in 90% and 3 minutes in 80% ethanol. This was followed by 2X 3 minutes in H_2O. Slides were immersed in Harris Hematoxylin for 10 minutes, dipped in tap water and then in acid alcohol (1% HCl, 70% ethanol) for 10 seconds. Sections were then washed in a coplin jar with continuous tap water flow for 5 minutes and treated in 1% ammonia for 6 minutes. Slides were again washed with continuous tap water flow for 2 minutes, dipped in alcoholic eosin Y (0.5% Eosin Y in 90% ethanol) for 10 minutes and washed again under tap water for 1 minute. Slides were dehydrated in 80% and 90% ethanol for 1 minute, 2X 95% and 2X 100%

ethanol for 1 minute. Slides were then treated in xylene (2X 3 minutes), the excess xylene was wiped away followed by mounting in permount. Microscopic photographs were obtained with a 35mm camera mounted to a Zeiss axiophot microscope using 100 ASA colour Tungsten film (Kodak).

5.2.6 IMMUNOSTAINING OF CRYOSECTIONED MUSCLE

Cryosections were dipped in dH_2O for 1 minute and then fixed in 4% PFA in 1X PBS for 3 minutes at room temperature. All antibody incubations and washes were performed in Stockholm (St.) PBS (8 mM Na_2HPO_4, 2.2 mM NaH_2PO_4, 137 mM NaCl, 2.7 mM KCl). Slides were washed 3X for 3 minutes with shaking. Specimens were blocked using 5% serum in St. PBS at room temperature for 15 minutes. Sections were incubated in a primary antibody solution containing 0.3 % TritonX-100 overnight at 4°C. The following day, slides were washed 3X 3 minutes with shaking and incubated with secondary antibody solution containing 0.3 % TritonX-100. Slides were washed 3X 3 minutes with shaking and mounted in antifade solution (Dako).

5.2.7 MUSCLE SECTIONING AND FIBRE SIZE DETERMINATION

Mouse hind limb muscle (including TA, EDL, gastrocnemius and soleus) samples were obtained from transgenic and wild type littermates following anaesthatization with somnitol (0.1 ml/100g) with care taken to ensure the soleus muscle of both animals were of similar length. Hind limb muscle was hemisectioned such that the soleus was bisected at the midline. Muscles were cryopreserved by attachment to a piece of acetate and submersion in isopentane cooled in liquid nitrogen for approximately 30 seconds. Frozen muscle samples were attached to a chuck with tissue tek at –20°C and sectioned using a cryostat. Muscle sections were adhered and melted to precleaned "Superfrost-Plus"

slides (VWR) and stored at –80°C. To ensure muscle sections were obtained from the centre 33% of the soleus muscle, sections were stained with α-bungarotoxin – CY3 (1:500, Molecular Probes) as described in the immunofluorescence section. Sections were stained with an antibody to type I myosin heavy chain (MHC) (courtesy of Dr. David Parry) as described in the immunofluorescence section except that the secondary antibody was conjugated to horseradish peroxidase. A brown precipitate resulting from horseradish peroxidase catalysis of the 3, 3'-Diaminobenzidine tetrahydrochloride (DAB) substrate indicated MHC type I positive muscle fibres. Type I (DAB positive) and type II (DAB negative) fibres from transgenic and wild type mice were measured using the ScionImage program (Scion corporation). Measurements were imported into the Microsoft Excel program for computation of statistics and graphs.

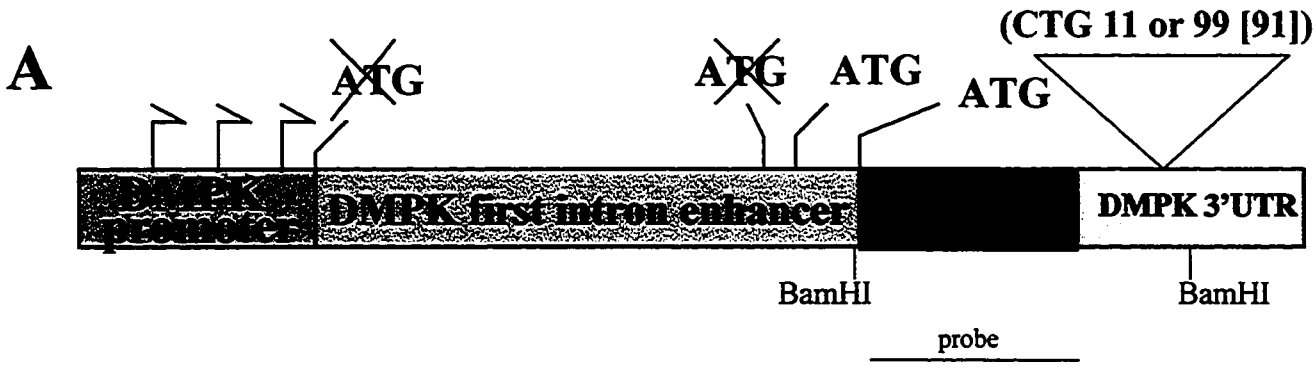
5.3 RESULTS

Although Myotonic dystrophy most noticeably affects skeletal muscle, it is a multisystemic disease also affecting heart, smooth muscle, brain and testes. The diversity of tissues affected in DM and the closely matching expression pattern of the myotonic dystrophy kinase suggests that perturbation of the function of the DMPK gene alone is responsible for DM. Consistent with this hypothesis, a number of DM features have been observed in transgenic mice overexpressing the human DMPK gene, including both the 5' and 3' UTR elements (Narang 2000). *In vitro* studies have implicated the DMPK 3' UTR (Sabourin et al 1997) and CTG repeat expansions contained within (Amack et al 1999; Bhagwati et al 1999; Usuki et al 1997) in causing defects in myogenic fusion, a prominent feature of cDM. To see if this observation could be carried over to an *in vivo* model and to assess any possible relevance of it to DM, it was of importance to study the

effects of DMPK 3' UTR overexpression in transgenic mice. In addition, the wide array of tissues in which DMPK is expressed suggests complex transcriptional regulation at this locus. We have characterized *cis* regulatory elements governing both basal and muscle specific expression of this gene in NIH 3T3 fibroblasts, human TE32 myoblasts and mouse 10T1/2 fibroblasts (Storbeck et al 1998). We wished to explore the functionality of sequences within the 5' UTR and first intron of the DMPK gene *in vivo*. Therefore, in order to study effects of DMPK 3' UTR overexpression *in vivo*, we combined the DMPK 3' UTR with the DMPK transcription regulatory elements. Transgenic mice expressing a GFP reporter gene driven by a 1.5 Kb fragment of the DMPK promoter and a 2.4 Kb fragment of the DMPK first intron were produced (figure 5-1A). The transgene also contained a section of the 3' end of the DMPK gene encompassing part of exon 12 to the end of exon 15 including the entire 3' UTR with either 11 or 91 CTG repeats (figure 5-1A). It was hoped that endogenous regulatory elements would direct transgene expression to specialized tissues where endogenous DMPK is localized. Following injection of the linearized transgene into fertilized oocytes, founder mice from mothers impregnated with injected oocytes were identified using southern blot analysis performed on DNA isolated from tail clippings (figure 5-1B).

In order to determine expression patterns conferred by the endogenous DMPK regulatory elements *in vivo*, total RNA was isolated from skeletal muscle, heart and brain from 5 lines of transgenic mice containing 11 CTG repeats. Expression of the transgene mRNA was highest in heart, then skeletal muscle and least in brain and this relative pattern was observed in all five lines (figure 5-2A, B). This expression pattern is

Figure 5-1. DMPK CTG 11 and CTG 91 transgenes and identification of transgenic mice by southern blot analysis. (A) Shown is a schematic representation of the transgene used in the construction of CTG 11 and CTG 91 transgenic mice. Indicated are the BamHI sites used in southern blot analysis and the location of the probe used for screening southern blots for transgene positive animals. (B) DNA was isolated from mouse tails, digested with BamHI, run on a 1% agarose gel, transferred to a charged nylon membrane and probed with the GFP cDNA. Several animals on the blot harbour the 91 CTG repeat transgene while a single animal is positive for the 11 CTG repeat transgene.


A
(CTG 11 or 99 [91])
ATG
ATG
ATG
ATG
DMPK promoter
DMPK first intron enhancer
DMPK 3'UTR
BamHI
BamHI
probe

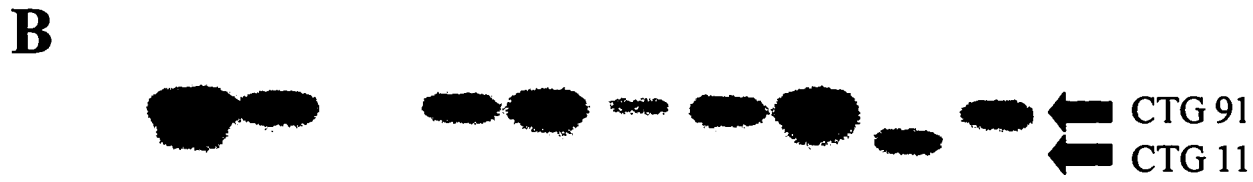
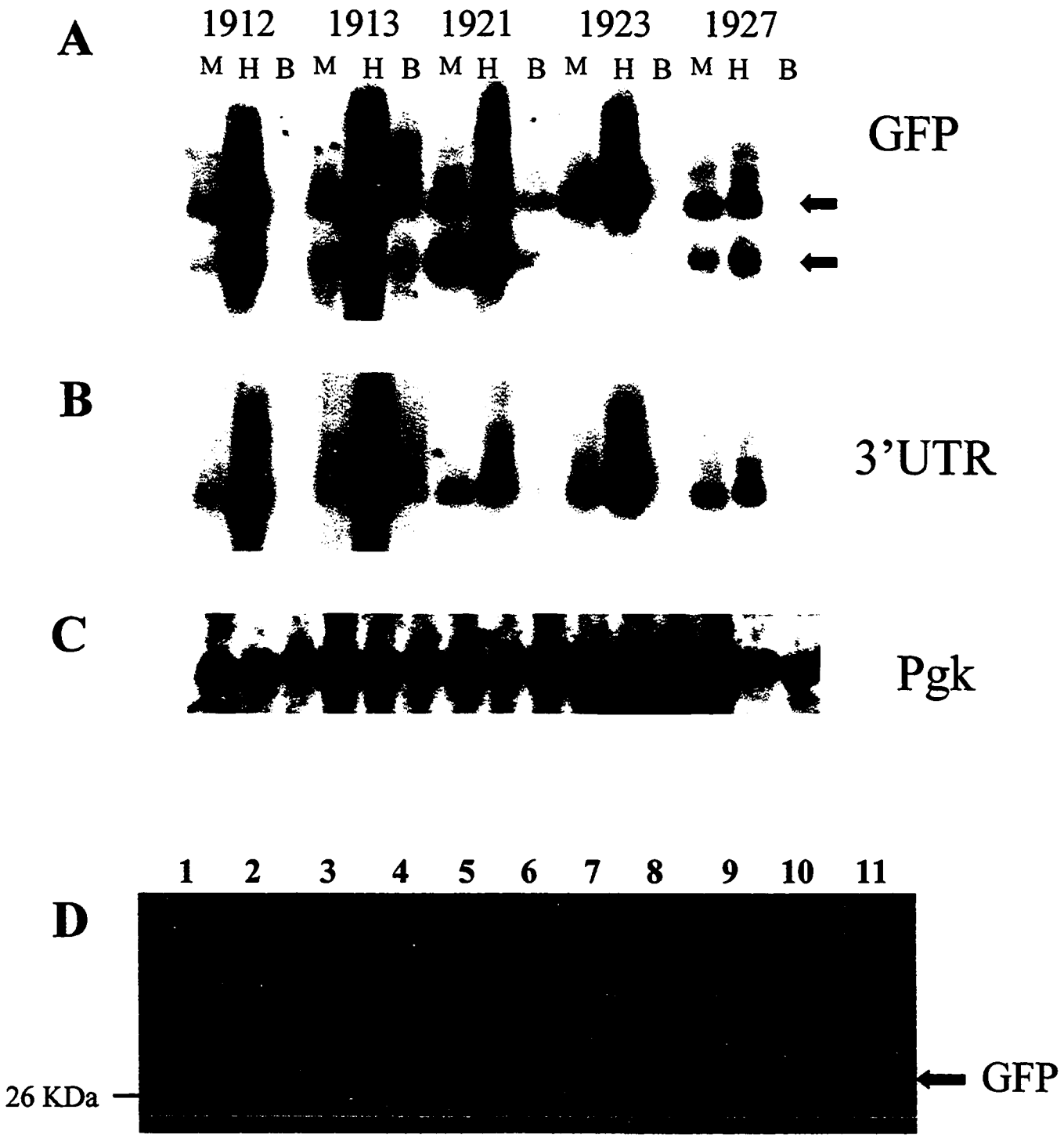

B
CTG 91
CTG 11

Figure 5-2. Expression analysis of mRNA and protein from various tissues of several lines of CTG 11 transgenic mice. (A) Northern blot analysis was performed on 15 μg of total RNA isolated from skeletal muscle, heart and brain of five CTG 11 transgenic mouse lines (1912, 1913, 1921, 1923 and 1927). Denatured RNA was run on a 1% formaldehyde agarose gel and transferred to a neutral nylon membrane. The northern blot was probed with GFP , the 3' end of the DMPK 3' UTR (B) and Pgk (C). The lower of the two bands indicated by the arrows represents a transcript truncated after the GFP segment of the transgene is transcribed because it is detectable with the GFP probe and not the 3' UTR probe. To control for RNA loading, the blot was probed with the Pgk cDNA. (D) Western blot analysis of protein isolated from transgenic and wild type littermates. 30 μg of protein lysate for each sample was run on a denaturing polyacrylamide gel (SDS-PAGE), transferred to a PVDF membrane and probed with a polyclonal GFP antibody. Lanes 1 and 2 respectively represent protein lysates from C2C12 cells transfected with a GFP expression vector or mock transfected. Lanes 3 and 4 are lysates obtained from tongue of a transgenic and wild type animal respectively. Lane 5 is skeletal muscle extract from a transgenic mouse, lanes 6 and 7 are skeletal muscle extracts from wild type littermates. Lanes 8 and 9 are extracts from brain of wild type and transgenic animals respectively and lanes 10 and 11 are extracts from heart of wild type and transgenic animals respectively. Expression of CTG 11 mRNA and protein is highest in cardiac tissue and is present but at lower levels in skeletal muscle.


A
1912
1913
1921
1923
1927
M H B M H B M H B M H B M H B
GFP
B
3'UTR
C
Pgk
D
1 2 3 4 5 6 7 8 9 10 11
26 KDa
GFP

consistent with the observed expression pattern of the endogenous gene [Jansen, 1992 #781]. When GFP cDNA was used to probe the northern blot, two prominent bands were observed (figure 5-2A). In contrast, when a probe from the 3' end of the DMPK 3' UTR was used to probe the northern blot, a single band was detected corresponding to the highest molecular weight band of the two observed using the GFP cDNA as probe (figure 5-2B). This clearly demonstrates premature termination is occurring *in vivo* because unlike the larger of the two transcripts identified with the GFP probe, the smaller transcript does not hybridize to the DMPK 3' UTR (figure 5-2A, B). To control for loading, the northern blot was probed with the mouse Pgk cDNA (figure 5-2C).

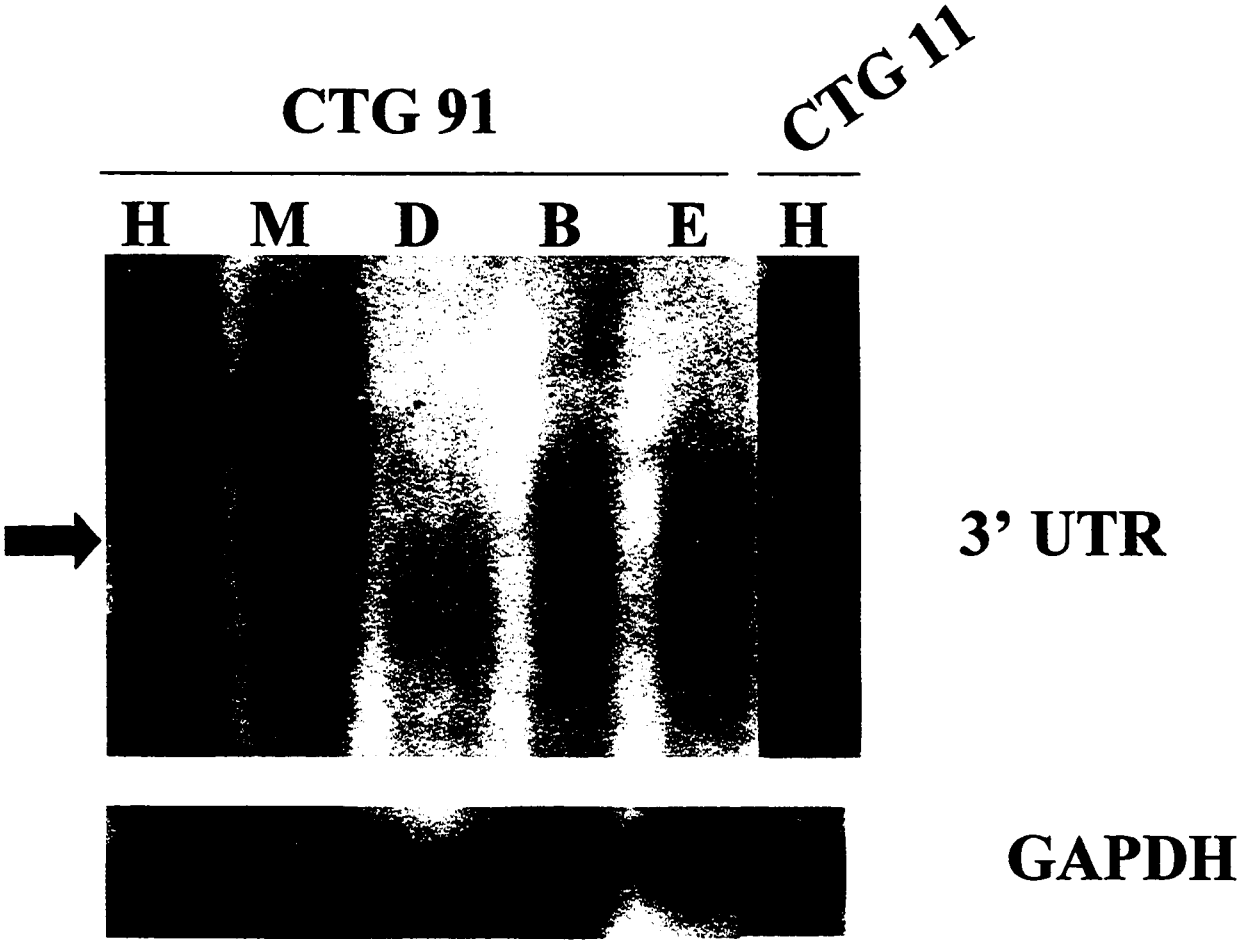
Expression of transgene mRNA from the DMPK promoter/enhancer mice was clearly observed in the expected tissues. However, in order to determine if this message was being translated and if protein expression reflected the mRNA distribution, western blot analysis was performed on tissue extracts obtained from one line of the CTG 11 mice. A 26 KDa band which reacted strongly with a GFP polyclonal antibody was detected in extracts from C2C12 myoblasts transiently transfected with a GFP expression construct (figure 5-2D, lane 1). This was in contrast to mock transfected C2C12 myoblasts where no GFP reactive species were detected (figure 5-2D, lane 2). Expression of GFP protein was observed in transgenic mouse tissue extracts isolated from tongue (figure 5-2D, lane 3), skeletal muscle (figure 5-2D, lane 5) and heart (figure 5-2D, lane 11) but not in brain (figure 5-2D, lane 9). Wild type littermate tissue extracts from tongue (figure 5-2D, lane 4), skeletal muscle (figure 5-2D, lanes 6 and 7), brain (figure 5-2D, lane 8) and heart (figure 5-2D, lane 10) were employed as negative controls. Clearly, expression of GFP protein mirrors that observed with the transgene

mRNA with highest expression in heart and lower levels observed in muscle. Expression of GFP protein was not detected in the brain extract of the transgenic animal analyzed, however this is not surprising considering the very low level of transgene mRNA observed in the five transgenic lines (figure 5-2 A, B and D).

We analyzed the relative expression of transgene mRNA isolated from various tissues obtained from three lines of 91 CTG repeat mice. Expression was highest in line 2043-1 (data not shown). We then compared expression levels between the 11 and 91 CTG repeat transgenic mice. Using northern blot analysis, total mRNA isolated from several tissues of a transgenic mouse from the 2043-1 line (91 CTG repeats) was run alongside heart mRNA isolated from a transgenic mouse from the 1912 line (11 CTG repeats). Expression of an mRNA containing the human DMPK 3' UTR was evident in skeletal muscle and heart tissue from the 91 repeat mouse (figure 5-3, CTG 91 lanes H, M). Expression was low or undetectable in eye, brain and diaphragm, although in the case of eye and diaphragm, GAPDH probing of the northern blot revealed significantly less RNA was loaded in these lanes (figure 5-3, CTG 91 lanes D, B, E). However, mRNA levels in muscle and heart of the CTG 91 mouse was significantly less than that observed in the control heart RNA sample from the 11 repeat mouse (figure 5-3, CTG 11 lane H). This data indicates that although the CTG 91 transgene is expressed, it is apparently expressed at levels significantly lower than observed for the CTG 11 transgenic mice.

We next sought to determine if the DMPK promoter/enhancer could direct expression of the transgene in the correct spatio-temporal pattern. Embryos from matings

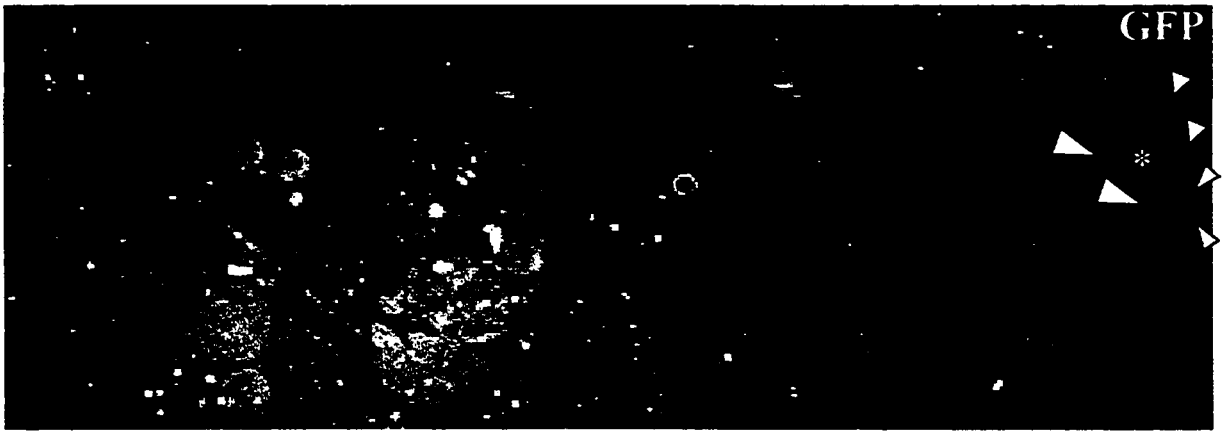
Figure 5-3. Expression analysis of CTG 91 transgenic mice. Total RNA was isolated from heart, muscle, diaphragm, brain and eye of CTG 91 mouse strain 2043-1 and 15 μg was run on a 1% formaldehyde agarose gel, blotted to neutral nylon membrane and probed with a portion of the DMPK 3' UTR or the mouse GAPDH cDNA. As a positive control, 15 μg of RNA isolated from heart of a CTG 11 transgenic mouse was included on the blot. CTG 91 mRNA, like CTG 11 mRNA, is expressed in heart and skeletal muscle but less so in brain and is expressed at lower levels than CTG 11 mRNA.


CTG 91
CTG 11
H
M
D
B
E
H
3' UTR
GAPDH

between transgenic and wild type mice at embryonic day 12 (day 12 dpc) were isolated, fixed, processed and sectioned. In order to identify transgenic embryos and to realize the developmental expression pattern conferred by the DMPK promoter/enhancer, embryo sections were analyzed by *in situ* hybridization with an antisense probe encompassing a portion of the GFP cDNA. The observed pattern with the GFP probe clearly revealed that only embryo 4 was transgenic (figure 5-4A). On a serial section, an antisense probe for myogenin was used as a positive control for the *in situ* technique. Also, because of its role in myogenesis, myogenin was used as a marker for developing muscle in the embryo. It is clear that all four embryos are positive for myogenin and that myogenin staining is observed in the somites (arrow) and developing muscles of the neck region (arrowhead)(embryo 3, figure 5-4B). Comparison of myogenin staining intensity between embryo 4 and the other three embryos indicates a noticeably weaker myogenin signal in embryo 4 and an absence of myogenin staining in neck muscles of embryo 4 (yellow asterisk, embryos 2-4, figure 5-4 B). The lack of myogenin staining in neck muscles (see yellow asterisk) of the transgenic embryo (embryo 4) compared with a wild type embryo (embryo 2) is evident despite similar planes examined (embryos 2 and 4, figure 5-4 B). When comparing GFP and myogenin staining in sections of embryo 4, it is evident that there are areas where expression of the two genes clearly overlap (arrowheads, figure 5-4A and B) and areas where GFP is expressed and myogenin is not (arrows and white asterisk, figure 5-4A and B). In addition, a segment of neck muscle, which is GFP and myogenin positive, located in the middle back of the day 12 embryo (green asterisk, figure 5-4 B) appears out of position in the transgenic embryo. In wild

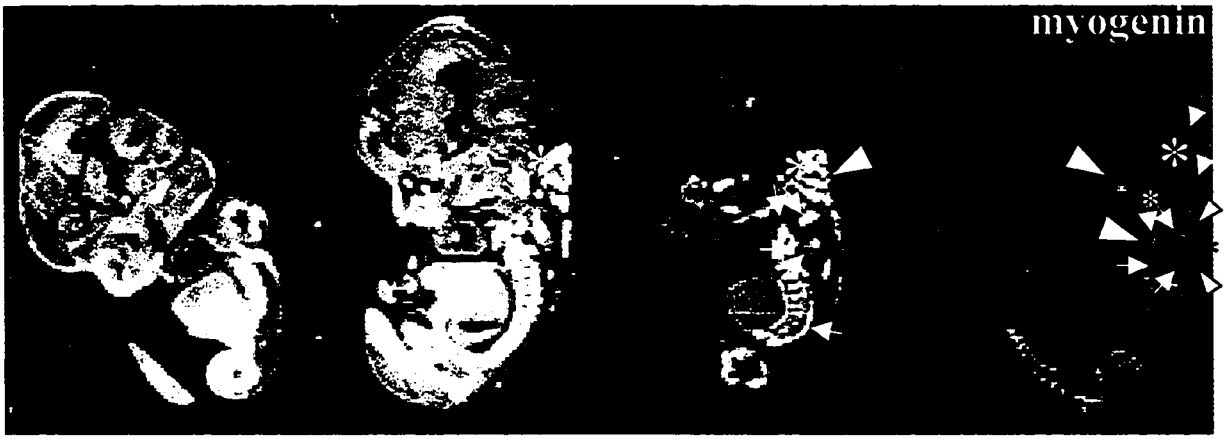
Figure 5-4. Expression of the CTG 11 mRNA during mouse embryogenesis. (A) Embryos were harvested from a wild type female that was mated with a transgenic male at 12 days post coitum (dpc). Embryos were fixed in formalin, processed, embedded in paraffin and sectioned using a microtome. Expression of the CTG 11 transgene on embryo sections was analyzed by *in situ* hybridization. Embryos are numbered from 1 to 4. (A) By using an antisense probe specific for GFP, transgenic embryos could be distinguished from wild type. (B) As a positive control, and to assay muscle differentiation, an antisense probe for myogenin, which is expressed in developing skeletal muscle, was utilized. At 12 d p.c., the CTG 11 transgene is expressed in skeletal muscle precursor cells of the somite and neck in a similar pattern to myogenin (confirming *in vivo* the muscle specificity of the regulatory elements in the DMPK first intron enhancer). In addition, myogenin levels are reduced only in the transgenic embryo and certain facial muscles fail to be detected by the myogenin probe in the transgenic embryo. White arrows in embryo 4 show areas of GFP transgene expression where myogenin expression is absent. Small white arrowheads in embryo 4 denote developing neck musculature positive for both GFP transgene and myogenin transcripts. Uppermost large white arrowhead in embryo 4 shows myogenin expression in a subset of musculature positive for GFP transgene transcripts. Lower large white arrowhead in embryo 4 illustrates muscle structure positive for both GFP transgene and myogenin transcripts. White asterisk in embryo 4 shows facial muscle positive for GFP transcripts but lacking myogenin transcripts. Yellow asterisks in embryos 2-4 indicate robust expression of myogenin in musculature of wild type embryos (2 and 3) and lack of myogenin expression in this area of the transgenic embryo (4). Green asterisk in embryos 2-4 illustrate the abnormal position of a muscle structure in the transgenic embryo (4) compared to wild type embryos (2 and 3). Yellow arrows in embryos 3 and 4 show normal positioning of 4 neck muscle structures in both the wild type (3) and transgenic embryo (4). White arrow in embryo 3 indicates myogenin staining of developing somitic musculature in a wild type embryo. DMPK 3' UTR mRNA linked to GFP shows appropriate spatial and temporal expression pattern during murine development and disrupts aspects of myogenesis *in vivo* as detected by abnormal myogenin *in situ* hybridization patterns .

A



1 2 3 4

B



type embryos, this muscle segment is positioned vertically in embryos 2 and 3, however, in the transgenic embryo (embryo 4), this segment of muscle is oriented higher and in a more horizontal position. Despite this difference, certain neighbouring structures are correctly positioned in the transgenic embryo (4) compared with normal embryo 3 (yellow arrows, figure 5-4 B).

Closer inspection of day 12 embryos highlights matching expression patterns between GFP transcripts in a transgenic embryo and myogenin transcripts in the developing somites of a wild type embryo (arrows). The discordance in staining of neck and face muscles between the transgenic and wild type embryos is also apparent (arrowheads) (figure 5-5A and B).

At embryonic day 20, just prior to birth, expression of both GFP and myogenin mRNA were analyzed by in situ hybridization. Like day 12 embryos, expression of the two genes overlapped significantly in various developing muscle structures like the tongue (T), intercostal muscles and muscles of the limb (arrows, figure 5-6A and B). Inspection of the head region reveals high levels of GFP and myogenin expression in the tongue (t), snout (*) and to a lesser extent in muscles of the neck and throat (m) (figure 5-6A and B). In contrast to myogenin expression (figure 5-7A), GFP was expressed in several regions of the brain including areas of the frontal lobe, the midbrain, the subventricular layer and the hindbrain (arrowheads, figure 5-7A and B). These data demonstrate that at least some regulatory elements required for expression of DMPK in the brain are included in the promoter/enhancer combination employed in the construction of these transgenic mice. Examination of the torso area of the 20 day embryo serial sections hybridized with myogenin and GFP probes revealed expression of

Figure 5-5. Expression of GFP and myogenin mRNA in day 12 CTG 11 embryo. Embryos were harvested from mothers at 12 dpc, processed, embedded in paraffin and sectioned. In situ hybridization with antisense probes to myogenin (A) and GFP (B) was performed. Expression of myogenin and the GFP containing transgene can clearly be seen in individual somites and also in developing muscle structures of the face and neck area

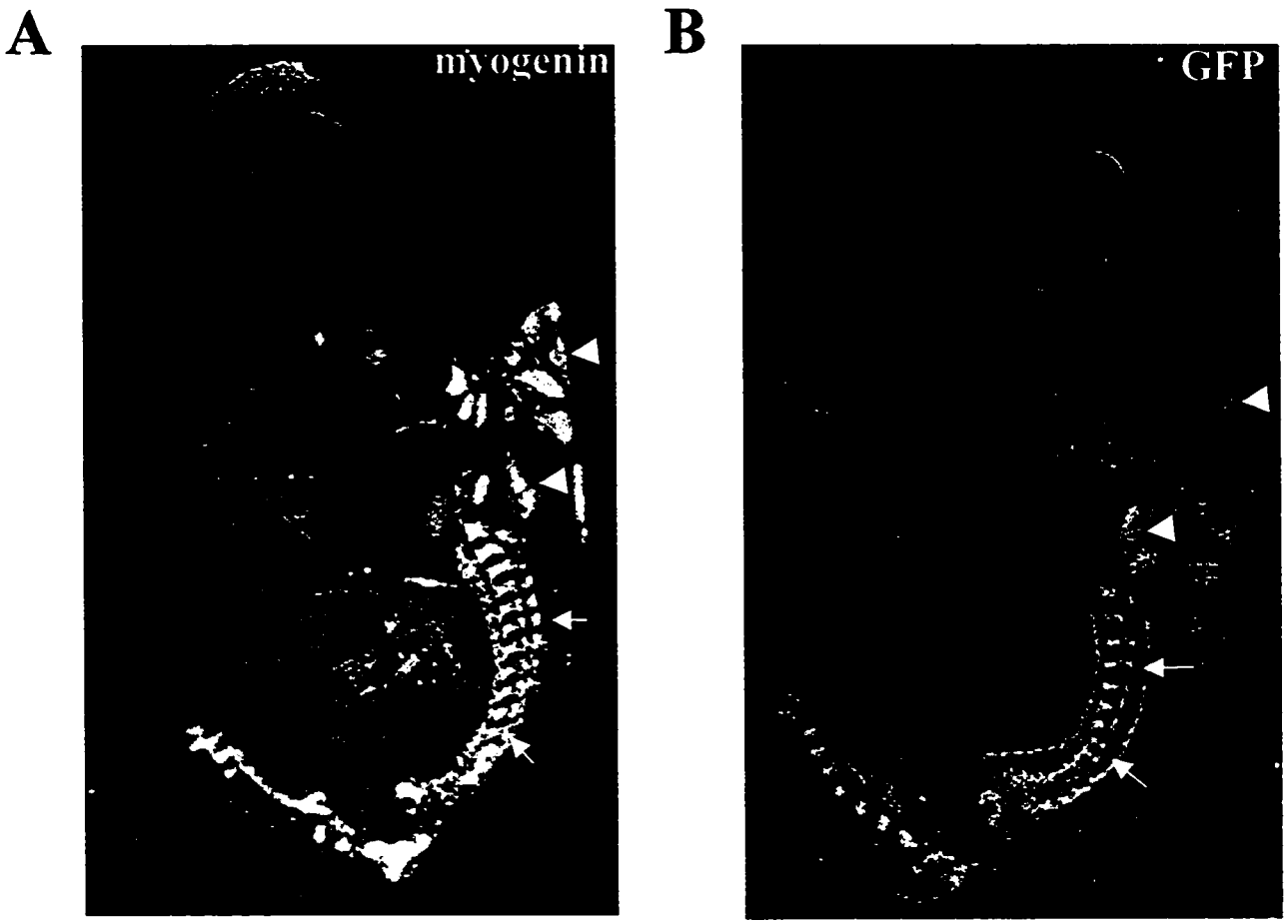

A
myogenin
B
GFP

Figure 5-6. Expression of CTG 11 transgene in mouse embryos at 20 dpc. Mouse embryos were obtained at 20 dpc from a wild type female mated with a transgenic male. Embryos were fixed in formalin, processed, embedded in paraffin and sectioned. In situ hybridization was performed on embryo sections with an antisense probe for (A) myogenin and (B) GFP. There are clear examples of expression of the CTG 11 transgene in the same areas as myogenin like the intercostal muscles (M) and the tongue (T) and also examples of expression of the transgene in areas distinct from myogenin like the brain , heart (H) and smooth muscle linings of blood vessels and intestines.

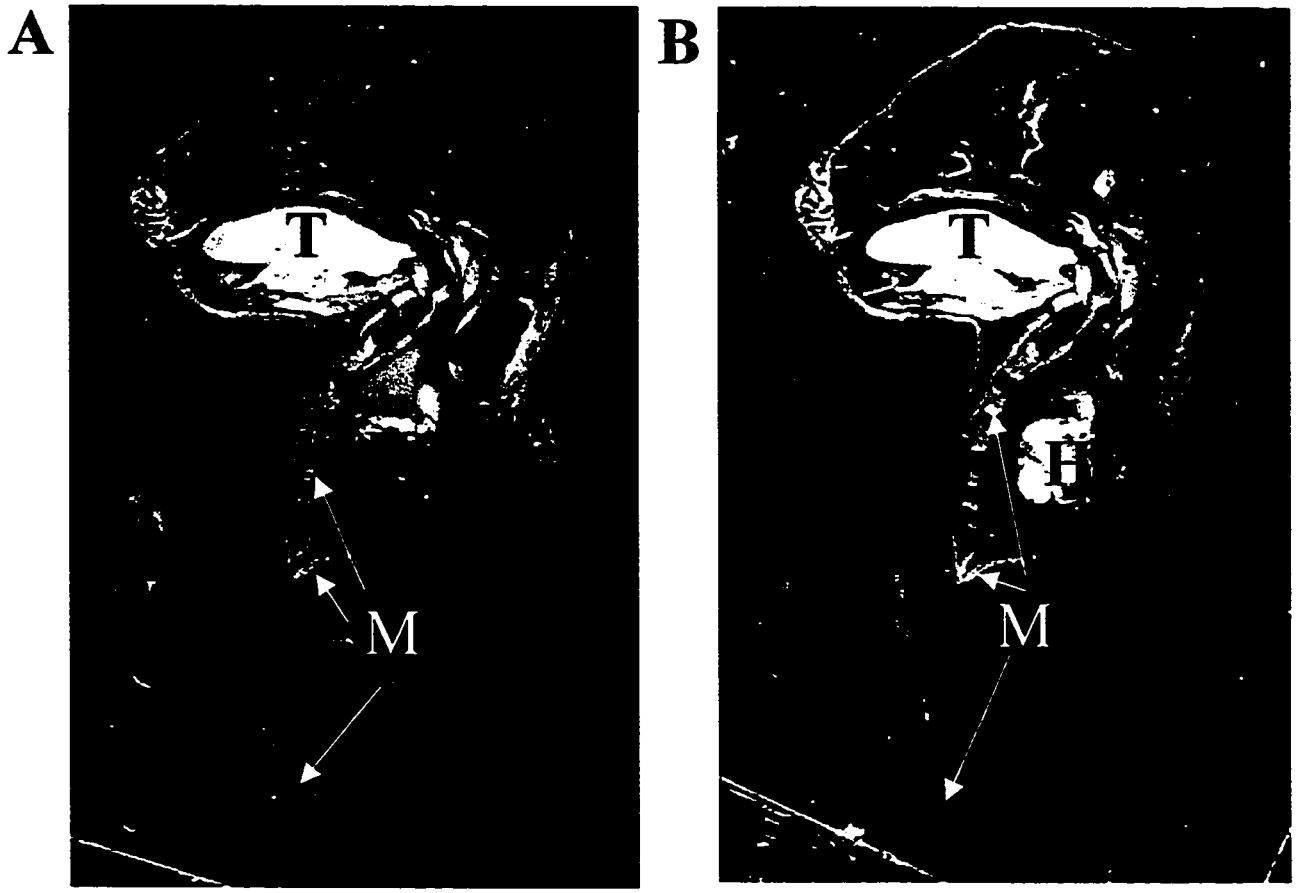
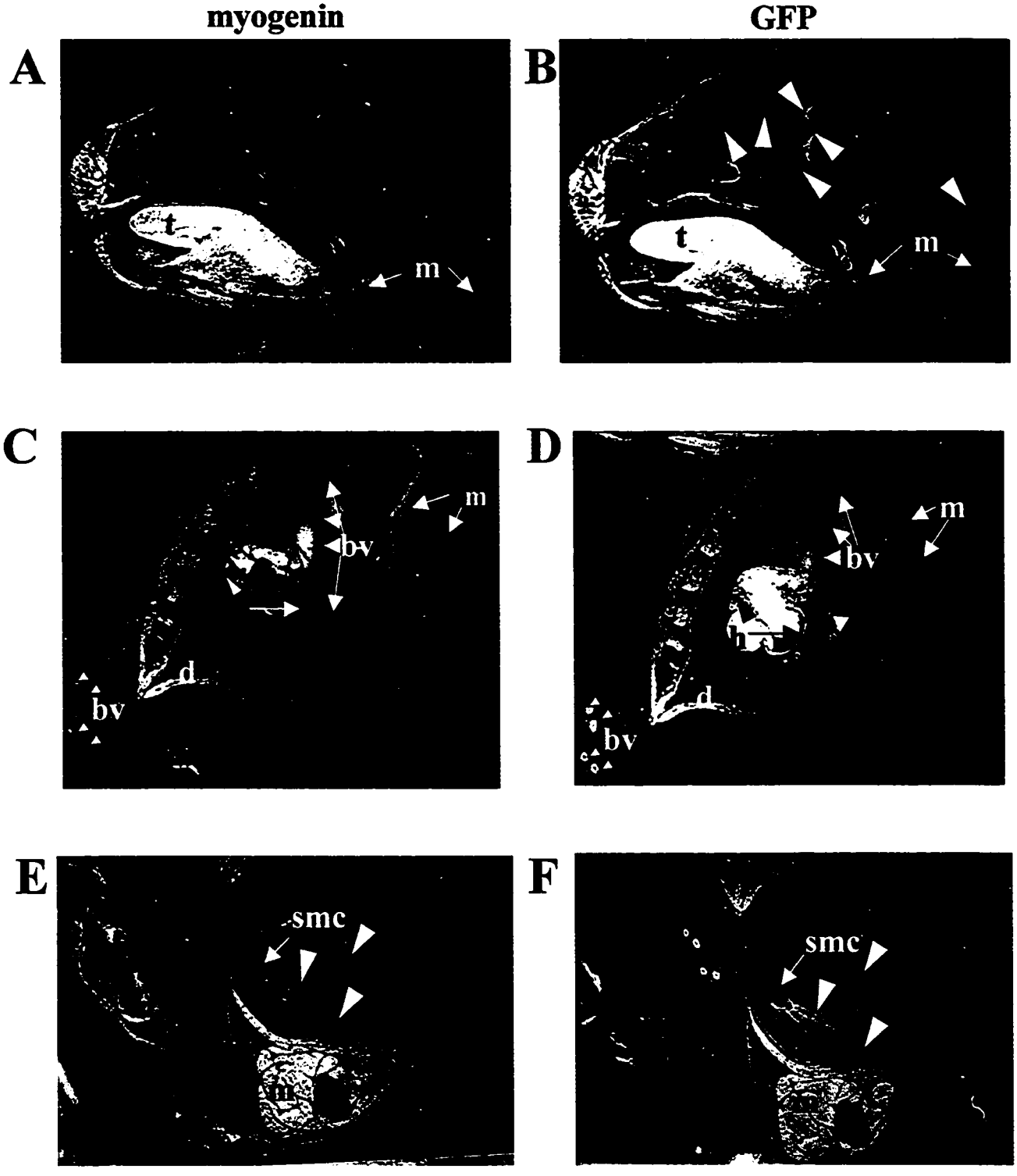

A
T
M
B
T
M

Figure 5-7. Expression of the CTG 11 transgene in d20 embryos. In situ hybridization was performed on a transgenic d20 embryo using antisense probes for myogenin (A, C, E) and GFP (B, D, F). (A, B) Expression of myogenin and the CTG 11 transgene is clearly seen in tongue and muscles of the head a neck area. The CTG 11 transgene is also seen in certain regions of the brain (arrowheads)(B). Expression of myogenin and the transgene are seen in the intercostal muscles (m), muscles of the back and neck (m)and in the diaphragm (d) (C, D). However, expression observed in smooth muscle cells lining the blood vessels (bv) and cardiac cells of the heart (h) is only seen with the transgene and not with myogenin indicating additional regulatory elements in the DMPK enhancer specifying expression of the transgene in these tissues. Expression of both myogenin and the CTG 11 transgene is observed in the muscles of the lower limb (m) (E, F). Only the CTG 11 transgene is expressed in the smooth muscle cell (smc) lining of the large intestines and amniotic blood vessels (arrows, arrowheads) (F). The CTG 11 transgene is expressed in skeletal muscle, heart, smooth muscle and brain at embryonic day 20. This pattern is indistinguishable from that of the endogenous gene and indicates that many of the regulatory elements required for tissue specific expression were included in the transgene.


myogenin
GFP
A
B
t
m
t
m
C
D
m
bv
d
bv
m
bv
d
bv
E
F
smc
smc

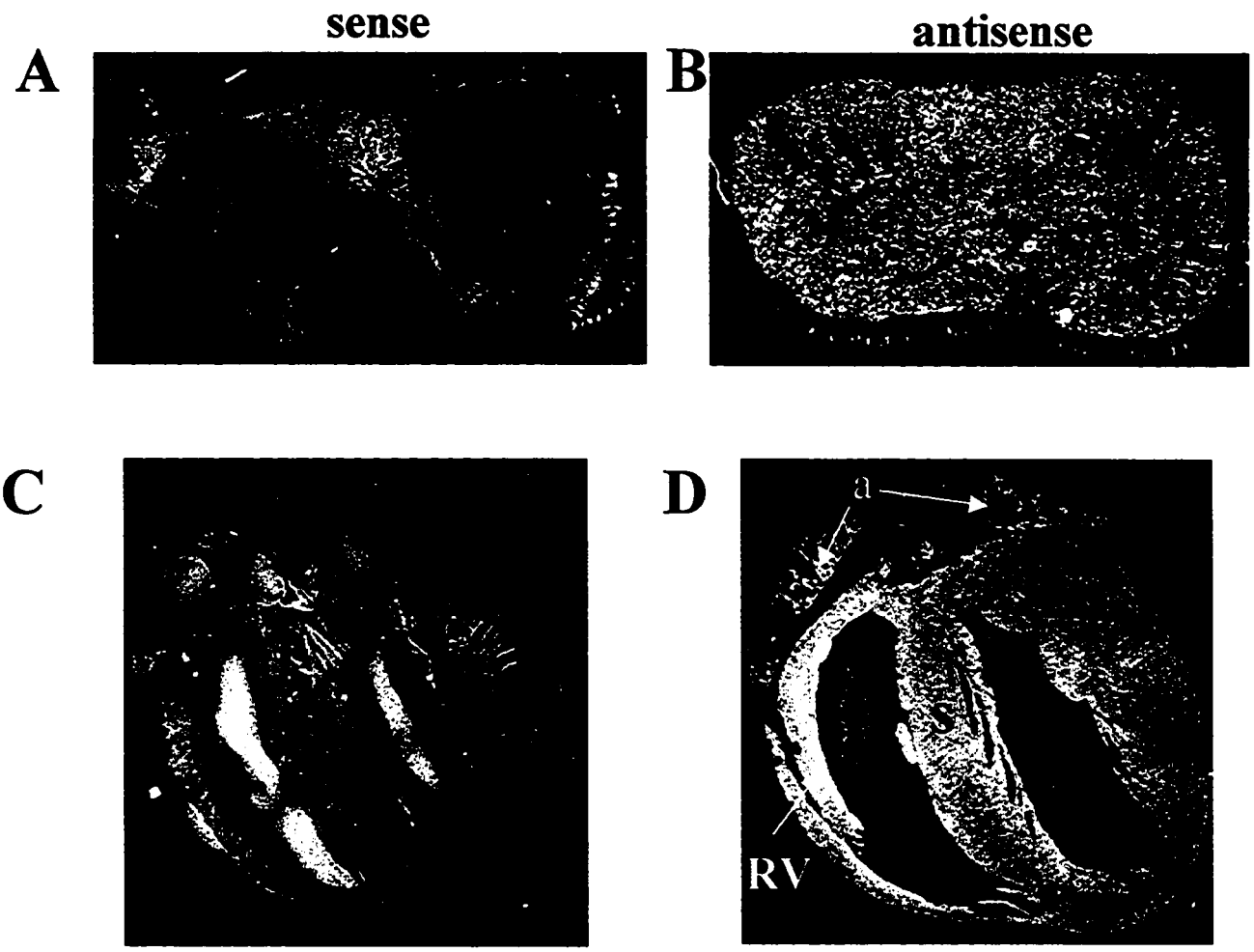
both genes in intercostal muscles (m), muscles of the back (m) and the diaphragm (d) (figure 5-7C and D). However, it was also evident that smooth muscle and cardiac expression was conferred to the GFP transgene by the DMPK promoter/enhancer elements. Expression of the GFP mRNA but not myogenin mRNA was observed in the atrial muscle (arrowhead, figure 5-7C and D) and in ventricular heart muscle (h, and arrow figure 5-7C and D). In addition, expression of GFP mRNA was observed in the smooth muscle lining of hepatic and amniotic blood vessels (bv, arrows and arrowheads) whereas myogenin was not expressed in these structures (figure 5-7C and D).

Finally, in the gut area of day 20 embryos, GFP and myogenin mRNA were observed in skeletal muscle of the lower limbs (m). In contrast, only GFP mRNA was localized to the smooth muscle layer lining the large intestines (smc, arrows and arrowheads figure 5-7E and F). These data are completely consistent with in situ hybridization data for the DMPK gene reported by Jansen et al (Jansen et al 1996) suggesting that most of the regulatory elements responsible for correct spatio-temporal regulation of the DMPK gene are contained within the fragments used in construction of our transgenic mice.

To investigate whether transgene expression persisted in adult mice, we examined expression of the GFP mRNA in a subset of adult tissues by *in situ* hybridization. Expression of the GFP transgene could be clearly detected in adult tongue and heart (figure 5-8B and D) whereas sense control probes were blank in both cases (figure 5-8A and C). Expression in the heart was strong in the atria (a), septum (s) and both ventricles (LV, RV, figure 5-8D).

Histological analysis of CTG 11 and CTG 91 six month old transgenic soleus muscle revealed only minor changes compared with wild type littermates. Hind limb muscles

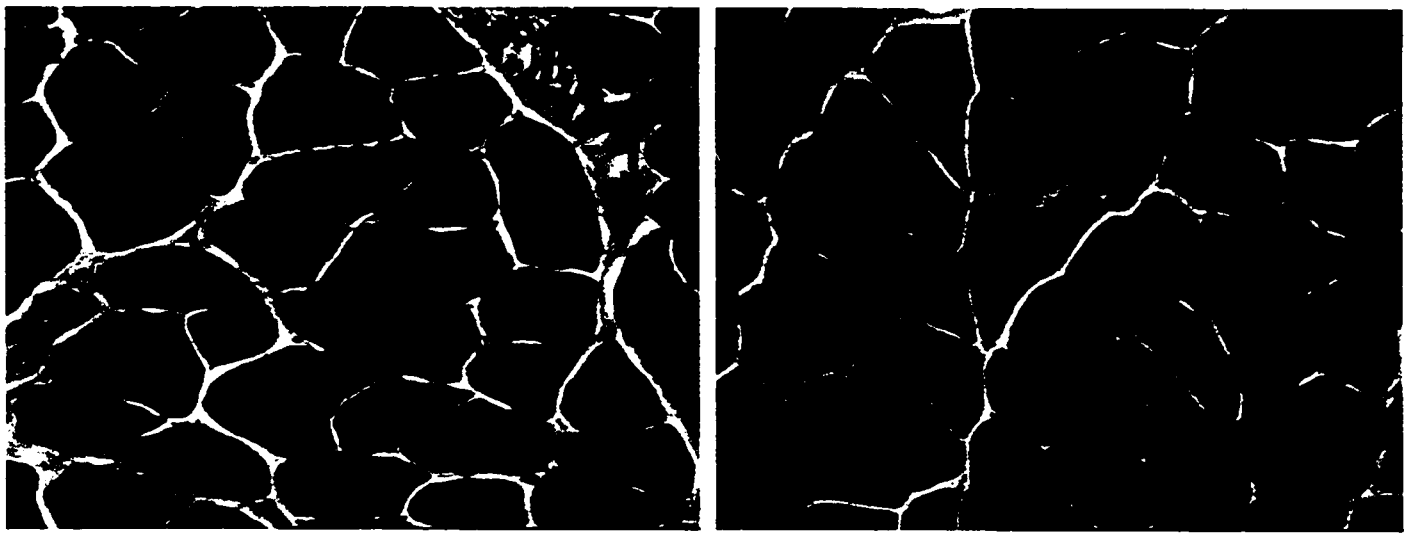
Figure 5-8. Expression of the CTG 11 transgene in adult heart and tongue. An adult transgenic CTG 11 mouse was sacrificed and heart and tongue tissue were recovered, fixed in formalin, processed, embedded in paraffin and sectioned. Sections of tongue (A, B) and heart (C, D) were analyzed by in situ hybridization using a GFP sense (A, C) or antisense (B, D) probe. Expression of the transgene mRNA is apparent in adult heart and tongue tissue.


sense
antisense
A
B
C
D
a
RV

were isolated from two separate transgenic lines of CTG 11 and three lines of CTG 91 mice as well as sex matched wild type littermates. In CTG 11 line 1923, it appears that fibres in the transgenic animal (figure 5-9 D) are larger than in the wild type (C). No major differences are evident between soleus fibres of transgenic and wild type animalof CTG 11 line 1913 (figure 5-9, A, B). Inspection of haematoxylin/eosin stained soleus from CTG 91 transgenic and wild type mice did not reveal any striking features of DM (figure 5-10). The random field from the transgenic sample of line 2043.1 contained two fibres with central nuclei, one feature of DM muscle pathology (arrows, figure 5-10 D). Also, it appears that muscle fibres in the transgenic sample of line 2038.3 are smaller in area than muscle fibres of the wild type control (figure 5-10 A, B).

We next investigated whether differences in mean fibre size indicative of atrophy or hypertrophy were evident in skeletal muscle of transgenic mice. Frozen sections were incubated with an antibody to type I MHC to distinguish type I and type II fibres within the soleus muscle. Type I and type II fibre areas were determined for wild type and transgenic mice (figure 5-11 A). In CTG 11 mice, which express similar levels of mRNA (figure 5-2) there were significant differences in type I fibre size but not in type II fibre sizes. Significant type I fibre atrophy in the transgenic muscle section was seen in CTG 11 line 1913, however, CTG 11 line 1923 had significant type I fibre hypertrophy in the transgenic muscle (figure 5-11 A). By contrast, two lines of CTG 91 mice displayed significant type I and type II fibre atrophy while one CTG 91 line showed significant type I hypertrophy but no significant differences in type II fibre sizes ($P<0.005$, figure 5-11 B). Expression levels of transgene mRNA are highest in the two lines displaying type I and II fibre atrophy (figure 5-8). Therefore it appears that despite lower expression of the

Figure 5-9. Cross-sections of soleus muscle from 6 month old 11 CTG repeat transgenic mice and wild type littermates. Frozen sections were obtained from the middle third of the soleus muscle confirmed by α-bungarotoxin staining and stained with haematoxylin and eosin. Stained cross-sections from wild type (A, C) mice are shown on the left and transgenic (B, D) cross-sections on the right. Two lines are shown in all, including 1913 (A, B) and 1923 (C, D).



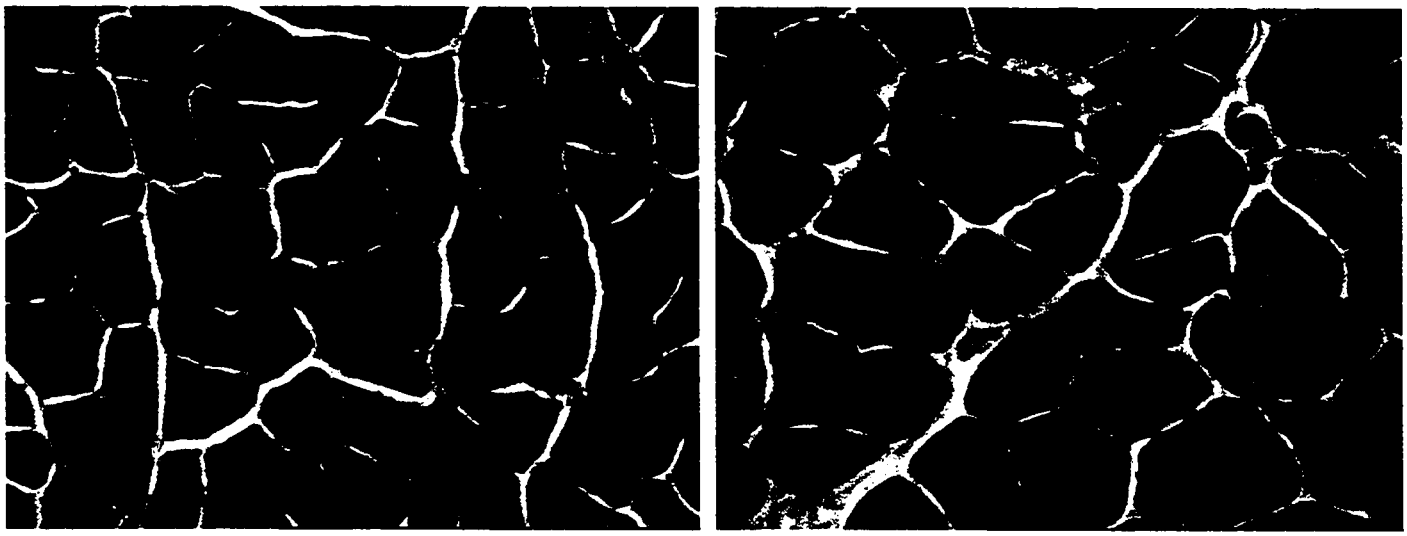


Figure 5-10. Cross-sections of soleus muscle from 6 month old 91 CTG repeat transgenic mice and wild type littermates. Frozen sections were obtained from the middle third of the soleus muscle confirmed by α-bungarotoxin staining and stained with haematoxylin and eosin. Stained cross-sections from wild type (A, C, E) mice are shown on the left and transgenic (B, D, F) cross-sections on the right. Three lines are shown in all, including 2038.3 (A, B), 2043.1 (C, D) and 2048.4 (E, F). Arrows in (D) indicate centrally located nuclei. Two of three lines of 91 CTG repeat transgenic mice show significant fibre atrophy in type I and type II muscle fibres ($P<0.005$).

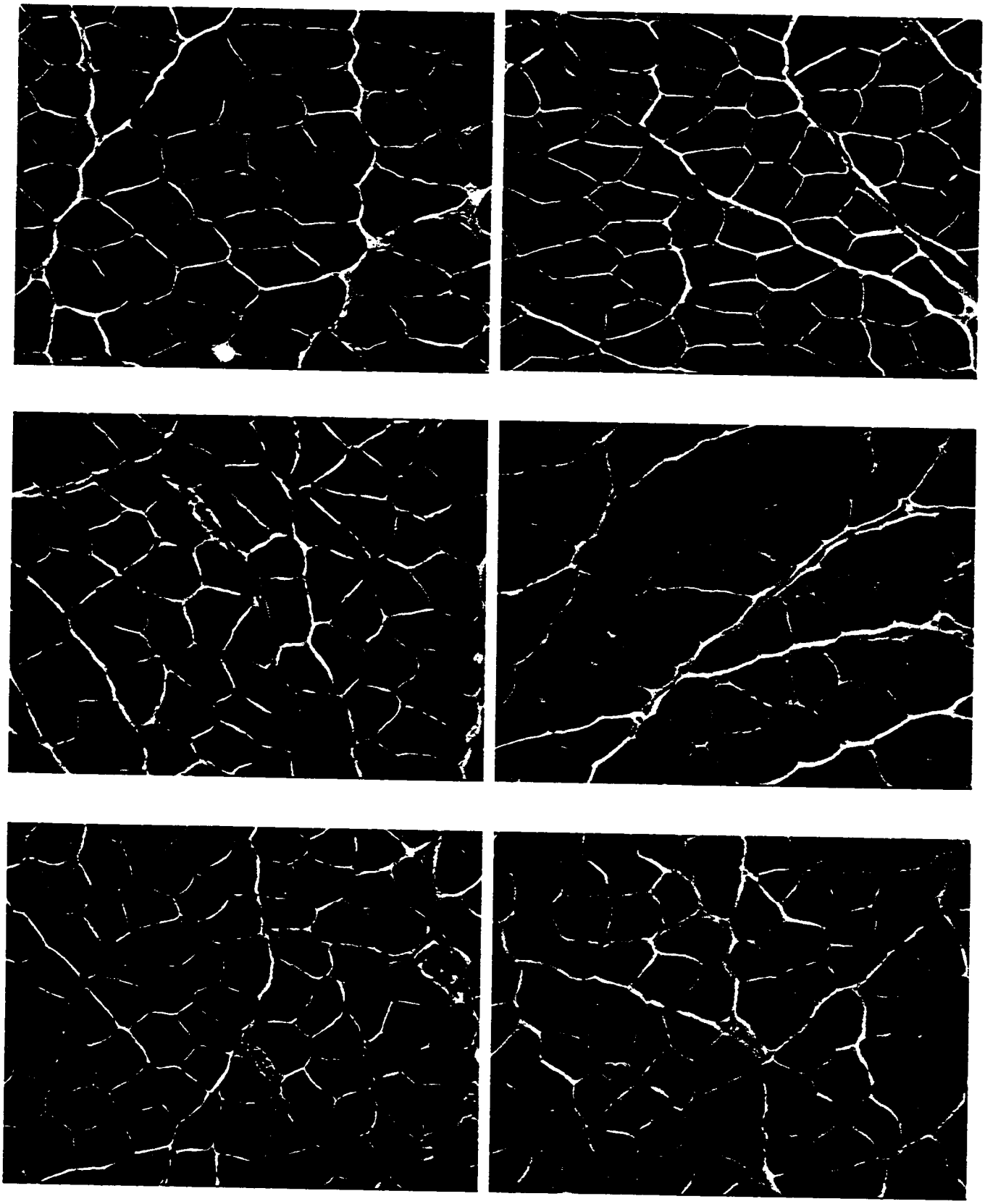
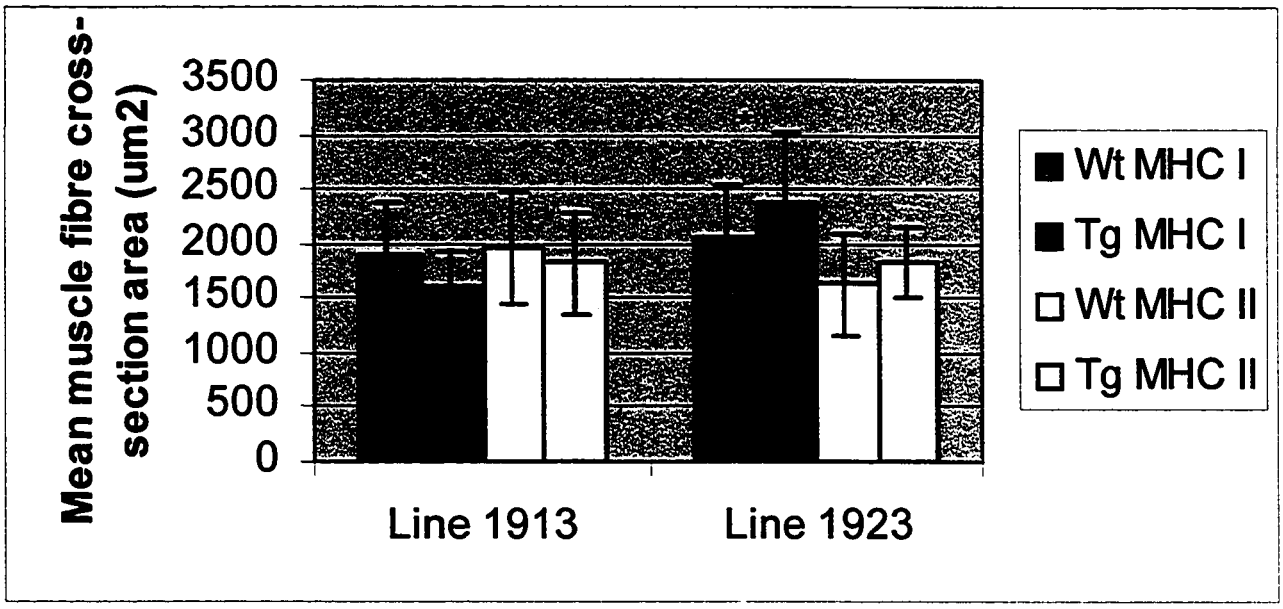
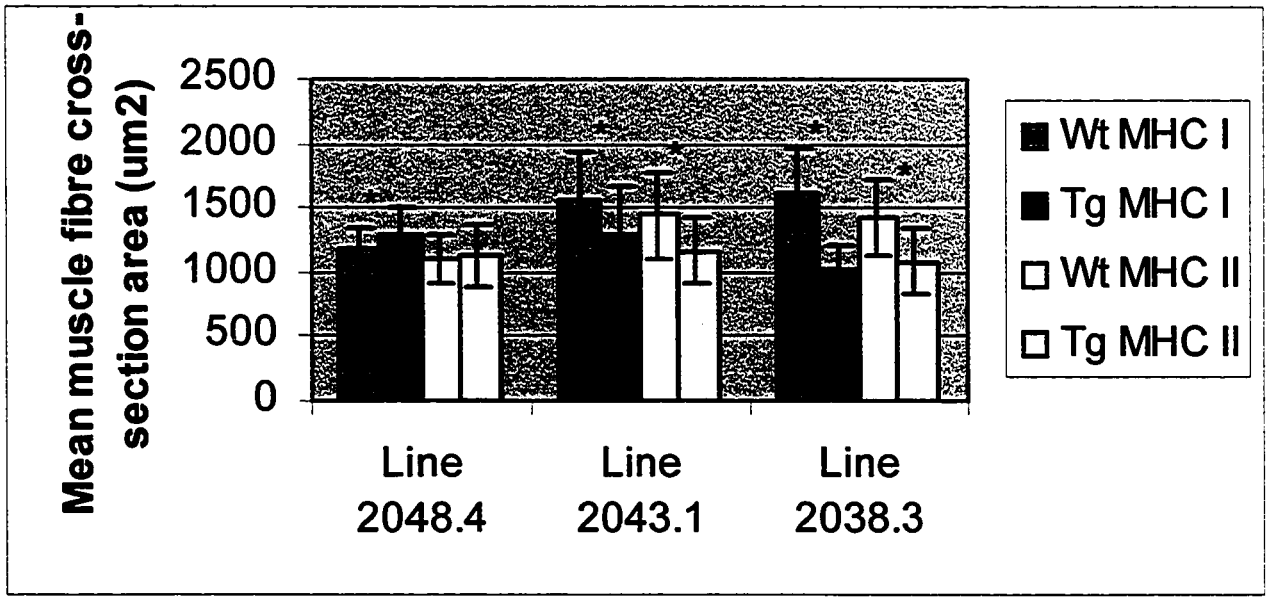


Figure 5-11. Determination of type I and II fibre sizes from cross-sectioned soleus muscle of CTG 11 and 91 transgenic mice. Frozen soleus muscle was cross-sectioned through the middle third of the muscle. This was confirmed using staining with α-bungarotoxin to detect neuromuscular junctions. Mean type I and II fibre sizes for two lines of CTG 11 repeat transgenic mice (A). Mean type I and II fibre sizes for three lines of CTG 91 repeat transgenic mice (B). One CTG 11 line (1913) has significant type I atrophy in the transgenic while the other line (1923) has significant type II hypertrophy in the transgenic. Two CTG 91 lines (2038.3, 2043.1) have significant type I and II atrophy in the transgenic while one line (2048.4) has significant type I hypertrophy in the transgenic. Significance is indicated with asterisks and levels of significance were accepted when $P<.005$ (type I fibres, n=100, type II fibres, n=50).

A


Mean muscle fibre cross-section area (um2)
3500
3000
2500
2000
1500
1000
500
0
Line 1913
Line 1923
Wt MHC I
Tg MHC I
Wt MHC II
Tg MHC II

B


Mean muscle fibre cross-section area (um2)
2500
2000
1500
1000
500
0
Line 2048.4
Line 2043.1
Line 2038.3
Wt MHC I
Tg MHC I
Wt MHC II
Tg MHC II

CTG 91 transgene compared with the CTG 11 transgene (figure 5-8), more consistent and marked atrophy is observed in the CTG 91 transgenic mice. In addition, atrophy in CTG 91 mice correlates with transgene mRNA levels.

5.4 DISCUSSION

My main research interest is in understanding the molecular mechanism(s) leading to DM. One simple key observation influencing our approach is that CTG repeat expansion is invariably linked with disease. In addition, it is emerging that the dominant nature of the DM mutation may be exerted at the RNA level. To test this hypothesis *in vivo*, we chose a transgenic approach whereby the DMPK 3' UTR with wild type (11) and mutant (99) CTG repeat lengths linked to a reporter gene were driven by endogenous DMPK regulatory elements (figure 5-1).

Endogenous DMPK regulatory elements were employed in this transgenic model so that the DMPK 3' UTR would be targeted to the appropriate tissues. Clearly, we have demonstrated that the transgene mRNA is expressed in muscle, heart and brain, tissues where endogenous DMPK is expressed (Jansen et al 1992)(figure 5-2 A). Expression is highest in heart, then skeletal muscle with the least mRNA observed in brain, consistent with endogenous DMPK expression (Jansen et al 1992) (figure 5-2 A). Taken together, this data indicates that the transgene is expressed in the appropriate tissues. However, in five different 11 CTG repeat containing transgenic mouse lines, expression levels in skeletal muscle and brain were not necessarily suggestive of "overexpression" (figure 5-2 A). Transgene expression levels in the heart were relatively higher, however, this high relative level is also the case for the endogenous gene (Jansen et al 1992). Interestingly, in all but one case, a smaller GFP containing transgene mRNA molecule lacking the

DMPK 3' UTR was produced from the transgene locus (figure 5-2 A, lower band). The smaller message is quite abundant in comparison to the larger message and because the 3' UTR is not present in the former molecule, it suggests that transcription is terminated early relatively often. This may suggest the selection of animals that have downregulated expression of the DMPK 3' UTR thus avoiding possible deleterious effects of expression of this element without compromising activity of the locus. Alternatively, in addition to integration into the cellular DNA of intact transgene molecules, transgenes truncated at the GFP/DMPK 3' UTR junction may have been introduced. However, based on southern blot analysis, this was not the case. Another possibility is the presence of a cryptic polyadenylation signal located between the reporter gene and the 3' UTR. Interestingly, this smaller message is only observed in stable myoblast cell lines expressing 99 CTG repeats but not in cell lines expressing 11 CTG repeats (figure 4-7). Regardless, it appears that in both transgenic mouse models and myoblast cell lines expressing 99 CTG repeats, production of a shorter transcript lacking the 3' UTR is more the rule than the exception. In addition, transgenic mice and stable cell lines both produce some longer transcripts indicative of read through transcription. This is perhaps due to a sub-optimal endogenous polyadenylation signal in the DMPK 3' UTR.

Regardless of transgene transcript sizes, only full length GFP protein of the appropriate size is detectable in extracts of heart, tongue and skeletal muscle (figure 5-2 B). This suggests that processing and translation of the transgene transcript is unaffected by variation in transcript size.

Interestingly, transgene mRNA in mice with 91 CTG repeats was expressed in a pattern identical to that observed with mRNA from mice with 11 CTG repeats except that

message levels were consistently lower (figure 5-3). This is in good agreement with the expression of similar constructs in stable cell lines suggesting similar selection mechanisms may be active in the two systems.

Analysis of GFP and myogenin transcripts by *in situ* hybridization in CTG 11 transgenic and wild type littermate control day 12 embryos indicates co-expression of the transgene and myogenin messages (figure 5-4). This data agrees well with endogenous transcript localization in mouse embryos and validates the *in vivo* utility of the DMPK promoter/enhancer fragments employed in this study (Jansen et al 1996). Interestingly, myogenin expression is less intense in a transgenic embryo compared to wild type embryos (figure 5-4 B, embryos 2-4) suggesting that expression of the transgene may interfere with muscle differentiation *in vivo*. Significantly, this reduced myogenin expression is observed in an embryo expressing the DMPK 3' UTR with only 11 CTG repeats. This is entirely consistent with *in vitro* data where overexpression of a DMPK 3' UTR with 5 CTG repeats in myoblasts delays both differentiation and accumulation of myogenin transcripts (Sabourin et al 1997). Reduction of myogenin levels and differentiation potential is observed when v-ras is overexpressed in quail myoblasts (Russo et al 1997) and differentiation of skeletal muscle is absent in myogenin knockout mice (Hasty et al 1993). Therefore, it would be of great interest to assay muscle differentiation status of transgenic embryos by staining with an antibody to myosin heavy chain to determine if differentiation is indeed delayed in comparison to wild type littermates.

There is not always complete agreement between expression of GFP and myogenin in day 12 embryos, particularly apparent in some facial and neck muscles

where GFP is expressed but myogenin is not (white asterisk, white arrows, embryo 4, figure 5-4 A, B). In the transgenic embryo (embryo 4), areas marked by the white arrows and white asterisk (embryo 4, figure 5-4, A, B) contain GFP transgene mRNA but no myogenin transcripts. In comparison, a wild type embryo (embryo 3) normally expresses myogenin in these areas of the neck and face (embryo 3, figure 5-4 B). One interpretation of this finding is that the onset of differentiation is delayed, marked by an absence of myogenin in a place where it should be present, because of expression of the DMPK 3' UTR. This result is very intriguing considering these muscle groups display significant involvement in DM (Harper 1989). For example, two key features of adult DM are an expression-less face due to weakness of facial muscles and wasting of the sternomastoid muscles. Also, DM patients often have difficulty holding their head up and have difficulty swallowing, all due to muscle wasting in this area (Harper 1989). A tent shaped mouth, open jaw and an expression-less facial appearance are characteristic features of congenital DM (Harper 1989). Also, cDM babies have difficulty sucking during nursing owing to facial muscle weakness (Harper 1989). Evidence of minor muscle developmental delays in day 12 transgenic embryos (green asterisks, figure 5-4 B) compared with wild type embryos suggests exertion of muscle differentiation inhibitory effects of 3' UTR expression at this stage despite the presence of only 11 CTG repeats. This abnormality might represent a delay in movement of this particular segment of embryonic tissue into the appropriate place and is particularly striking in that certain neighbouring structures are correctly positioned in the transgenic embryo (4) compared with a normal embryo 3 (yellow arrows, figure 5-4 B).

Myogenin is only expressed in skeletal muscle whereas DMPK is expressed in smooth muscle, brain and heart in addition to skeletal muscle (Jansen et al 1992). These differences come to light when analyzing embryos at day 20 (figure 5-6, 5-7). Expression of the transgene is clearly observed in brain structures, smooth muscle within linings of blood vessels and large intestine and in the heart, which are all negative for myogenin (figure 5-7, A-F). The utility of a 3.7 Kb fragment of DMPK 5' UTR sequence as a promoter was tested *in vivo* and found to express exclusively in neuronal tissues (King et al 1996). Therefore, neuronal expression observed in our experiments is likely the result of activity from the 1.5 Kb of endogenous DMPK promoter used in our construct. Our data suggests that the size of the minimal brain specific *in vivo* promoter can be reduced from 3.7 to 1.5 Kb. Moreover, the DMPK promoter/enhancer used in our transgene construct appears to contain all the necessary elements for expression of a reporter to compartments where endogenous DMPK mRNA is known to be expressed (Jansen et al 1996).

The presence of fibre atrophy upon histological analysis of CTG 91 transgenic mice is suggestive of a minor myopathy in these animals due to expression of a mutant DMPK 3' UTR. The lack of consistent atrophy in CTG 11 transgenic muscle fibres indicates that despite higher expression levels, 11 CTG repeats is not sufficient to cause significant atrophy. In addition, lack of consistent muscle atrophy in CTG 11 mice indicates that these mice are able to develop hindlimb muscle normally despite lower levels of myogenin mRNA observed during development. The CTG 91 transgenic lines with atrophy in both type I and type II soleus muscle fibres expressed more transgene mRNA than the line that did not show type I fibre atrophy. Type I and II fibre atrophy is

partially consistent with features of DM, where type I atrophy is primarily observed, whereas type II fibres are either unaffected or are sometimes hypertrophic (Harper 1989). The relationship between expression level and atrophy observed in CTG 91 transgenic mice is consistent with greater numbers of CTG repeats present in the DMPK 3' UTR mRNA mediating pathology. The occurrence of type II atrophy in the soleus muscle of the transgenic mice might be the result of ectopic expression of the transgene in type II fibres. Endogenous DMPK is expressed in type I fibres exclusively (van der Ven et al 1993). Currently we are investigating fibre type distribution of the transgene product using immunohistological techniques.

Because such low amounts of transgene are produced in these mice, it seems unlikely that non-specific effects resulting from GFP protein expression cause the observed pathology. The fact that fibre atrophy is observed in two lines of CTG 91 transgenic mice argues against integration effects being the cause of this pathology. Therefore, we have demonstrated that expression of just the DMPK 3' UTR in combination with an unrelated reporter *in vivo* can result in a reduction of myogenin levels in day 12 embryos. Furthermore, this may result in a delay in differentiation and fibre atrophy in adult mouse muscle, reminiscent of DM. A recent study by Mankodi and colleagues (Mankodi et al 2000) found that 250 CTG repeats integrated into the 3' UTR of an α-skeletal actin transgene resulted in numerous features of DM. The range of DM features included myotonia, variability of muscle fibre size, centrally located nuclei, ringed fibres and loss of fibre type distinction (Mankodi et al 2000). This study confirms that over-expression in muscle of CUG repeats within an mRNA molecule is sufficient to cause pathological features of DM. This is consistent with our findings that expression of

just a mutant DMPK 3' UTR can result in features of DM in a transgenic mouse. We observed fewer features of DM in our model possibly due to fewer CTG repeats present in our transgene and because we employed a much weaker promoter, likely resulting in lower levels of transgene mRNA. Thus, our transgenic model may represent a milder form of the Mankodi model. Because our model utilizes endogenous regulatory elements, we aimed to maximize pathological events which depend on correct temporal and spatial appearance of the transgene mRNA. Therefore, subtle effects of CTG repeat pathology may be lost in favour of gross, more non-specific changes in the Mankodi model owing to the use of the α-skeletal actin regulatory elements. In addition, α-skeletal actin expression is initiated at day 8.5 in the mouse embryo (Buckingham et al 1992) whereas endogenous DMPK is not activated until day 10 (Jansen et al 1996). Overexpression of a CUG repeat containing mRNA at this time may mediate pathogenic effects unrelated to normal DM pathology which may not necessarily be observed if transgene mRNA expression initiated at day 10.

It is interesting to note that we observed consistently lower levels of the 91 CTG repeat transgene mRNA than 11 CTG repeat mRNA in skeletal muscle. This suggests that the transgene is somehow toxic to skeletal muscle and embryos expressing higher levels of CTG 91 transgene mRNA are subjected to negative selection. The fact that myogenin expression was reduced throughout and altered in future face and neck musculature in embryos of CTG 11 transgenic mice supports this idea. It is interesting to note that Mankodi *et al.* observed in some longer (250) CTG repeat lines fairly robust expression compared with wild type CTG repeat length lines in skeletal muscle indicating that somehow these transgenic animals escaped the negative selection described above

(Mankodi et al 2000). This may be due to the use of different mouse strains or differences in promoter function.

We have clearly shown that the DMPK promoter/enhancer is active in the appropriate tissues during development and in adult transgenic mice. In addition, we have shown that fibre atrophy, consistent with DM, is present in mice expressing a DMPK 3'UTR with 91 CTG repeats. In addition, delayed muscle differentiation resulting from over-expression of the DMPK 3' UTR previously observed *in vitro* can be recapitulated *in vivo* as we have observed in developing transgenic mouse muscle. This clearly demonstrates that over-expression of just the DMPK 3' UTR in association with an unrelated coding sequence *in vivo* can result in some features of DM. Based on the findings of Mankodi et. al discussed above, it is likely that a complete set of DM muscle pathology was not observed in our study because of lower expression and relatively fewer CTG repeats in our transgene. More analysis will have to be performed to confirm delayed muscle development *in vivo* and extend these observations to the CTG 91 mice. Further assessment of muscle atrophy and the presence of myotonia in adult transgenic mice would be desirable. Furthermore, because the transgene is expressed in brain, heart and smooth muscle in addition to skeletal muscle, a unique opportunity exists to examine the effects of DMPK 3' UTR expression in these tissues.

CHAPTER 6. GENERAL DISCUSSION

Myotonic dystrophy (DM) is a complex multisystemic inherited neuromuscular disorder. DM affects both adults and children and varies considerably in age of onset and penetrance (Harper 1989). Adult DM is marked by myotonia and progressive muscle weakness and wasting. Skeletal muscle effects in congenital DM (cDM) are potentially fatal due mainly to the involvement of respiratory muscles (Harper 1989). Individuals born with cDM are hypotonic and as a result are often referred to as "floppy babies" due to the lack of musculature in the limbs. Because hypotonia disappears by age 3-4, this feature of cDM seems to represent a delay in muscle development. In addition, facial muscle weakness and immaturity result in a characteristic facies marked by an open mouth and a tented upper lip. cDM individuals are usually mentally retarded and will develop adult DM features early in life if they survive the neonatal period (Harper 1989).

The genetic basis for DM is the expansion of a CTG repeat tract in the 3' UTR of the DMPK gene. The presence of the DM mutation in the 3' UTR precludes any effect of the mutation on the coding sequence of the gene and presented a unique challenge in understanding the molecular pathogenesis of this disorder. Based on observations in our laboratory indicating elevated expression levels of the DMPK mRNA in patient muscle and brain samples of cDM individuals, we hypothesized that a dominant gain of function of the CTG repeat expansion might contribute to DM pathology (Sabourin et al 1993). Subsequent studies in our laboratory revealed that over-expression of the DMPK 3' UTR containing only 5 CTG repeats could inhibit C2C12 myoblast differentiation (Sabourin et al 1997). Our hypothesis regarding molecular pathogenesis of DM, being an RNA

mediated dominant gain of function, was strengthened by the discovery of mutant DMPK transcripts in abnormal foci within nuclei of patient myoblasts (Taneja et al 1995).

The main objective of my thesis was to understand the molecular pathogenesis of myotonic dystrophy and to reproduce aspects of DM pathology in the mouse. We utilized the C2C12 myoblast cell culture system to address specific aspects of the dominant gain of function hypothesis. We chose to employ endogenous transcription regulatory elements in our studies and therefore cloned sections of the human DMPK 5' region and first intron in order to map these elements. We identified a fairly complex promoter containing several sites for transcription initiation that functions in cooperation with a myoblast specific enhancer element located within the first intron. Not surprisingly, the first intron enhancer was responsive to MyoD and responsiveness to this muscle specific transcription factor was lost when cognate binding sites for this protein, E-boxes, were deleted from reporter constructs. The expression pattern of DMPK and the transcription factor MEF 2C are similar suggesting that this factor may contribute to DMPK expression. A MEF 2C-like element is present in the DMPK promoter but MEF 2C binding sites are lacking in the DMPK enhancer element. It remains possible that this factor might contribute to expression of DMPK in muscle, heart and brain. Because of the complexity of the DMPK promoter/enhancer and our lack of understanding of how it functions, we chose to employ a large 1.5 Kb fragment containing the promoter and a 2.4 Kb fragment containing the myoblast specific enhancer in the construction of transgenes. This strategy proved satisfactory because expression of a reporter gene directed by these transcription control elements in a cell culture system and in transgenic mice emulated expression of the endogenous gene accurately (figures 5-2, 5-5) (Jansen et al 1992).

Previous studies have established that over-expression of the DMPK 3' UTR could delay myogenesis *in vitro* (Sabourin et al 1997). Continuing this line of investigation, we explored possible dominant gain of function mechanisms of DM by establishing stable C2C12 myoblast cell lines. These cell lines expressed the DMPK 3' UTR linked to the reporter gene GFP under control of endogenous DMPK regulatory elements. Empirical observations suggested that cells expressing the DMPK 3' UTR may have been dying during the selection process. When stable cell lines were subjected to low serum conditions during differentiation, an increase in apoptosis was noted in cell lines expressing the DMPK 3' UTR compared to control cell lines expressing the Pgk 3' UTR (figure 4-4 B, C). A certain amount of apoptosis during myoblast differentiation is a normal occurrence both *in vitro* and *in vivo* (Mampuru et al 1996; McClearn et al 1995), however, expression of the DMPK 3' UTR elevates this differentiation associated apoptosis.

We observed similar levels of apoptotic sensitivity in stable cell lines expressing the DMPK 3' UTR with 11 or 99 CTG repeats (figure 4-6). However, stable DMPK 3' UTR cell lines with 11 CTG repeats expressed about ten fold higher levels of the DMPK 3' UTR mRNA compared with the 99 CTG repeat cell lines (figure 4-8). We propose that in C2C12 myoblasts subjected to apoptotic triggers, higher expression of a DMPK 3' UTR with fewer CTG repeats results in effects similar to that found with lower expression of a DMPK 3' UTR having greater repeat numbers. Interestingly, the difference in mRNA expression level of the DMPK 3' UTR in 11 and 99 CTG repeat cell lines was about ten fold, roughly the same as the difference in repeat number. This

suggests that C2C12 cells tolerate a certain amount of CUG repeat sequence in mRNA beyond which the cells cannot survive.

We suggest the possibility that apoptosis might play a role in both congenital and adult DM. In the case of congenital DM, increased apoptosis during myoblast differentiation might reduce the number of available myoblasts to numbers below a threshold required for differentiation resulting in poorly differentiated muscle at birth. The CUG repeats in the DMPK mRNA would likely be the apoptotic trigger and it is significant that DMPK mRNA production increases throughout myoblast differentiation (Davis et al 1997; Skerjanc et al 1994; Storbeck et al 1998). Therefore, as the process of differentiation proceeds within a single myoblast, the amplitude of the trigger increases. In adult DM, apoptosis of myonuclei in skeletal muscle is observed at an elevated rate (Yamada et al 2000). Apoptosis is a normal adaptive response in skeletal muscle. For example, in muscles of rats subjected to space flight (Hikida et al 1997) or hind limb unweighting (Allen et al 1997), loss of myonuclei occurs as the metabolic needs of the muscles decline. The net result is a smaller muscle mass with identical nuclear to muscle mass ratio. We have shown that patient myoblasts and amniocytes are more susceptible to staurosporine mediated apoptosis than control cells (figure 4-13). Therefore, it is not unreasonable to propose that myonuclei in muscle of DM patients may have a lower apoptotic threshold than normal myonuclei leading to an elevated rate of myonuclear loss throughout life resulting in progressive muscle weakness and wasting in DM.

CUG repeats within a mutant DMPK 3' UTR mRNA molecule have been shown to form dsRNA hairpin structures (Gacy et al 1995; Tian et al 2000). PKR becomes activated following binding to ds RNA (Williams 1999). We demonstrated a repeat

length dependent activation of PKR in C2C12 myoblasts expressing DMPK 3' UTR constructs with 11 and 99 CTG repeats (figure 4-11). Although it has been shown that PKR becomes activated in a repeat length dependent manner when bound to CUG repeats *in vitro* (Tian et al 2000), this is the first demonstration of PKR activation in cultured cells expressing the DMPK 3' UTR. PKR, when over-expressed in C2C12 myoblasts, results in enhanced differentiation (Kronfeld-Kinar et al 1999) which is seemingly paradoxical considering cDM is marked by delayed differentiation. However, when PKR is activated by ds RNA, the activation profile is "bell-curve" shaped, or, at higher concentrations of ds RNA, PKR activity is inhibited (Williams 1999). Therefore, it is possible that in congenital DM, where CTG repeats are the largest, the large CUG hairpin structure may actually result in inhibition of PKR activity and a delay in differentiation. When a dominant negative form of PKR is over-expressed in C2C12 myoblasts, a delay in myoblast differentiation is observed (Salzberg et al 2000) indicating that delayed differentiation in cDM is consistent with ablation of PKR activity. In the case of adult DM, PKR may be hyper-activated. This may not necessarily have an adverse effect on muscle development. When PKR is over-expressed in C2C12 myoblasts, enhanced differentiation is observed (Kronfeld-Kinar et al 1999). However, a chronic elevated level of activated PKR in skeletal muscle of DM patients might lower the apoptotic threshold of myonuclei leading to progressive muscle loss and weakness. Indeed, PKR activation can lead to apoptosis (Gil & Esteban 2000). The CUG repeats in the mutant DMPK mRNA are for the most part sequestered within the nuclei of patient cells (Taneja et al 1995). Approximately 20% of PKR is nuclear (Jeffrey et al 1995) and it remains a possibility that apoptotic signals can be initiated from the nucleus (Moll & Zaika 2001).

Certainly, many viruses transcribe their genomes within the nuclei of host cells (Becker et al 1999). As a result, nuclear PKR may become activated and would be required to transduce antiviral signals to the cytoplasm. Therefore, it is plausible that PKR might recognize CUG hairpin structures in mutant DMPK transcripts within patient nuclei resulting in kinase activation and transduction of signals to the cytoplasm.

Interestingly, another ds RNA binding protein was recently reported to bind CUG repeats in a length dependent manner. Muscleblind, which was originally identified in Drosophila, is required for normal muscle and eye development in this species (Miller et al 2000). The human version of this protein is present in differentiated myoblasts and is localized within distinct foci in patient myoblasts (Miller et al 2000). Thus muscleblind represents an attractive candidate for a factor sequestered by the CUG repeats in DM and elucidation of its normal function is of great importance.

Our laboratory has previously reported that over-expression of the DMPK 3' UTR can inhibit myogenesis *in vitro* (Sabourin et al 1997) and that over-expression of the entire human DMPK gene containing 22 CTG repeats in transgenic mice can replicate many features of DM muscle pathology (Narang 2000). Therefore, we set out to demonstrate that expression of the DMPK 3' UTR without the DMPK coding sequence could replicate pathological features of DM in transgenic mice. Remarkably, many features observed *in vitro* in the C2C12 mouse myoblast model were also observed in transgenic mice. For example, transgenic mice expressing 91 CTG repeats expressed the transgene at about 10% of the level observed in mice with 11 CTG repeats (figure 5-8). This observation reinforces the contention that expression of high levels of > 90 CTG repeats within the DMPK 3' UTR is incompatible with survival whether in cell lines or

mice. In addition, a reduction in myogenin mRNA production in day 12 embryos expressing the transgene with 11 CTG repeats was noted in comparison to wild type embryos by *in situ* hybridization. Remarkably, a similar reduction in myogenin was noted in C2C12 myoblasts over-expressing the DMPK 3' UTR suggesting that similar mechanisms are responsible for this phenotype *in vitro* and *in vivo*. This is entirely consistent with delayed muscle development observed in congenital DM. However, it is not clear if reduced accumulation of myogenin in CTG 11 transgenic day 12 mouse embryos has adverse consequences on the remainder of development because we only analyzed transgenic embryos with myogenin and GFP probes at day 20. Analysis of wild type day 20 embryos with a myogenin probe would reveal any muscle developmental deficiencies present in transgenic embryos. At birth, transgenic mice are relatively indistinguishable from wild type littermates indicating that if any muscle deficiencies exist, they are relatively minor. This is in contrast with cDM where effects on muscle at birth are severe. This suggests that reduced myogenin accumulation observed at day 12 in CTG 11 mice is a relatively mild consequence of expression of the DMPK 3' UTR in developing muscle. This is not surprising considering that the CTG 11 transgene is perhaps only mildly over-expressed and that the CTG repeat is only 11 repeats in length. It would interesting to speculate what the effects on developing muscle in transgenic mice might be if one could achieve high level expression of the 3'UTR with >90 CTG repeats.

In addition to lower myogenin expression, certain facial and neck muscle structures fail to be detected by myogenin in CTG 11 transgenic embryos at day 12 (figure 5-3). This is particularly striking considering that weakness and lack of facial and

neck musculature is almost always a noted feature of DM. Adult DM patients have a characteristic expression-less appearance due to lack of tone in facial muscles (Harper 1989). Furthermore, DM patients experience problems swallowing due to weakness of muscles lining the esophagus and difficulty raising their head due to weakness of sternomastoid muscles (Harper 1989). Also, cDM patients have a characteristic expression-less facial appearance, a tent shaped upper lip and an open mouth appearance owing to facial and jaw muscle weakness (Harper 1989). The observed delay or lack of myogenin staining in muscles of the neck and face in transgenic mice is strongly suggestive that expression of the DMPK 3' UTR is sufficient to result in this particular subset of features of DM.

In addition to developmental defects, expression of the DMPK 3' UTR in transgenic mice resulted in fibre atrophy only in CTG 91 mice (figure 5-10). Two of three lines displayed reduced fibre cross-section areas in both type I and type II fibres. The CTG 91 transgenic line with lowest transgene expression showed no evidence of fibre atrophy suggesting that expression of the 91 CTG repeat containing transgene is responsible for this phenotype. In this regard, a recent study has shown that expression in mice of 250 CTG repeats inserted into the 3' UTR of an α-skeletal actin mini-gene resulted in numerous features of DM (Mankodi et al 2000). These features included myotonia, central nuclei, nuclear foci, ringed fibres, loss of fibre type specificity and fibre size variability. This data clearly supports our hypothesis that over-expression of a component of the DMPK 3' UTR, the CTG repeat, in the absence of the DMPK coding sequence, can replicate features of DM in transgenic mice. The two main differences between the two models are the choice of promoter and the inclusion in our study of the

DMPK 3' UTR in addition to the repeats. It is likely that the inclusion of a greater number of CTG repeats and use of the stronger α-skeletal actin promoter resulting in greater expression of the transgene allowed for a wider variety of DM features to be observed in the Mankodi model. We have not yet examined our mice for other DM features including myotonia and nuclear foci. Given the poor expression observed in CTG 91 transgenic lines, it is puzzling that Mankodi *et. al* were able to achieve appreciable expression with 250 CTG repeats. This may be due to the fact that the DMPK 3' UTR was not included in their transgene and this sequence and not the CTG repeats which is the toxic element to muscle development leading to selection of low expressing animals. Our finding that expression of a wild type DMPK 3' UTR impacts myogenesis *in vivo* supports this concept. However, the poor expression observed in CTG 99 transgenic mice compared to CTG 11 transgenic mice suggests that a combination of CTG repeats and the DMPK 3' UTR contribute to the observed toxicity.

In conclusion, my thesis has provided evidence for a dominant gain of function mechanism that may contribute to the DM molecular mechanism. This mechanism results in the apoptosis of target cells because of the inappropriate activation of PKR. In addition, we have demonstrated that expression of the DMPK 3' UTR in association with a reporter gene without the DMPK coding sequence in transgenic mice can replicate certain pathological features of myotonic dystrophy. These features being overall reduction and changes in myogenin mRNA accumulation and distribution in facial and neck muscles during development and fibre atrophy in adult soleus muscle. Finally, the endogenous regulatory elements identified in the DMPK 5' UTR and first intron have been identified and characterized. As a result, transgenic mice constructed with these

regulatory elements has allowed for the expression pattern of the reporter gene to parallel closely the expression of the endogenous DMPK gene.

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