INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

Université d'Ottawa · University of Ottawa

REGULATION OF MYOD INDUCED MYOGENESIS IN P19 CELLS

by
Christine Armour

Thesis submitted to the Department of Biochemistry
in partial fulfilment for the degree
of
Master of Science

University of Ottawa
Ottawa, Ontario, Canada
May, 1997

Abstract

MyoD is a member of the myogenic regulatory family of transcription factors which play a pivotal role in the formation of skeletal muscle. Stable expression of MyoD in the P19 embryonal carcinoma cell line leads to enhanced muscle formation only after aggregation of these cells. To study how MyoD and cell aggregation cooperate to affect muscle differentiation, a myc epitope tagged version of the protein was generated which functioned like the native MyoD protein. The production of an expression construct containing an internal ribosome entry site (IRES) was required to achieve efficient expression of mycMyoD using a drug resistance gene. With this expression construct it was determined that the level of mycMyoD protein did not increase after aggregation. Similarly no changes in cellular localization were observed as MycMyoD was located in the nucleus both before and after aggregation. There was no apparent change in dimerization partners upon aggregation as mycMyoD was found bound to E2A proteins in both the non-aggregated and aggregated cells. In a DNA binding assay mycMyoD from both cell types was able to bind to an E-box containing oligonucleotide. Thus, the effect of cell aggregation is not upon mycMyoD but rather is believed to be either at the point of DNA accessibility by mycMyoD:E2A protein heterodimers or at the stage of transcriptional activation. *In vivo*, these means of regulation are likely to ensure that myogenesis occurs only when proper cell contacts are achieved.

Acknowledgements

First, I would like to thank the good Dr. McBurney who accepted me into his lab despite having known me for three summers as a summer student. A character building experience, his supervision ensured never a dull moment and has taught me how to think more critically and analytically. Even though he has a busy schedule, his door has always been open for discussions. I thank him for all his guidance and support (and for giving me thicker skin).

I am thankful to the other professors for any suggestions and guidance they have given.

The technicians I owe great thanks, especially Jane and Karen, who have endured my constant pestering for almost five years.

I also thank Beth who has provided me with much support both administrative and emotional. I can always count on a smile (or box of kleenex or cup of tea...).

A Big thanks to beer club - beer and commiserating makes everything go down a little easier.
Thanks to the McBurney lab for all the wonderful treats.

Thanks to all my lab mates, both and present - [I would like to write a personalized thanks to everyone, but MSc theses are capped at 120 pages ....]- who have given me technical advice but more importantly proven to be wonderful interesting friends whom I shall miss very much.

I would like to thank my family - they are probably just as happy as I that the end is almost in sight.

A huge thank you to Chris - my biggest supporter.

Last but not least, I would like to thank Dr. Ken Garson. He has contributed an incredible amount of technical advice and knowledge without any hesitation both in the lab and for this thesis. I have no doubt that his support made this experience infinitely better. Technical assistance aside, I would like to thank him most for being such a good friend. It has meant a lot to me. I shall remember this the most.

Table of Contents

Chapter Three
Results

Chapter Four
Discussion

List of Figures

Abbreviations

A	aggregated
bHLH	basic helix-loop-helix
bp	base pair
D	Daltons
DMSO	dimethylsulfoxide
EDTA	ethyldiamine tetra-acetic acid
FGF	fibroblast growth factor
hr	hour
kb	kilobase
kD	kilodaltons
min	minute
MRF	myogenic regulatory factor
N	non-aggregated
oligo	oligonucleotide
PBS	phosphate buffered saline
PAGE	polyacrylamide gel electrophoresis
Pgk	phosphoglycerate kinase
RA	retinoic acid
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
TBS	Tris buffered saline
TGF	transforming growth factor
UTR	untranslated region

Chapter One

Introduction

Skeletal myogenesis is the process by which undifferentiated cells develop into striated muscle. It involves a sequence of carefully orchestrated events in which each step is tightly controlled and regulated by a myriad of cellular signals. Determining the nature of such regulatory processes is crucial to understanding mammalian development. The myogenic regulatory family of transcription factors plays an integral role in initiating the formation of skeletal muscle from the otherwise undefined mesodermal cell. Regulation of *MyoD* activity is a pivotal step in myogenesis. The aim of this thesis is to gain an understanding of myogenesis by examining the regulation of *MyoD* mediated skeletal muscle formation in the P19 embryonal carcinoma system.

1.1 The myogenic regulatory factors

MyoD, a member of the myogenic transcription factors, was one of the first examples of a mammalian "master control" gene that is able to initiate differentiation of a cell into a specific lineage. MyoD was isolated by virtue of its ability to convert 10T1/2 fibroblasts into myoblasts (Davis et al., 1987). Since then, three other related myogenic regulatory factors, *myf-5, myogenin and MRF4* (also known as *herculin* and *myf-6*) have been described (Wright et al., 1989; Rhodes and Konieczny, 1989; Miner and Wold, 1990; Edmondson and Olson, 1989; Braun et al., 1989; Braun et al., 1990). All of these four factors are able to activate transcription of muscle specific genes and induce the myogenic program to varying degrees when ectopically expressed in a variety of cell

types. The degree of myogenic conversion depends both on the background of the recipient cell and the factor being expressed.

1.1.1 Expression Patterns

The four MRF genes have characteristic expression patterns with expression beginning in the somites, the embryonal mesodermal structures from which skeletal muscle arises (reviewed in Lassar and Munsterberg, 1994; Molkentin and Olson,1996). The first MRF to be expressed in the mouse is myf-5 at day 8 in the anterior somites, followed by myogenin at day 8.5. MRF-4 appears at day 9 while MyoD is the last to be expressed and appears at day 10.5. This ordered pattern of MRF expression is repeated in more posterior somites later during differentiation. In this way, differentiation progresses in each somite in a rostrocaudal fashion along the neural tube with the most differentiated being at the anterior end. The muscle in the developing limbs develops from somitic cells which migrate to the limbs where they express the MRF genes in an order similar to that in the somites.

The temporal regulation of MRF gene expression suggests that each factor may serve a different role in the myogenic pathway. Indeed, gene knockout experiments in mice support this hypothesis. The *MyoD* null mouse has normal skeletal muscle but has elevated amounts of *myf-5* mRNA (Rudnicki et al., 1992). Likewise, the *myf-5* knockout mouse has normal skeletal muscle but dies at birth due to an underdeveloped rib cage (Braun et al., 1992). However, the double knockout mouse of *myf-5* and *MyoD* completely lacks myoblasts and skeletal muscle suggesting that *myf-5* and *MyoD* have overlapping roles which are necessary for forming myoblasts. In addition, mice lacking

myogenin form myoblasts which fail to differentiate, resulting in a severe lack of skeletal muscle and embryos which are not viable (Nabeshima et al., 1993; Hasty et al., 1993). This implies that both *MyoD* and *myf-5* are required for myoblast formation while *myogenin* (and perhaps *MRF4*) is required for the further development of those myoblasts (Weintraub, 1993).

1.1.2 MRF Sequence Features

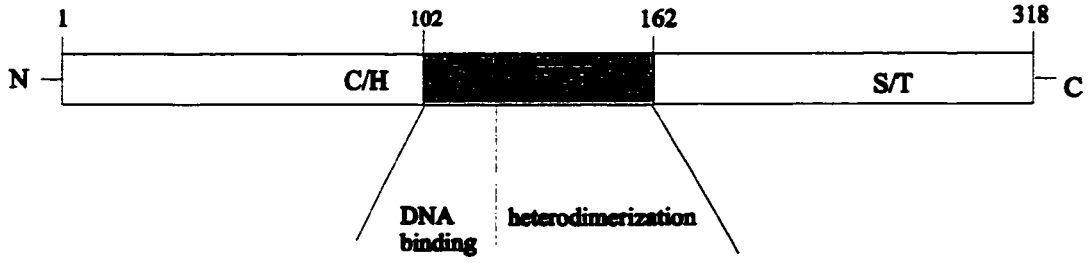
The four MRFs belong to the basic helix-loop-helix (bHLH) superfamily of transcription factors whose most notable sequence feature is the bHLH domain (Fig 1A). This superfamily contains numerous transcription factors; some are specific for growth, some are specific for lineage specific differentiation while others are relatively ubiquitous. The bHLH domain was originally termed the myc similarity region since this was one of the first proteins to be characterized which included this domain (Lassar et al., 1989; Stone et al., 1987). The bHLH domain encodes a basic region (in MyoD, 13 amino acids) followed by a conserved region which forms an amphipathic α helix (15 amino acids) - loop- (5-20 amino acids)-amphipathic α helix (15 amino acids) structure (Ma et al., 1994; Murre et al., 1989). The basic domain is involved in DNA binding while the HLH region is involved in dimerization (Voronova and Baltimore, 1990; Davis et al., 1990). Not surprisingly, the removal of the bHLH domain from MyoD completely abolishes its myogenic activity (Tapscott et al., 1988). In addition to the conservation of the bHLH domain (80% similarity), the four MRF proteins encode a cysteine/histidine rich region N-terminal to the bHLH and a serine/threonine rich region in their C-terminus (Dias et al.,

Fig. 1 Schematic representations of MyoD sequence features and muscle gene transcription by MyoD

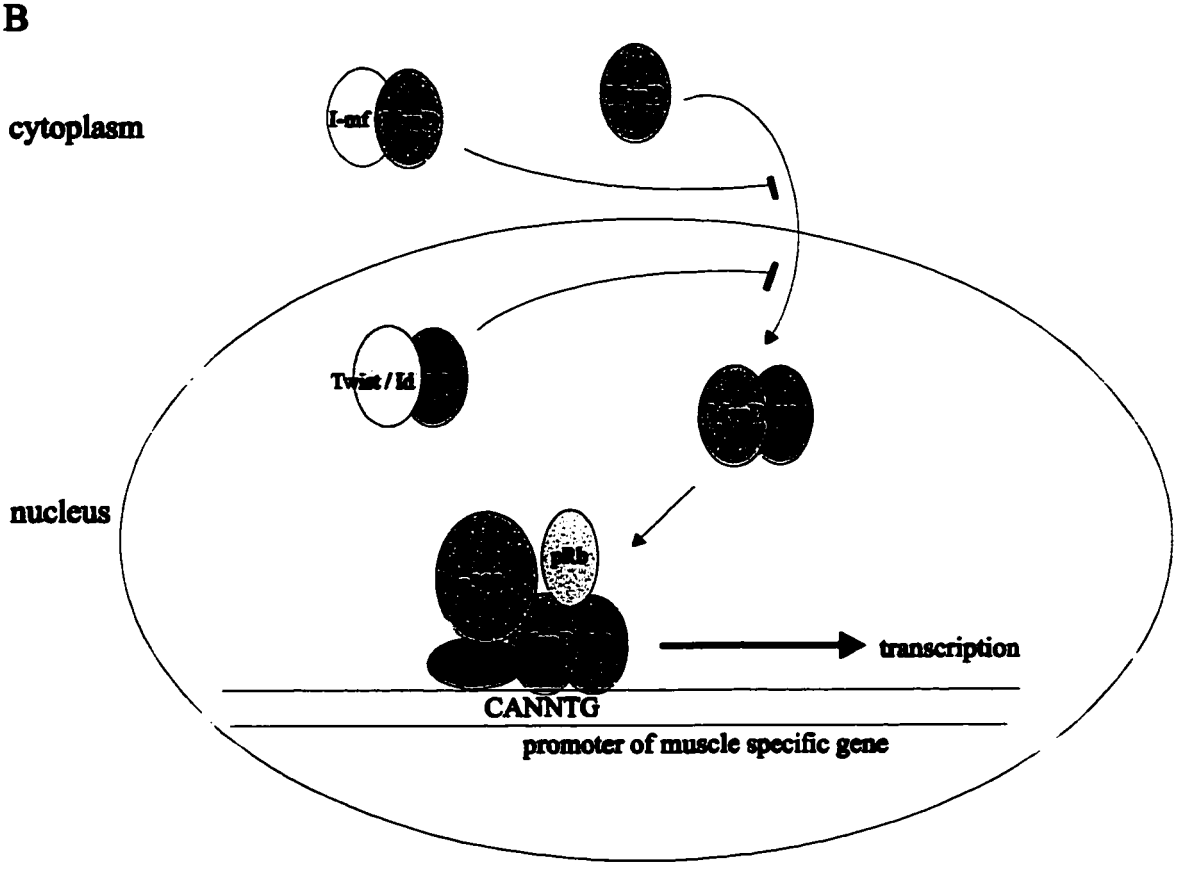
A) A linear representation of the MyoD with the common MRF features shown. The most notable features are the basic (b) and helix-loop-helix (HLH) motifs. The basic domain mediates DNA binding and the HLH heterodimerization. Also found in the MRFs are the cysteine/histidine (C/H) and serine/threonine rich (S/T) regions. The purpose of these regions is unclear. Numbers indicate amino acid positions.

B) A summary of some interactions involved in transcription of muscle specific genes by MyoD. Shown are interactions which do not necessarily occur together or in the same cell. MyoD is translocated from the cytoplasm to the nucleus but may be sequestered in the cytoplasm by I-mf. Once in the nucleus, it must heterodimerize with an E2A protein to be active. This heterodimerization may be prevented by the proteins Mtwist or Id which preferentially bind E2A proteins. The MyoD:E2A heterodimer binds to an E-box (sequence = CANNTG). Other factors thought to co-operate with MyoD to activate transcription include p300, pRb and members of the MEF2 family. It is not known whether these factors bind to the transcription complex at the same time.

A


1
102
162
318
N
C/H
S/T
C
DNA
binding
heterodimerization

B


cytoplasm
I-mf
Twist / Id
nucleus
transcription
CANNTG
promoter of muscle specific gene

1994) (Fig. 1A). MyoD, the MRF of interest in this thesis, comprises 318 amino acids while myf-5, myogenin and MRF-4 comprise 255, 224 and 242 respectively (Olson, 1990).

1.1.3 Heterodimerization

In order to be active, an MRF must dimerize with another bHLH protein. Although MyoD is able to form homodimers, the more active form of MyoD results from heterodimerization with one of the E2A gene products (Lassar et al., 1991)(Fig 1B). The E2A gene encodes two gene products, E12 and E47, which arise from alternative splicing (Henthorn et al., 1990; Murre et al., 1989). E12 and E47 also contain the bHLH motif and are ubiquitously expressed (Aronheim et al., 1993). The increased activity of MyoD heterodimers may in part be due to the DNA binding affinities which have been reported be ten times greater than MyoD homodimers (Sun and Baltimore, 1991; Murre et al., 1989).

1.1.4 DNA binding : the E-box

As early as 1985 it was known that the sequence CANNTG, or E-box, was an *in vivo* site in immunoglobulin enhancers for binding by numerous factors (Murre et al., 1989b; Ephrussi et al., 1985b). In the same year, studies performed with myocyte extracts demonstrated that a myocyte specific nuclear factor also bound to an E-box containing sequence from the mouse creatine kinase enhancer (Buskin and Hauschka, 1989). Subsequent studies demonstrated that this nuclear factor was a MyoD: E-protein heterodimer (Fig. 1B). The MyoD:E2A heterodimer showed high affinity for the E-box

and could bind to two E-boxes present in the mouse creatine kinase enhancer (Lassar et al., 1989; Murre et al., 1989). The entire bHLH domain of MyoD was required for E-box binding (Lassar et al., 1989).

E-boxes are often present in multiple copies in muscle specific promoters (Sartorelli et al., 1990; Sartorelli et al., 1992) and are also found in the promoters of the MRFs, allowing for autoregulation and positive feedback (Thayer et al., 1989; Braun et al., 1989). Such positive feedback is likely to be necessary for achieving the differentiated state.

1.1.5 E-box specificity

The E-box sequence is a relatively simple and short sequence which would be expected to be found relatively commonly throughout the genome and within the enhancers/promoters of non-muscle genes. Specificity of muscle gene transcription is believed to be achieved by a number of means. One such means is the context of E-boxes within muscle -specific genes (Blackwell and Weintraub, 1990; Yutzey and Konieczny, 1992; Huang et al., 1996). While MyoD is able to bind the sequence CANNTG, where NN is any two base pairs, the MyoD:E protein heterodimer is reported to prefer certain sequences such as internal cytosines as well as 5 and 3' thymidines (Blackwell and Weintraub, 1990). In addition, *cis*-acting repression elements flank both sides of E-boxes present within the enhancers of non-muscle genes presumably preventing inappropriate transcription (Weintraub et al., 1997). Perhaps the most predominant means of controlling specificity is through distinctive MRF characteristics.

While numerous transcription factors contain the bHLH region, only the

myogenic bHLH proteins are able to activate muscle specific gene transcription. Switching the basic domain of MyoD and myogenin with that of either E12 or *achaete-scute*, a Drosophila neurogenic bHLH, allowed the altered MyoD and myogenin to still heterodimerize and bind DNA but they were unable to activate muscle -specific transcription (Brennan et al., 1991; Davis et al., 1990). The specificity endowed by the basic region was narrowed down to two necessary adjacent amino acids, an alanine and a threonine, which are conserved in all in MRFs (Davis and Weintraub, 1992). These two amino acids may ensure proper protein conformation necessary for myogenic specificity. Substitution of these two amino acids and third amino acid from the junction between the basic region and helix 1 from MyoD into the corresponding positions of E12 allowed the converted E12 protein to activate the myogenic program. Thus, the transcriptional specificity of the MRF genes is achieved both by sequence characteristics of the enhancers/promoters and by features of the MRF genes.

1.1.6 Transcription by MyoD

Although DNA binding and heterodimerization are mediated by the bHLH domain, a 53 amino acid acidic domain in the N-terminus of MyoD is necessary for transcriptional activation (Weintraub et al., 1991). When this domain is fused to a heterologous DNA binding protein such as the Gal4 gene it confers a transactivation potential of 25-50 fold greater than the entire MyoD fused to the Gal4 gene (Weintraub et al., 1991). It is postulated that this domain is normally masked within the MyoD protein and co-regulators may be required to unmask this domain and increase transcriptional activity.

1.2 **Regulation of the MRFs**

Since the MRFs serve a pivotal role in the formation of skeletal muscle, their activity is highly regulated. Indeed, evidence of regulation exists for almost every step that must occur before MyoD transcribes muscle specific genes; from transcription of MyoD itself, to the binding of MyoD to target promoters and consequent transactivation of muscle specific genes. Since there exists more information on regulation of MRFs than can be addressed within the scope of this thesis, an overview of the topic is given with attention paid to types of regulation relevant to this thesis.

1.2.1 Transcription

The upstream signals which initially activate transcription of the MRFs are not yet known. Recent evidence however places *MyoD* under the control of the homeobox gene *Pax-3* (Maroto et al., 1997; Tajbakhsh et al., 1997). A current model for the regulatory interactions which induce the myogenic pathways suggests that the *Wnt* family and *sonic-hedgehog* (Shh), which are both secreted proteins, induce *Pax-3* and *myf-5* both of which in turn activate *MyoD* and the rest of the myogenic pathway (Rawls and Olson, 1997). *Pax-3* has also been shown to activate transcription of *myf-5* (Maroto et al., 1997; Tajbakhsh et al., 1997). However, whether Pax-3 binds to the promoters of *myf-5* and *MyoD* to activate their transcription is unknown (Wang et al., 1997; Leyns et al., 1997; Hoppler et al., 1996). Evidence also supports the model that MyoD and myf-5 regulate each others expression. It has been noted that myf-5 and MyoD are generally not expressed in the same cell line (Braun et al., 1989a) and that often their expression

patterns are reciprocal (Peterson et al., 1990) suggesting that they may downregulate one another. MyoD knockout mice overproduce myf-5 (Rudnicki et al., 1992), supporting the suggestion that these genes do regulate each others' transcription. In addition to these pathways, both FGF and TGF have been shown to repress MyoD transcription (Vaidya et al., 1989).

1.2.2. Post Transcriptional Regulation - Translational Control

Translational regulation is a common point of regulation for numerous proteins (reviewed in Melefors and Hentze, 1993). While such regulation does not appear to be prevalent in controlling the MRFs, a number of examples of such regulation exist. Myogenin mRNAs are detected in embryonic somites as early as day 9.5, yet the protein does not appear until day 10.5, suggesting lack of translation in early somites (Cusella-De Angelis et al., 1992). The MEF2A 3' UTR serves as a cis-acting translational repressor, restricting the expression of MEF2A to specific tissues (Black et al., 1997). In addition a number of muscle structural proteins have been shown to be translationally regulated (Sarkar, 1984).

1.2.3 Localization

The next level of regulation which has been demonstrated to be active in controlling the activity of the MRF proteins is that of regulating the intracellular localization. Control of nuclear import has been shown to be a method of regulation commonly utilized in cells to control the activity of a variety of transcription factors

ultimately influencing differentiation and growth (reviewed in Vandromme et al., 1996).MyoD contains two nuclear localization signals (NLS), one present in the basic domain and the other in helix-1 (Vandromme et al., 1995). These two signals have been shown to be active in promoting nuclear transport and can function independently of one another (Vandromme et al., 1995). In *Xenopus*, MyoD has been demonstrated to be regulated at the level of nuclear localization. *Xenopus* embryos injected with mouse MyoD (mMyoD) but not Xenopus MyoD (XmyoD) initiate the myogenic program (Rupp et al., 1994). It appears this difference arises from the fact that mMyoD is imported into the nucleus while XmyoD remains in the cytoplasm, presumably because XMyoD is specifically inhibited from being translocated to the nucleus (Rupp et al., 1994).

In rat embryo fibroblasts, the nuclear localization of MyoD has been shown to be dependent on c-AMP dependent protein kinase (PKA) (Vandromme et al., 1994). However, the activity of PKA was believed to be directed not at MyoD itself since mutagenesis of all putative PKA phosphorylation sites was unable to prevent nuclear import (Vandromme et al., 1994).Recently, nuclear localization has also been found to be modulated by a novel factor termed I-mf (Inhibitor of MyoD Family) which was shown to inhibit the activity of the MRFs (Chen et al., 1996). During embryogenesis I-mf is expressed throughout the sclerotome, a somitic derivative which does not give rise to skeletal muscle. I-mf acts by sequestering the MRFs in the cytoplasm by masking their NLS (Fig. 1B). In addition, I-mf can also interfere with the DNA binding activity of the MRFs. I-mf is expressed in sclerotome, the same area of the somite in which two other inhibitors of MRFs, Mtwist and Id, are expressed (Wang et al., 1992; Wolf et al., 1991;

Chen et al., 1996). These inhibitors are not expressed in the myotome, the portion of the somite which forms myoblasts. It is believed that I-mf acts in concert with Id and Twist to control patterning in the somite by preventing improper commitment to the myogenic pathway (Chen et al., 1996).

1.2.4 Dimerization

As previously mentioned, a necessary step of MyoD activation is the formation of a heterodimer with a product of the E2A gene. Given that this dimerization is crucial in the activation of MyoD, it is not surprising that a number of regulatory mechanisms act to promote or repress this dimerization. The most well- characterized of these mechanisms involves the Id family of proteins. This family includes at least four members; Id1, Id2, Id3 and Id4 (Riechmann et al., 1994; Sun et al., 1991; Benezra et al., 1990; Jen et al., 1992)which possess the HLH domain but lack the basic domain. Without the basic domain, the Id proteins may still dimerize to other bHLH proteins but the heterodimer is unable to bind DNA (Benezra et al., 1990). While the Id proteins are able to bind MyoD and the other MRFs, they preferentially bind to E proteins (Jen et al., 1992), and in this way sequester the E-proteins from the MRFs consequently preventing myogenesis (Fig. 1B). Id expression is high in proliferating myoblasts presumably to allow cell division and delay differentiation(Benezra et al., 1990). Another bHLH inhibitory protein which has been demonstrated to moderate dimerization is MTwist (Spicer et al., 1997).Like Id, MTwist preferentially binds E-proteins and sequesters them from binding to the MRFs (Fig. 1B). It is also believed to prevent DNA binding by

MyoD and to inhibit trans-activation by MEF2, another myogenic factor (Spicer et al., 1997).

Dimerization may also be regulated by the formation of disulfide bridges that are able to form spontaneously between E2A proteins resulting in cross-linked E2A homodimers(Benezra, 1994). This disulfide bond is required for DNA binding by E2A homodimers at physiological temperatures and is believed to be the reason that a disulfide bond cross-links E2A homodimers in B cells, which require E2A homodimers for DNA binding. Dissociation of these disulfide bridges through a reducing agent or an activity in muscle cell lysates results in monomeric E2A proteins which are then able to heterodimerize with MyoD. Such a mechanism has been proposed to regulate dimerization and consequently myogenesis (Benezra, 1994).

Phosphorylation has also been implicated in controlling dimerization status. The Mos kinase is expressed to high levels in skeletal muscle, and has been demonstrated to bind and phosphorylate MyoD but not E2A proteins (Lenormand et al., 1997). This Mos mediated phosphorylation of MyoD promotes the formation of MyoD:E-protein heterodimers (Lenormand et al., 1997). Thus, the Mos kinase acts to promote the transcriptional activity of MyoD.

1.2.5 DNA binding

It is well documented that exposure of myoblasts to growth factors, such as FGF(fibroblast growth factor) and TGFβ (transforming growth factor) inhibits myogenesis (reviewed in Dias et al., 1994). These growth factors act through the activation of various kinases. The activity of these kinases is thought to be directed at the

MRFs themselves or at one of their co-regulators. Such phosphorylation mediated regulation of the MRFs has been demonstrated to occur at the level of MRF binding to DNA. For example, FGF phosphorylates a conserved protein kinase site in the DNA binding domain of myogenin (Li et al., 1992b). This site is phosphorylated *in vivo* and causes myogenin to become inactive presumably because the myogenin heterodimers are unable to bind DNA. In the case of MRF4, potential serine and threonine targets of PKA or PKC phosphorylation were mutated without affecting the PKA/PKC mediated inhibition of MRF4, indicating that the observed inhibition was not due to direct phosphorylation of MRF4 (Hardy et al., 1993). Thus, it was presumed that PKC or PKA was phosphorylating another protein possibly E12, which has been shown to be phosphorylated by PKC *in vitro* (Hardy et al., 1993). Phosphorylation can also stimulate myogenesis. Phosphorylation of E47 by casein kinase II has been demonstrated to increase the transcriptional activities of MyoD and MRF4 by inhibiting DNA binding by E47 homodimers (Johnson et al., 1997).

Recent evidence suggests that regulation of myogenesis via modulation of DNA binding also occurs when MRFs are unable to access E-box binding sites due to a repressive chromatin conformation. MyoD was found to possess the ability to remodel chromatin at its respective binding sites and activate transcription from these previously inaccessible sites (Gerber et al., 1997). MyoD's ability to disrupt the chromatin structure was inhibited by TGF-β, bFGF and sodium butyrate (Gerber et al., 1997). The activity of these growth factors may be aimed at the additional proteins which are required for chromatin rearrangement. In this way, signalling pathways may influence the DNA

binding ability of MyoD:E protein heterodimers and ultimately regulate myogenesis.

1.2.6 Transcriptional Activity :Activation and Repression

The most well-characterized co-regulators involved in regulating MRF mediated transcription are the myocyte enhancer binding factor-2 (*MEF-2*) family of transcription factors. This family consists of four genes *Mef2 a,b,c* and *d* which bind an A/T rich conserved sequence in muscle-specific promoters. MEF2 proteins have homology for an amino terminal MADS domain (for MCM1, agamous, deficiens and serum-response factor) and a nearby domain named the Mef2 domain which mediates DNA binding (reviewed in Olson et al., 1995). *In vivo*, MEF2s are expressed after the MRFs and are unable to initiate muscle-specific differentiation on their own. However, when co-expressed with the MRFs, myogenic conversion is greatly enhanced. The increased transcriptional activity is a result of a direct interaction between the MRFs and the MEF2s. As a result of these interactions only one of the two factors , either an MRF or a MEF2 need be bound to their respective DNA binding sites (Molkentin et al., 1995). This interaction allows the activation of those muscle-specific genes which do not contain E-boxes and also provides a strong auto-regulatory loop as MEF2 sites are found within the promoters of the MRFs.

An additional regulatory layer has been revealed with the finding of a direct interaction of p300 (and CBP) with MyoD and MEF2C *in vivo* (Sartorelli et al., 1997). p300 binds to the activation domain of MyoD, and is believed to serve as the "unmasking"factor which enhances MyoD's transcriptional activity. The p300 and MEF2

interaction occurs via the MADS box. The effect of p300's binding is to positively influence myogenesis since it increases the transactivation ability of both MyoD and MEF2C and a dominant negative p300 abrogates myogenesis (Sartorelli et al., 1997). In this way p300 is believed to reinforce the auto-regulatory loop established by the MRFs and the MEF2 factors.

At least two kinases have been shown to have negative effects upon transcription by the MRFs. PKA has been reported to suppress the transcriptional activities of the MRFs both directly (Winter et al., 1993) and indirectly (Li et al., 1992a). [These PKA mediated suppression effects are different from those mentioned previously which affect DNA binding.] The indirect mechanism is unknown, but as with other regulatory mechanisms, may involve phosphorylation of a co-factor. The direct mechanisms was reported to be the result of phosphorylation of the bHLH domain which does not inhibit DNA binding but suppresses transcriptional activity (Winter et al., 1993). Another phosphorylation event by an unknown kinase has been demonstrated to occur only upon dimerization of myogenin (Zhou and Olson, 1994). The dimerization of myogenin with E2A gene products is shown to potentiate phosphorylation of myogenin's transcriptional activation domains, diminishing myogenin's transcriptional activity (Zhou and Olson, 1994). This method of control may permit rapid downregulation of myogenin's activity.

The effect of bone morphogenetic protein -2 (BMP-2), upon MyoD and myogenin is likewise negative; it inhibits MyoD/myogenin from initiating transcription (Katagiri et al., 1997). The mechanism of this inhibition remains unclear, although it is

known that BMP acts at a point following DNA binding of the MRF heterodimer.

1.3 Interaction of MyoD and the cell cycle

The proper formation of fully differentiated muscle cells from primitive mesodermal cells requires a precise balance between cell cycle progression and the initiation of differentiation. The MRFs were found to play a role in linking these two processes. Their role first became evident with experiments which demonstrated that MyoD could inhibit growth independently of differentiation (Sorrentino et al., 1990; Crescenzi et al., 1990). This link between growth and differentiation was further elucidated with the finding of a direct interaction between the retinoblastoma (pRb) protein and MyoD (Gu et al., 1993). Briefly, during the cell cycle pRb is phosphorylated by key cell cycle enzymes, the cyclin dependent kinases (eg. CDK4 and CDK6) and, primarily the cyclin D, family (for review see Weinberg, 1995). When hyperphosphorylated (late G1) pRb is unable to bind the mitogenic E2F family of transcription factors, the cell cycle proceeds. Just after mitosis, pRb becomes hypophosphorylated and is able to bind E2F members and arrest the cell cycle. The direct interaction of pRb with MyoD is necessary both for mediating the exit of the cell cycle during myogenesis and for transcription of muscle specific genes (Gu et al., 1993). p107, an pRb family member is able to substitute for pRb as a differentiation co-factor with MyoD but cannot replace pRb in suppressing growth (Schneider et al., 1994). In addition to binding pRb, MyoD has been demonstrated to induce *Rb* gene expression during myogenesis (Martelli et al., 1994).

The interplay of MyoD and cell cycle is complicated with the finding that Cdk/cyclin-D themselves inhibit the activity of MyoD in proliferating cells and this corresponded with a change in MyoD's phosphorylation state (Skapek et al., 1995). Complimentary to this mechanism is the finding that forced expression of p21, a Cdk inhibitor, allows MyoD to become active and transcribe muscle specific genes (Skapek et al., 1995). In addition, p21 is up-regulated in cells undergoing myogenic differentiation and in fibroblasts maintained in low serum which express MyoD(Halevy et al., 1995; Parker et al., 1995).

1.4 **Embryonal Carcinoma Cells**

This thesis studies the regulation of myogenesis in the P19 cell line. The P19 cell line is a pluripotent cell line with a stable diploid karyotype (McBurney and Rogers, 1982). It was derived from a teratocarcinoma which was created by implanting a 7.5 day C3H/He embryo into the testis of an adult mouse (McBurney and Rogers, 1982). Differentiation of P19 cells mimics early embryonic development and they can form a variety of cell types when injected into blastocysts (Rossant and McBurney, 1982). Little differentiation occurs upon aggregation, although the early mesodermal gene Brachyury T is induced (Vidricaire et al., 1994). However, when aggregated and exposed to a specific drug a variety of cell types may be formed. Dimethylsulfoxide (DMSO) treatment and aggregation leads to the formation of both cardiac and skeletal muscle along with other mesodermal and endodermal types (McBurney et al., 1982). Cardiocytes begin to appear around day 6 of DMSO treatment while skeletal muscle appears later

around day 9 (Edwards et al., 1983). Treatment with retinoic acid during aggregation directs the P19 cells into the neuroectodermal derivates including neurons, glia and fibroblast- like cells (Rudnicki and McBurney, 1987; McBurney et al., 1982; Berg and McBurney, 1990).

1.5 **MyoD mediated conversion of P19 cells into skeletal muscle**

While most types of differentiation in P19 cells require induction by drug exposure and aggregation, skeletal muscle can be formed when P19 cells stably expressing MyoD (termed [MyoD]P19 cells) are aggregated (Skerjanc et al., 1994). P19 cells were stably transfected with an expression construct encoding the MyoD gene. These cells were then cultured in monolayers, termed non-aggregated, or were aggregated. The non-aggregated MyoD expressing P19 cells formed skeletal muscle at only a very low frequency (<4% of colonies following selection). The majority of the non-aggregated [MyoD]P19 cells, which have not developed into skeletal muscle, express markers characteristic of undifferentiated stem cells as well as those found in myoblasts, such as myf-5 and myotonic dystrophy kinase (Skerjanc et al., 1994). Such transcripts are normally absent from growing P19 cells. Aggregation of these cells leads to a marked increase in myogenesis since approximately 30% of the cells in these cultures become skeletal muscle. Transcripts found in developing skeletal muscle such as myogenin and desmin, are detected by day 6 in the aggregated [MyoD] P19 cells. The day 6 differentiated cells appear as bipolar myocytes, but later fuse into multinucleated myotubes by day 10. These [MyoD]P19 cells possess characteristics of embryonic

differentiated muscle since they express both embryonic and slow isoforms of myosin heavy chain.

As mentioned previously, aggregation of P19 cells results in inefficient differentiation and [MyoD] P19 cells only efficiently form skeletal muscle following aggregation. This suggests that aggregation plays a role in regulating the MyoD directed differentiation.

1.6 **Area of Investigation**

Previous studies have shown that MyoD can dominantly induce myogenesis in a variety of cell types. In the P19 cell model described above, aggregation potentiates the ability of MyoD to induce myogenesis. The aim of this thesis was to examine whether the increase in myogenesis following aggregation of [MyoD]P19 cells was the result of the direct up-regulation of MyoD activity. In order to facilitate the investigation of changes in MyoD activation following aggregation, an improved model system was developed which included the generation of an active MyoD protein fused to the myc-epitope recognized by the monoclonal antibody 9E10. Preliminary results had suggested that the expression of MyoD protein increased following aggregation. Experiments were designed to address the possibility of translational regulation mediating this effect. However, once it was determined that translational control and up-regulation of MyoD protein was not occurring following aggregation, other mechanisms of control were examined. The following points were then evaluated as possible means of MyoD

regulation:

1) Regulation of nuclear import

2) Ability to heterodimerize with an E2A protein

3) Ability of MyoD:E2A heterodimers to bind DNA

Chapter Two

Materials and Methods

2.1 Plasmid constructs

The construct Pgk-MyoD[+UTR] is as described previously (Skerjanc et al., 1994). It contains the phosphoglycerate kinase [*Pgk-1*] promoter driving an *EcoRI* fragment containing the full-length MyoD cDNA (Davis et al., 1987) followed by a Pgk3' fragment which directs 3' end processing of the resulting mRNA (McBurney et al., 1991; Adra et al., 1987). To construct the plasmid pCA1, [Fig. 2A] a *BsrI-BglII* fragment from Pgk-MyoD [+UTR], containing MyoD without any MyoD untranslated region (including only two base pairs 5' to the start ATG, effectively removing any MyoD untranslated region) and a portion of *Pgk*3' was ligated into the *KpnI/BglII* sites of pPOP (Hannan et al., 1993) using an adaptor. The adaptor, shown below, permitted ligation of the *BsrI* end of the fragment to the *KpnI* site of pPOP. The adaptor also contained an internal *XbaI* site which was useful for making a second plasmid, pCA2 which would fuse a 6-myc-epitope tag to the N-terminus of MyoD. The adaptor is shown as follows:

```
        KpnI XbaI TfiI
        5' CTCTAGAATCGG 3'
3' CATGGAGATCTTAG 5'
```

The construct pCA2 [Fig 2B] was made using an *Xba-BglII* fragment from pCA1 which was ligated in-frame into pKJ1ΔF, a *Pgk* containing plasmid which contains six myc epitope tags. The resulting plasmid, pCA2, was expected to drive the expression of a N-terminal 6-myc MyoD under the control of the Pgk promoter.

Fig. 2A **A schematic of the pCA1 plasmid**

To construct pCA1, the coding region of MyoD without any MyoD untranslated region was ligated into the *Pgk* driven expression plasmid pPOP using an adaptor as described in the text. The blue panel represents the entire MyoD coding region. Important restriction sites used for constructing all MyoD expression plasmids are shown in their approximate positions. (not drawn to scale)

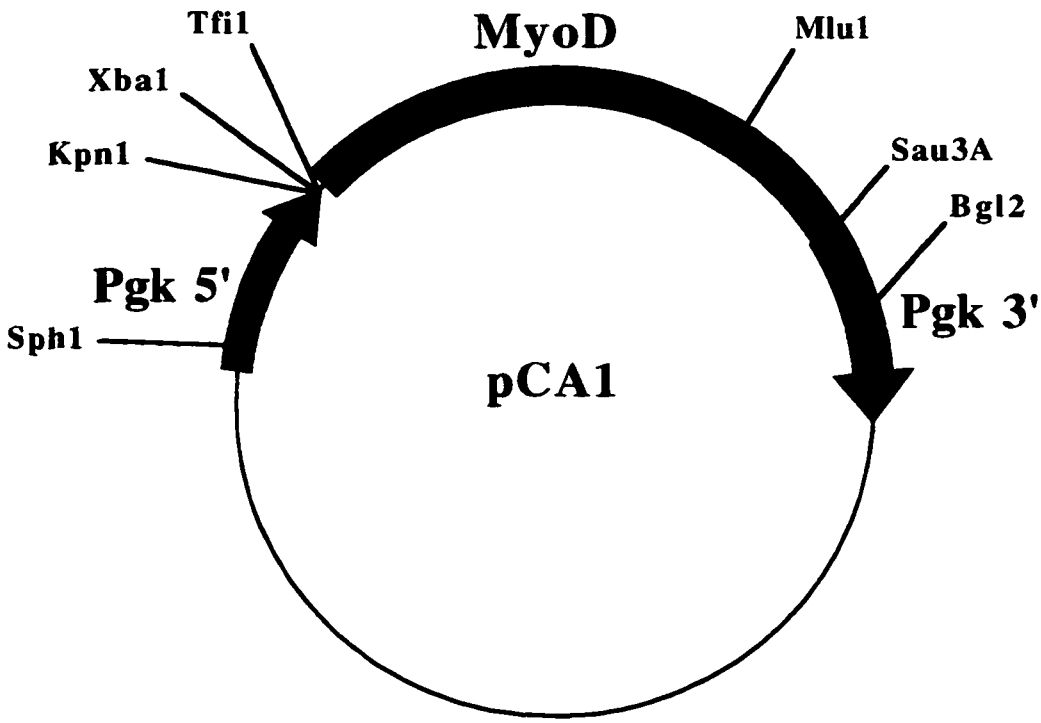

Tfi1
MyoD
Mlu1
Xba1
Kpn1
Sau3A
Bgl2
Pgk 5'
Pgk 3'
Sph1
pCA1

Fig. 2B **A schematic of pCA2**

Construction of pCA2 was performed by ligating the *Xba-BglII* fragment in-frame into the myc epitope in plasmid pKJ1ΔF. The result is a *Pgk* driven MyoD fused to 6 myc epitope tags at the N-terminus. The MyoD ATG transcription start site was removed and transcription begins from the ATG at the 5' end of the myc tag. Restriction sites relevant for the construction of MyoD expression constructs are shown in approximate locations. (not drawn to scale).

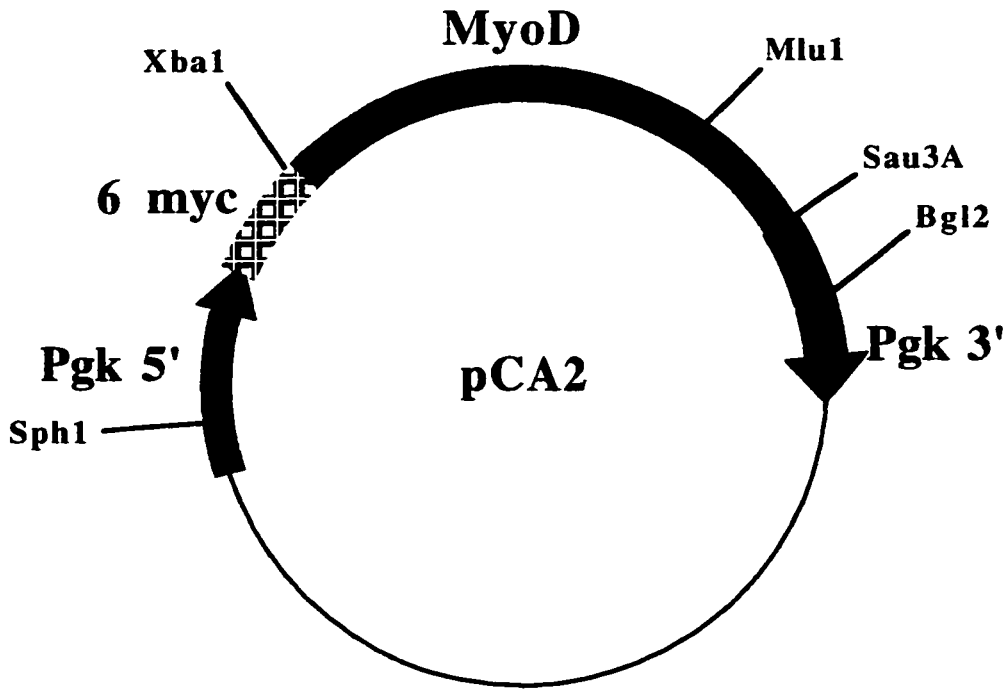
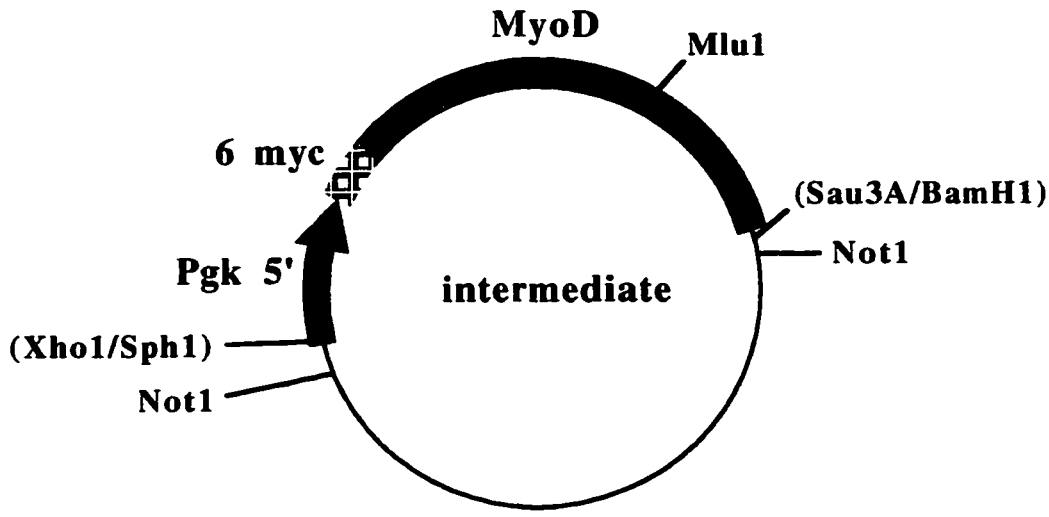
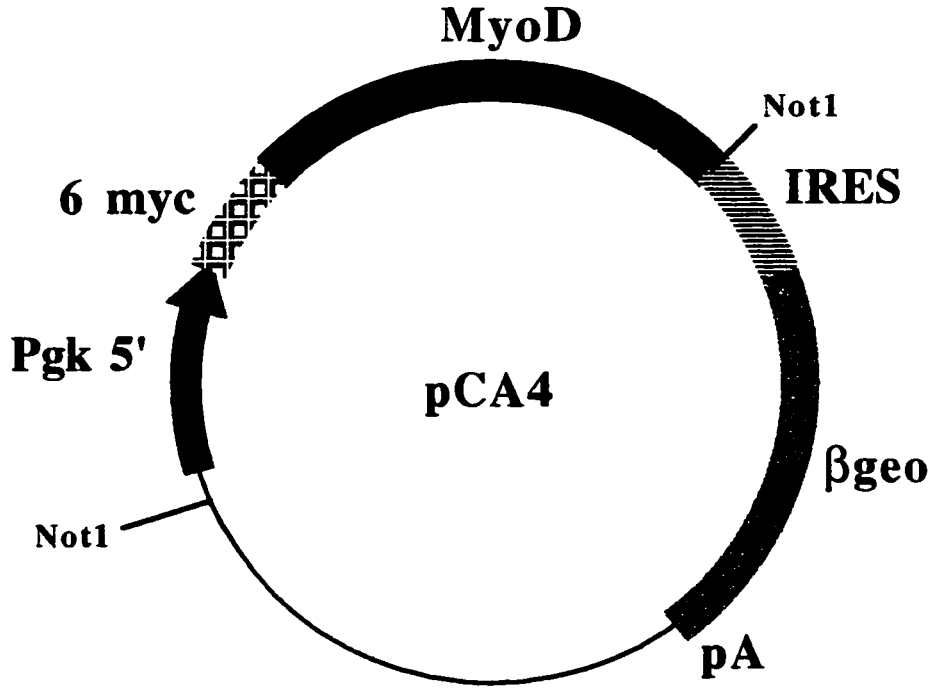

MyoD
Xba1
Mlu1
Sau3A
6 myc
Bgl2
Pgk 5'
pCA2
Pgk 3'
Sph1

Fig. 2C **Schematic of pCA4 and the intermediate plasmid used in its construction** An intermediate plasmid shown above was first constructed to facilitate production of pCA4. Two fragments from pCA2, a *Sph1-Mlu1* and *Mlu1-Sau3A*, encompassing most of *Pgk5'* and all of mycMyoD, were ligated into tgCMVHygTK to generate the intermediate plasmid such that *Not1* sites flanked *Pgk* 5' and mycMyoD. The Not1 fragment from the intermediate was ligated into IRES-βgeo which contained only one unique site, *Not1*, 5' to the IRES sequence. The resulting plasmid, pCA4, has Pgk5' driving expression of mycMyoD followed by the internal ribosome entry site (IRES) sequence and the βgeo fusion gene. An SV-40 polyadenylation (pA) sequence is present after βgeo to replace Pgk3' and allow proper 3' processing. Relevant restriction sites are shown in approximate locations. (not drawn to scale)


MyoD
Mlu1
6 myc
(Sau3A/BamH1)
Not1
Pgk 5'
intermediate
(Xho1/Sph1)
Not1


MyoD
Not1
6 myc
IRES
Pgk 5'
pCA4
βgeo
Not1
pA

To construct pCA4, an intermediate plasmid was first generated (Fig. 2C). The MyoD and *pgk5'* containing *SphI-MluI* and *MluI-Sau3A* fragments from pCA2 were ligated into the *XhoI-BamHI* sites of tgCMV-Hy-Tk (Immunex Research and Development Corp., Seattle, WA). Once this intermediate was obtained, the *NotI* fragment from this plasmid was ligated into the *NotI* site of the 7.84kb IRES-βgeo plasmid (Mountford et al., 1994). The resulting plasmid encoded the *Pgk* promoter driving mycMyoD followed by the internal ribosome entry site (IRES) and βgeo gene, which encodes a fusion protein of the bacterial β-galactosidase gene and the drug resistance gene, neomycin (Fig. 2C). Instead of *Pgk3'* an SV-40 polyA termination signal is present after the βgeo gene.

The plasmid encoding Pgk-puro (Vara et al., 1986) contains the *Pgk* promoter driving expression of the drug selection gene puromycin. Stable transfection of this plasmid allows selection of expressing cells in puromycin. Similarly, Pgk-neo, constructed by Karen Jardine, expresses the selection gene neomycin driven by the *Pgk* promoter. The plasmid B17 enhances integration of transfected DNA and consists of intragenic regions of the murine *Pgk* gene (McBurney et al., 1994). Pgk-lacZ, another *Pgk* plasmid, drives expression of the bacterial β-galactosidase gene and is described in Pari et al., 1991. The plasmid Pgk-neo:CA-CAT was constructed by Giovanna Pari (MSc thesis) and the relevant portion contains the cardiac actin (CA) promoter driving expression of the chloramphenicol acetyl-transferase (CAT) gene. Other plasmids used include the Pgk-CAT plasmid, in which CAT gene expression is driven by the *Pgk*

promoter [Karen Jardine] and the Pgk-mycpuro gene [Helen Tai] containing a myc epitope tagged version of the puromycin gene driven by the *Pgk* promoter. The plasmid containing the IRES sequence followed by the β-geo fusion genes was as described in Mountford et al (1994) and the corresponding plasmid Pgk-IRES- βgeo, [Karen Jardine], contains the IRES sequence and β-geo gene expressed by the Pgk promoter. The plasmid pGEM was obtained from BioCan (Mississauga, Ont)and is used as a control plasmid. The plasmid CMV-p300 contains the human cytomegalovirus (CMV) promoter driving the expression of p300 (Eckner et al., 1994b).

Plasmids were propagated in *Escherichia coli* strain DH5α or strain TG1 and were isolated by the sodium dodecyl sulfate (SDS)/alkaline lysis method (Birnboim, 1983) or by use of QIAGEN Maxi prep kit (QIAGEN). Manipulation of plasmid DNAs and subcloning methods were essentially according to Sambrook et al. (1989).

2.2 Cell culture and transfections

The P19 cell line was maintained as described (Rudnicki and McBurney, 1987). Cells were transfected by the calcium phosphate method (Chen and Okayama, 1987) or by Lipofectamine (Gibco BRL, Burlington Ont.) with the modifications described below. For transient transfections, 10^6 cells were plated in 5ml media in a 60mm dish 24 hrs before transfection. Transient transfections utilizing Lipofectamine were followed according to the manufacturer's directions (Gibco BRL, Burlington Ont.) with 10 μl Lipofectamine, 1 μg Pgk-lacZ, 2 μg CA-CAT and 2 μg of a MyoD containing plasmid depending on the experiment. For transient transfections utilizing the calcium phosphate method 3 μg Pgk-lacZ, 5 μg CA-CAT and 5 μg of MyoD containing plasmid in 0.5ml 2.5

M $CaCl_2$ was mixed with 0.5ml 50mM BES buffer at pH 6.88 and incubated at 37°C, 5% CO_2 until a very fine precipitate formed. The solution was then added to the cells and incubated for 6-8hrs after which cells were washed and given fresh media. Cells were harvested 48 hrs after transfection for β-galactosidase activity (Skerjanc and McBurney, 1994) or for CAT activity using FAST CAT (Molecular Probes) according to the manufacturer's direction followed by detection and quantification using a phosphorimager (Molecular Dynamics) and Excel 5.0.

To isolate P19 cells with transfected DNA stably integrated, cells were transfected using one of the methods described above, except with different amounts of DNA. For calcium phosphate transfection 2 μg Pgk-puro, 8 μg B17 (McBurney et al., 1994) and 8 μg MyoD containing plasmid were used and with Lipofectamine 1 μg Pgk-puro, 3 μg B17 and 3 μg MyoD containing plasmid were used. Plasmid use varied from experiment to experiment as described in results, eg. Pgk-puro was replaced with Pgk-neo. After 24 hrs, cells were trypsinized and approximately $2x10^6$ cells were selected in the required drug, either 2 μg/ml puromycin (Sigma-Aldrich, Oakville Ont), or 400ug/ml G418 (Gibco BRL, Burlington Ont). When colonies began to appear (6-10 days depending on drug) they were either picked and used as clones, or colonies were pooled and used as a mixed population. Pooled clones were used for all experiments prior to the construction of pCA4 after which only clones were used. Cells stably expressing MyoD are referred to by the MyoD protein they contain, i.e. [mycMyoD]P19 are cells stably transfected with a mycMyoD expression construct . Some results shown in this thesis (as described in results) were from cell cultures in which stable lines which were transfected

with pCA4 and selected for G418 resistance, were then co-transfected with Pgk-puro and pCA2 and selected for resistance to both puromycin and G418.

Stably expressing cells were either cultured in tissue culture dishes, (non-aggregated), or in 60mm bacterial dishes so that aggregates could form, (aggregated). Cells were aggregated for 5 days at which point they were plated onto tissue culture dishes and harvested, the next day, day 6. Non-aggregated cells were maintained in culture dishes for the same period of time and passaged as required. Under both conditions cells were maintained in the presence of selective drug. On day 6, cells were harvested for immunofluorescence or for isolation of either RNA or protein.

2.3 **Immunofluorescence**

Cells were plated onto gelatin coated coverslips and fixed in -20°C methanol for 5 min and then re-hydrated in phosphate buffered saline (PBS) for 15 min at room temperature. After re-hydration cells were washed once more with PBS and then incubated for one hour with the appropriate primary antibody. For myc tag immunodetection, 50 µl of the culture supernatant from the mouse monoclonal 9E10 hybridoma was used (Chan et al., 1986); for myosin heavy chain detection 50 µl of the mouse anti-myosin monoclonal antibody supernatant MF20 was used (Bader et al., 1982); for β-galactosidase staining the rabbit polyclonal was diluted 1:500 (Cappel, Cooper Biomedical, Westchester PA). Each coverslip was washed three times in PBS for 2min and then incubated for one hour with either a 1:100 dilution of the goat anti-mouse immunoglobin G cy3-linked antibody (Jackson Immunoresearch Labs Inc, Westgrove PA) or with a 1:50 dilution of the goat anti-rabbit immunoglobin G flourescein

isothiocyanate (FITC) linked antibody (Amersham Oakville Ont). Coverslips were then washed 2 min each for three times in PBS and mounted on slides for viewing with the Zeiss Axiophot microscope.

For double labelling, the same procedure as described above was followed except it was repeated with second set of antibodies [described above] before mounting the coverslips on slides. For example, the β-galactosidase primary antibody and corresponding secondary antibody were used in sequence followed by the MF20 primary antibody and corresponding secondary antibody. Pictures of the cells were taken with a Close-Coupled Device (CCD) camera and the Snappy™ image grabber. Colour tones and saturation of images were adjusted with Corel PhotoPaint.

2.4 **Northern blot analysis**

Total RNA was harvested using Trizol (Gibco BRL, Burlington Ont.) according to the manufacturer's instructions. Northern blot analysis was performed as described in Sambrook et al. (1989) with the following modifications. 10ug of RNA from each sample were separated on a 1% agarose gel containing formaldehyde. RNA was transferred to Hybond-N (Amersham, Oakville Ont) by capillary blotting and was cross-linked by UV irradiation. Hybridization was done with either a MyoD probe made from the *Bsr-BglII* fragment described previously (section 2.1) or a tubulin probe made from a tubulin cDNA containing plasmid. Probes were made with the multiprime labelling kit (Amersham, Oakville Ont) and labelled to $>10^9$cpm/μg with [α-^{32}P]dCTP (Amersham Oakville Ont). Hybridization was carried out for 16hrs at 42°C followed by three 10min washes at 42°C in 2X SSC (Sambrook et al., 1989) and three 20 min washes in 0.2X SSC

at 65°C. Images were captured either by autoradiography or by use of a phosphorimager (Molecular Dynamics, CA).

2.5 **Western blot analysis**

Western blots were performed as described in Sambrook et al. (1989), with the modifications described below. Cells were harvested in 2X harvesting buffer [1M Tris pH 6.8, 200mM dithiothreitol, 4% sodium dodecyl sulfate (SDS), 20% glycerol] at 4°C and sonicated for thirty seconds. Protein was then quantified using the Biorad (Mississauga, Ont) protein assay kit and equal amounts of protein from each sample (usually 15 - 30 μg) were separated on 8% or 10% PAGE. Proteins were then electrophoretically transferred to Hybond-C Extra (Amersham Oakville, Ont). Blots were blocked in 5% powdered milk in Tris-buffered saline (TBS)(10mM Tris-HCl pH7.5, 150mM NaCl) and all antibody immunodetection was also done in 5% milk :TBS solution. For immunodetection of MyoD, a mouse monoclonal MyoD(5.8A) antibody was generously donated by Dr. Peter Houghton (St. Jude's Children's Research Hospital, Memphis Tennessee) and was used at 1:100 dilution (Dias et al., 1992). The myc epitope was detected using a 1:100 dilution of the mouse monoclonal 9E10 supernatant (American Type Culture Collection, ATCC, Rockville, MA). Detection of myosin heavy chain was with the mouse anti-myosin monoclonal antibody MF20 supernatant, undiluted (Bader et al., 1982). β-tubulin was detected with 1:10 dilution of the mouse β-tubulin monoclonal hybridoma supernatant (Developmental Studies Hybridoma Bank, University of Iowa, Iowa). β-galactosidase was detected with undiluted supernatant from the β-galactosidase mouse monoclonal JIE7 (Developmental Studies Hybridoma Bank,

University of Iowa, Iowa). The E2A gene products E12 and E47 were both detected with the mouse monoclonal antibody Yae 416 (Vierra et al., 1994)(SantaCruz Biotechnology, Santa Cruz CA). The secondary antibody used was a goat anti-mouse anti-IgG heavy and light chain, horse-radish peroxidase conjugated antibody at a dilution of 1:5000 (Biorad, Mississauga,Ont.). Following incubation of the blot in each antibody solution, the blot was washed four times for 10 min in TBS. Detection was performed with the Kirkegaard Perry Laboratories (Gaithersburg, MA) Lumiglo™ chemiluminescence substrate kit as per manufacturer's directions. Luminescence was detected by autoradiography and exposures varied from blot to blot but were in the range of 10s to 5min.

2.6 S^{35}-Methionine *in vivo* labelling and immunoprecipitation

Confluent plates of P19 cells stably expressing pCA4 or a control plasmid Pgk-IRES-βgeo (both non-aggregated and aggregated) were treated as described in Lassar et al. (1991) with the following modifications. Cells were starved of methionine and cysteine by incubating them for 1 hour at 37°C in α-MEM methionine and cysteine free media (Gibco BRL, Burlington Ont.) containing 10% dialyzed serum (3:1 ratio Donor bovine: Fetal calf serum) plus 292 mg/L L-glutamine. Cells were then given 70.15 μCi/ml of Pro-Mix™ (Amersham, Oakville Ont.), an *in vivo* cell labelling mix containing L-[^{35}S]-methionine and L-[^{35}S]cysteine (Pro-Mix™ contains10mCi/ml [^{35}S]-methionine and 4.3mCi/ml [^{35}S]cysteine) and incubated for another four hours. Cells were then rinsed four times in TBS and then dislodged in 2ml TBS +10mM ethyldiamine tetra-acetic acid (EDTA) and pelleted. Cells were then lysed for 1 hr at 4°C in approximately 24 000 cell equivalents/uL (≈0.5mL) of buffer containing 20mM Tris-HCl pH 7.5,

150mM NaCl, 2mM EDTA, 1% TritonX-100, 1mM PMSF, 1mM aproteinin, 1mM leupeptin (Frack's buffer). For each sample, 60 µl of a 50% slurry of Protein G-Plus Sepharose beads (Gibco BRL, Burlington Ont.) were washed in Frack's buffer and then incubated for 1 hr in the same buffer with 2.4 µg High Performance Liquid Chromatography (HPLC) purified 9E10 myc antibody (Chan et al., 1986) kindly donated by Dr. Ken Garson. After 1 hr the beads were washed once again to remove unbound 9E10 antibody and then incubated with the labelled cell lysate overnight at 4°C. The following day, the unbound protein supernatant was removed and the beads were washed four times in Frack's buffer. 50 µl 2X SDS harvesting buffer (Sambrook et al., 1989) was added to each sample followed by incubation at 100°C for 3 minutes after which the supernatant from each sample was separated on a 10% SDS-PAGE gel (Sambrook et al., 1989). Following separation the gel was dried and exposed on the phosphorimager (Molecular Dynamics, CA) until signal was detected.

2.7 **Nuclear Lysates**

Nuclear lysates were obtained using the method described by Dignam et al. (1983) although many modifications were necessary for the cells used and are as follows. Confluent plates of both non-aggregated and aggregated cells, including controls were trypsinized, washed three times in PBS, quickly rinsed in 1ml cold 10mM NaCl and then gently pelleted at 2000 rpm, 4°C. The supernatant was removed and cells were then incubated in 2ml of cold lysis buffer (20mM Hepes pH7.9, 10mM NaCl, 1.5mM $MgCl_2$, 0.2 mMEDTA, 0.1%NP-40, 1mM PMSF, 1mM aproteinin, 1mM leupeptin) until lysis of the cytoplasm was observed by microscopy. Nuclei were pelleted at 2000rpm at 4°C and

then quickly rinsed in lysis buffer without NP-40. After removal of the supernatant, nuclei were resuspended in ≈ $4x10^4$ cell equivalents/µl Buffer C (20mM Hepes pH7.9, 20% glycerol, 420mM NaCl, 1.5 $MgCl_2$, 0.2 mM EDTA) and incubated on ice for 1 hr. During incubation the nuclei were sheared with a $26^{3/8}$ gauge needle until complete destruction of the nuclei was observed by microscopy. The nuclear lysate was centrifuged for 15min at 14,000rpm at 4°C and the supernatant was dialyzed overnight at 4°C in Buffer D (20mM Hepes pH 7.9, 5%glycerol, 0.1M KCl, 0.2mM EDTA) using microcollodion tubes with a molecular weight cut off of 3400 D (Sartorius, Micro-collodion bags, Germany). The next day the lysates were centrifuged at 14,000rpm for 15min at 4°C, the protein concentration determined and the lysates stored at -80°C until needed.

2.8 **Immunoprecipitation - Western**

The non-radioactive immunoprecipitations were performed using a slightly different method from the immunoprecipitations described for the *in vivo* labelling experiments. In order to obtain cleaner results, nuclear lysates were used. Immunoprecipitations were performed as described in section 2.6 with the following modifications. The Sepharose beads were pre-incubated with either 1 µg E12/E47 Yae 416 antibody, 1 µg of Bcl2 antibody (DAKO, Denmark), or no antibody. Following washing, 20 µg of the appropriate nuclear lysate was incubated with the antibody bound beads for 3 hrs at 4°C with occasional mixing. The beads were washed four times with Frack's buffer, extracted in the 2X SDS harvesting buffer (Sambrook et al., 1989) and separated on a 10% SDS-PAGE gel. Transfer and immunodetection of

immunoprecipitated mycMyoD was performed as described previously for Western blotting (section 2.5) with a 1:100 dilution of the mouse monoclonal 9E10 supernatant (American Type Culture Collection, ATCC, Rockville, MA).

2.9 **Electrophoretic Mobility Shift Assays**

Electrophoretic mobility shift assays (EMSAs) were performed as described by Buskin and Hauschka (1989) with the following modifications. The oligonucleotides used were the MEF-1 consensus oligonucleotides (Santa Cruz Technology, Santa Cruz CA.) identical to sequences of the mouse muscle creatine kinase (MCK) promoter around the high affinity E-box (Buskin and Hauschka, 1989) and a MEF-1 mutant oligonucleotide with an identical sequence to that found in the MCK promoter with the exception of a six base pair substitution within the E-box (Santa Cruz Technology, Santa Cruz CA). Both oligonucleotides used were 25 base pairs long and double stranded. The top strand of each is shown below with the E-box shown in bold and mutated base pairs underlined.

MEF-1 consensus oligonucleotide:
5'- GAT CCC CCC AA**C ACC TG**C TGC CTG A -3'

MEF-1 mutant oligonucleotide:
5'- GAT CCC CCC AA**C AC**<u>**G GTA AC**</u>C CTG A -3'

Oligonucleotides were end-labelled with polynucleotide kinase and [γ ^{32}P]ATP as described in Sambrook et al. (1989) and purified on an HR-200 spin-column (Pharmacia, Baie d'Urfé, Que.) to yield a probe with a specific activity of approximately 200,000 cpm/ng of oligonucleotide. Binding reactions contained 20 µg of nuclear lysate,

20mM Hepes pH 7.8, 0.05 M KCl, 10%glycerol, 1mM dithiothreitol (DTT), 0.2 mM EDTA and were pre-incubated with 2.0 μg poly (dI-dC) (Bohreinger Mannheim, Laval, Que.) for 15 min at room temp to inhibit non-specific binding of the labelled probe to nuclear proteins. Samples to be supershifted contained 1.0 μg of the appropriate antibody (antibodies as described in sections 2.3 and 2.5) and were otherwise identical to other samples. Following this incubation, 1.0-2.0 ng of labelled probe was mixed into each sample for a total volume of 25 μl. The reactions were incubated for 15 min at 37°C after which 0.5 μl of a 0.05% bromophenol blue/xylene cyanol solution was added. DNA-protein complexes were resolved on a 5% polyacrylamide gel (29:1 polyacrylamide:Bis-NN-acrylamide) in 50mM Tris-base, 50mM boric acid and 1mM EDTA (same solution in running buffer) which had been pre-run for 30 min at 100Volts . The gel was then run at 100 Volts at room temp with water cooling until the bromophenol blue was approximately two thirds of the way down the gel. The gel was removed, wrapped in plastic and exposed to the phosphorimager (Molecular Dynamics, Sunnyvale, CA) for 15 min for visualization.

Chapter Three
Results

3.1 Expression of MyoD in P19 cells

To investigate the mechanism by which MyoD expressing P19 cells undergo myogenesis, P19 cells stably expressing the wild type *MyoD* cDNA were generated. The cells were co-transfected with Pgk-MyoD, B17 and Pgk-puromycin and selected in puromycin. Colonies were pooled and then grown in monolayers (non-aggregated) or aggregated for five days. P19 cells transfected with a control plasmid, Pgk-CAT, and with Pgk-puromycin, did not form bipolar myocytes in either the non-aggregated or aggregated samples (as shown with myosin heavy chain immunodetection). MyoD expressing P19 cells formed bipolar myocytes only in the aggregated sample (Fig 3A). The proportion of aggregated [MyoD]P19 cells which formed skeletal muscle was approximately 30%.

To determine the levels of *MyoD* mRNA expressed from the Pgk-MyoD vector (+UTR), a northern analysis of total RNA from pooled [MyoD]P19 cells was performed. Figure 3B shows that comparable levels of the *MyoD* mRNA are present both before and after aggregation, while no signal was detected in the control lanes. The length of *MyoD* mRNA expressed from Pgk-MyoD was of the expected size. Endogenous *MyoD* mRNA was not detected in controls.

Fig. 3A **Expression of MyoD induces myogenesis following aggregation of P19 cells**

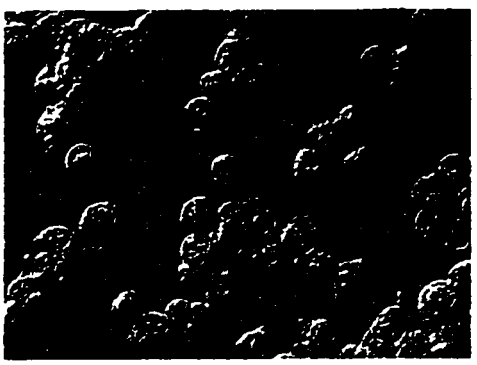
Immunofluorescent detection of striated muscle specific myosin heavy chain with MF20 monoclonal antibody in non-aggregated and aggregated control and [MyoD+UTR]P19 cells. Control cell lines were co-transfected with Pgk-puromycin and Pgk-CAT. Differential interference contrast microscopy (left panels) shows the same field of view as shown for immunofluorescence (right panels). Bar = 40μm

α myosin heavy chain

NAgg
P19

Agg
P19

NAgg
[MyoD]P19



Agg
[MyoD]P19

Fig. 3B **Levels of Pgk-MyoD mRNA do not increase during aggregation**
Total RNA from [MyoD+UTR]P19 or [CAT]P19 stable cell lines was extracted on day 5 from both non-aggregated and aggregated cultures and examined by Northern blot assay. The blot was first hybridized with a *MyoD* cDNA probe and then stripped and hybridized with a tubulin cDNA probe. The lanes are as marked. The full length Pgk-MyoD message is expected to be approximately 1833 bases not including the poly(A) tail while the 18s and 28s ribosomal RNAs are 1869 and 4712 bases respectively.


MyoD
CAT
non-aggregated
aggregated
non-aggregated
aggregated
28s
18s
MyoD
tubulin

3.2 **Removal of the MyoD untranslated region has no effect on myogenesis**

Given that the levels of Pgk- MyoD mRNA did not change during aggregation, it was postulated that MyoD protein expression was up-regulated during aggregation resulting in a corresponding increase in myogenesis. Preliminary experiments had suggested that MyoD protein expression increased following aggregation despite the observation (Fig 3B) that Pgk-MyoD mRNAs remained constant. Since myogenin and MEF2A (Cusella-De Angelis et al., 1992; Black et al., 1997), are translationally regulated, it was possible that MyoD might be similarly regulated. Commonly the 5' or 3' UTR of a gene may interact with the translational machinery to inhibit or slow the translation of the mRNA into protein (Melefors and Hentze, 1993; Blau 1993). It is conceivable that translation of *MyoD* mRNA is inhibited by a portion of the UTR in the non-aggregated cells, resulting in insufficient amounts of the protein to induce myogenesis. Under this hypothesis, MyoD would become adequately translated in the aggregated cells allowing them to become muscle. To examine this possibility, the entire 5' and 3' UTR from the *MyoD* cDNA were removed so that any effect of these regions would be lost. This *MyoD* fragment is referred to as *MyoD*-UTR and is expressed by the Pgk promoter in the plasmid pCA1. To verify that MyoD encoded by this plasmid was functional, transient transfections were performed. A reporter construct, consisting of the human cardiac actin promoter driving expression of the CAT gene, was used to determine activity of the *MyoD* gene encoded by pCA1. Co-transfections of this reporter with pCA1 indicated that the MyoD protien produced from pCA1 was functional as it was able to transactivate transcription from this promoter (Fig. 6A).

The ability of pCA1 to induce myogenesis was determined as described in section 3.1. Stably expressing [MyoD- UTR] P19 cell lines were generated and pooled clones were grown in either the non-aggregated or aggregated state. As shown previously, control cell lines expressing Pgk-puromycin and Pgk-CAT did not show muscle formation before or after aggregation. The removal of the 5' and 3' UTRs did not affect the phenomenon of muscle formation since the [MyoD-UTR] P19 cells formed significant amounts of muscle only after aggregation (Fig. 4A).

To determine if the time course of muscle formation was similar to the [MyoD+UTR] P19 cells, a Western blot analysis of protein lysates from the [MyoD-UTR] P19 cells was performed. As with the [MyoD+UTR]P19 cells, significant muscle was formed after five days of aggregation as shown by immunodetection of myosin heavy chain (Skerjanc et al., 1994) (Fig 4B). Northern analysis of mRNA from these cell lines showed that no appreciable change in *MyoD*-UTR mRNA levels either before or after aggregation (Fig 4C). Without the UTR the *MyoD* mRNA runs below the 18s ribosomal RNA.

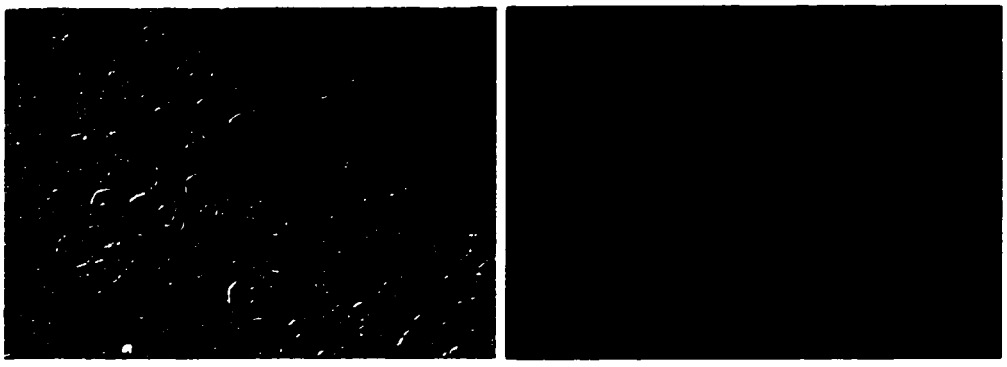
Since the deletion of the *MyoD* untranslated regions had no observable effect on muscle formation in P19 cells, we interpreted this to indicate that translational regulation mediated by the 5' and 3' UTRs was not responsible for the phenomenon of increased myogenesis following aggregation of [MyoD]P19 cells.

Fig. 4A **Removal of the 5' and 3' untranslated region has no effect on *MyoD* induced myogenesis**

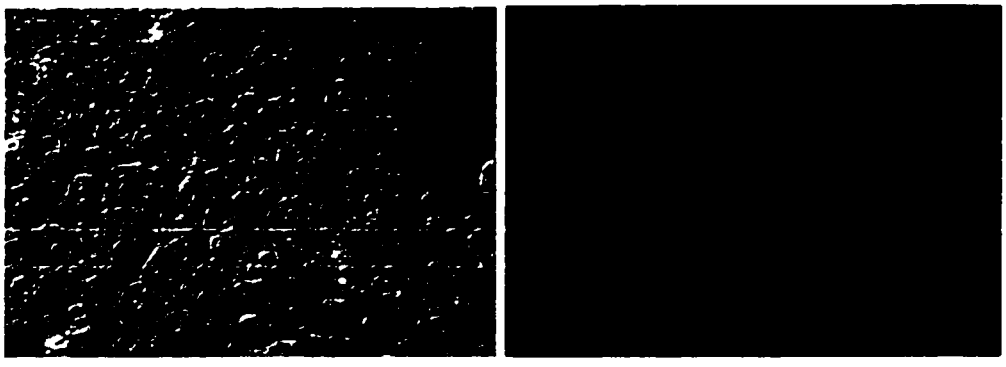
Immunofluorescent detection of myosin heavy chain in non-aggregated and aggregated control and [MyoD-UTR]P19 cells. Control cell lines were co-transfected with Pgk-puromycin and Pgk-CAT. Differential interference contrast microscopy shows the same field of view as shown for immunofluorescence. Bar = 40μm

α myosin heavy chain

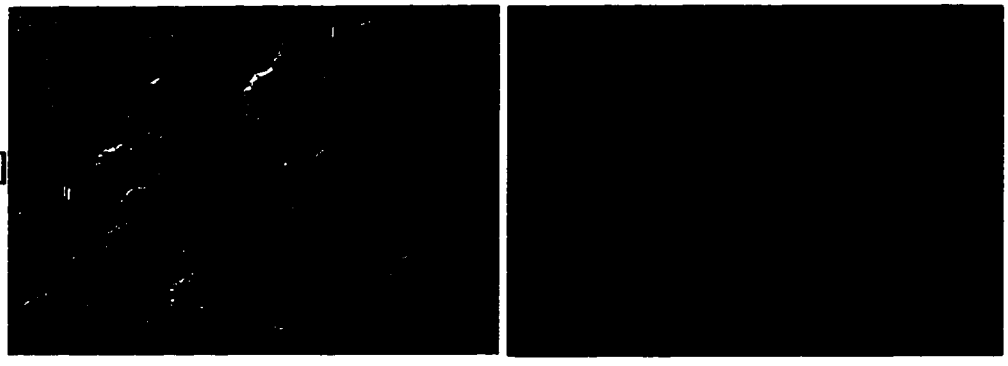
NAgg
P19



Agg
P19



NAgg
[MyoD-UTR]
P19



Agg
[MyoD-UTR]
P19

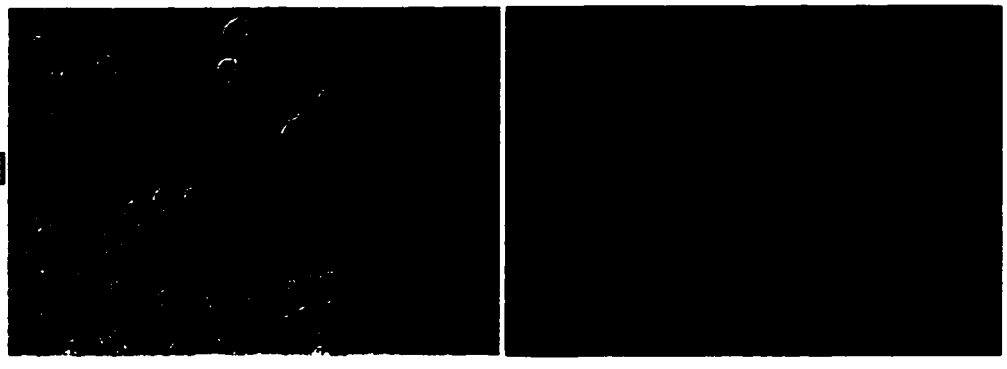
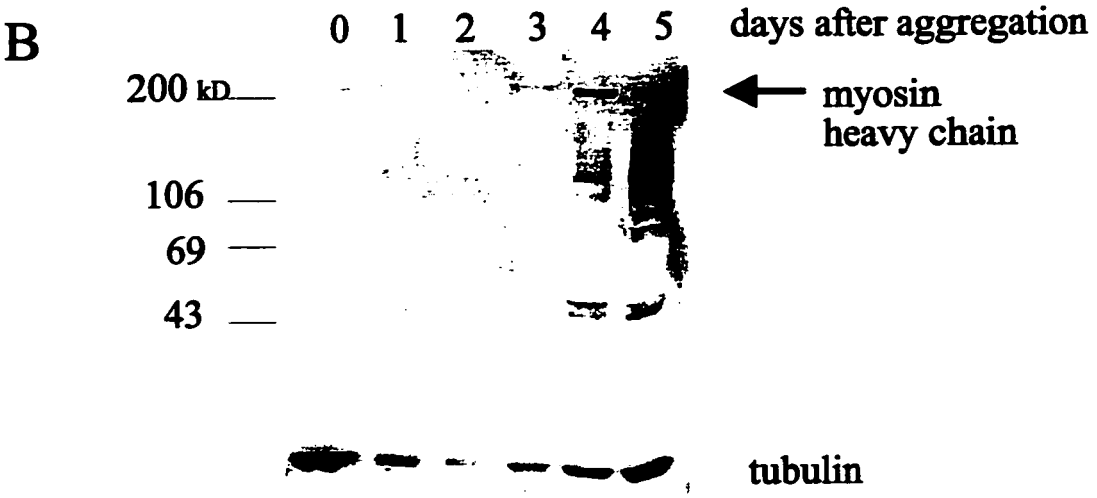
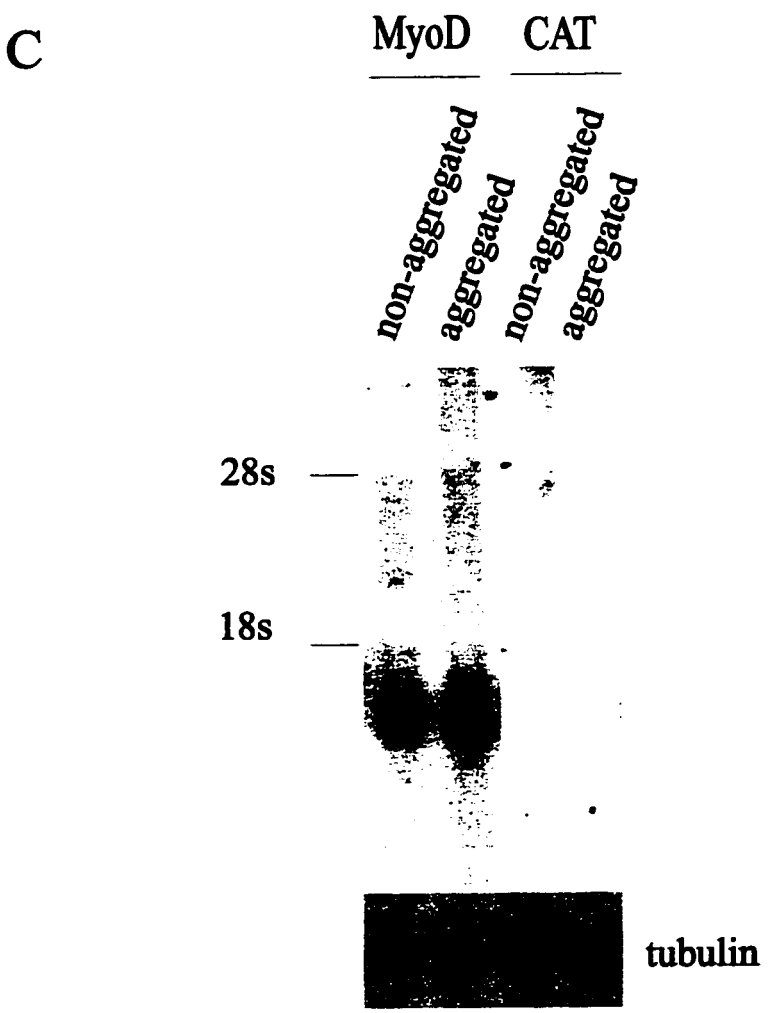


Fig. 4B & 4C **Removal of the *MyoD* untranslated region does not influence levels of mRNA or the timing of muscle formation**

4B Protein harvested daily from stable [MyoD-UTR]P19 cells before aggregation and for five days of aggregation were analysed by Western blot assay using an anti-myosin heavy chain antibody. The β-tubulin loading control is shown below and was performed using an anti-tubulin antibody. Size markers are as shown in kilodaltons.

4C Total RNA from both non-aggregated and aggregated [CAT]P19 and [MyoD-UTR] P19 cells was harvested and analysed by Northern blot assay. The blot was hybridized with a *MyoD* probe followed by a β-tubulin probe. *MyoD* message lacking the UTR is predicted to be 958 bases not including the poly (A) tail and appears below the 18s rRNA (1869 bases).


B
0 1 2 3 4 5
days after aggregation
200 kD
106
69
43
myosin
heavy chain
tubulin


C
MyoD
CAT
non-aggregated
aggregated
non-aggregated
aggregated
28s
18s
tubulin

3.3 pCA2 expresses mycMyoD

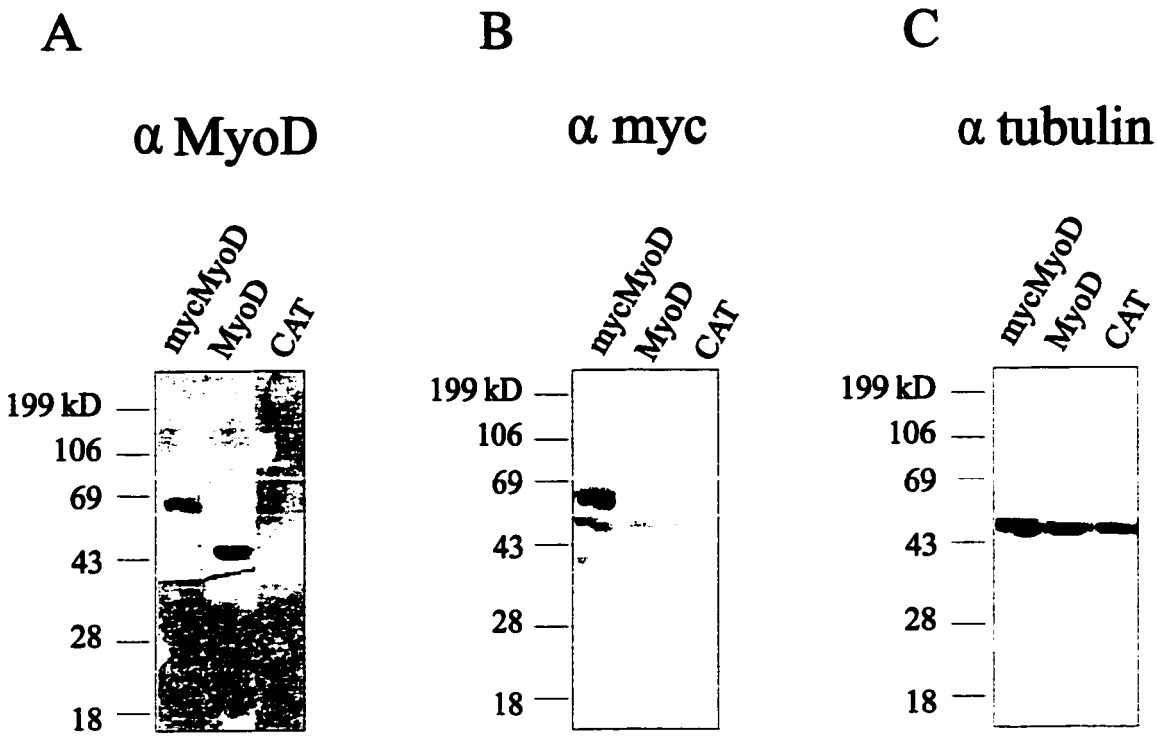
Although *MyoD*'s activity was unaffected by the removal of the 5' and 3' UTRs, a number of other types of regulation may have been responsible, as reviewed in the introduction. Determination of MyoD protein expression, localization, etc., requires a reliable supply of antibody. At the time these experiments were conducted, MyoD antibody was not commercially available, although a small amount was generously donated by Dr. Peter Houghton. Consequently, it was convenient to epitope tag MyoD with six myc tags at the N-terminal end to facilitate detection of the MyoD protein. This protein, referred to as mycMyoD, lacks the *MyoD* UTRs. Like other plasmids constructed for this thesis, expression of *mycMyoD* was driven by the Pgk-promoter. The Pgk-mycMyoD expression plasmid is referred to as pCA2.

A Western blot analysis of [mycMyoD]P19, [MyoD]P19, and [CAT]P19 stable cell lines was performed to confirm both MyoD and mycMyoD protein expression. As shown in Fig. 5A, mycMyoD protein was expressed well in the [mycMyoD] P19 cells as was the MyoD protein in the [MyoD]P19 cells. Control cells showed no MyoD expression. It is expected that a mycMyoD protein would be larger than the wildtype MyoD protein due to the addition of the myc epitope tag. The MyoD protein is 318 amino acids with an apparent molecular weight of 45-48kD (Tapscott et al., 1988; Dias et al., 1992). The myc epitope tag represents an additional 102 amino acids which adds approximately 12kD to the MyoD protein, yielding a mycMyoD protein which should be approximately 60kD. The apparent molecular weight of mycMyoD is 65kD, slightly higher than predicted.

In addition to being immunoreactive with the MyoD antibody, the mycMyoD protein should be detectable using the anti-myc 9E10 antibody. As expected, a band in the [mycMyoD]P19 lysate was detected in same position as that detected by the MyoD antibody (Fig 5B). No bands were detected in the [MyoD]P19 or [CAT]P19 lanes with the anti-myc 9E10 antibody. The faint band located at approximately 47kD in all lanes of the anti-myc 9E10 immunoblot represents the remaining signal from the tubulin detection which was performed prior to the myc detection. A Western blot detecting tubulin showed that equal amounts of protein were loaded for each sample (Fig. 5C). From these results we concluded that the pCA2 vector expressed the mycMyoD protein, although these experiments did not address the biological function of this fusion protein.

Fig.5 **A mycMyoD protein is expressed from pCA2**

A Western blot of lysates from P19 cells stably transfected with *Pgk-* expression constructs encoding MyoD, mycMyoD and CAT was used for three consecutive immunodetections. In panel A, an anti-MyoD antibody was used for immunodetection. Panel B shows the same blot used for immunodetection with the anti-myc 9E10 antibody. Panel C is the immunodetection performed with an anti-tubulin antibody to indicate relative amounts of protein loaded in each lane. Size markers are as shown in kilodaltons.


A
α MyoD
mycMyoD
MyoD
CAT
199 kD
106
69
43
28
18
B
α myc
mycMyoD
MyoD
CAT
199 kD
106
69
43
28
18
C
α tubulin
mycMyoD
MyoD
CAT
199 kD
106
69
43
28
18

3.4 mycMyoD functions like MyoD

It was essential for future experiments to verify that the mycMyoD protein was fully functional. To determine if mycMyoD could perform the same myogenic activities as MyoD, it was tested in a number of assays. MyoD has been demonstrated to transactivate the cardiac actin (CA) promoter in non- muscle cells, including P19 cells (Skerjanc et al., 1997; Skerjanc and McBurney, 1994; Pari et al., 1991). To test mycMyoD activity in this assay, as well as that of the MyoD lacking UTR, all three expression constructs, Pgk-MyoD, Pgk-MyoD-UTR and Pgk-mycMyoD were transiently transfected into P19 cells. Also co-transfected were the CA-CAT reporter as well as the control plasmid, Pgk-lacZ, to allow for normalization of transfection efficiencies. After transient transfection, cells were assayed for both CAT activity and β-gal activity and the results from three independent assays were compared (Fig. 6A). A transfection containing only the control plasmids showed negligible CAT activity whereas all three *MyoD* expression plasmids were active (Fig. 6A).

MyoD is able to induce formation of muscle following transient transfections although the induction is inefficient since less than 3% of transfected cells become muscle (Skerjanc et al., 1994). To test mycMyoD in this assay, the three MyoD expression constructs used in the previous assay as well as a control plasmid, pGEM, were transiently transfected into P19 cells with the expression plasmid Pgk-lacZ to measure transfection efficiency. The relative efficiencies of muscle formation were determined (Fig. 6B). Spontaneous muscle formation in pGEM transfected controls was less than 0.1% of transfected cells, while MyoD expression constructs induced

myogenesis in 0.75 - 2.0% of transfected cells. The ability of each of the plasmids to induce myogenesis was comparable and confirmed that the mycMyoD fusion protein had retained MyoD activity.

Finally, it was necessary to confirm that mycMyoD would efficiently induce myogenesis in P19 cells following aggregation as described earlier for MyoD. Thus, stable cells lines expressing mycMyoD were generated following co-transfection with Pgk-puromycin. As with the other MyoD stable cell lines, the cells which survived selection and were presumably expressing mycMyoD, showed enhanced myogenesis after aggregation (Fig. 6C). Because the mycMyoD functioned equivalently to MyoD with respect to its myogenic abilities in P19 cells, and because the myc 9E10 antibody was readily available, mycMyoD expression constructs were used in all subsequent experiments.

Fig. 6A **MycMyoD transactivates the human cardiac actin promoter**
Transient assays to examine transactivation of the cardiac actin (CA) promoter by the MyoD and mycMyoD proteins expressed from different constructs were performed. Each of the constructs were co-transfected with CA-CAT and Pgk-lacZ. CAT activity was assayed for each transfection and normalized for transfection efficiency by measuring β-galactosidase activity. Each transfection was repeated three times. The control transfection contained only CA-CAT and Pgk-lacZ.

Transactivation of the cardiac actin promoter by genes encoding MyoD and mycMyoD

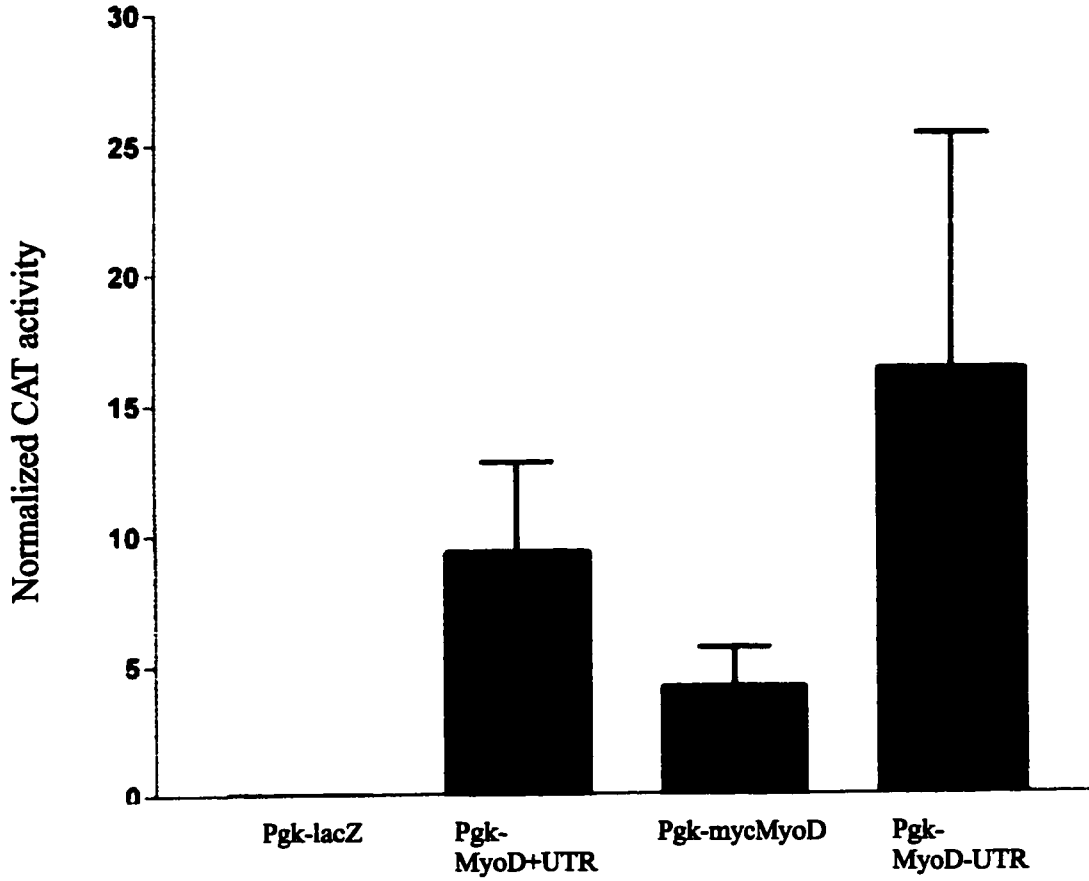


Fig. 6B **MycMyoD induces muscle formation in a transient assay**
MyoD expression plasmids were co-transfected with Pgk-lacZ into P19 cells. 48hrs after transfection 100, 000 cells were plated on coverslips and allowed to adhere. Cells were then stained for either β-galactosidase activity and for myosin heavy chain, and the number staining for each was determined. The myosin heavy chain positive cells is expressed as a fraction of the cells transfected (number of cells β-galactosidase positive). Approximately 3-5% percent of cells were β-galactosidase positive. Each bar represents three independent assays. The control sample included pGEM.

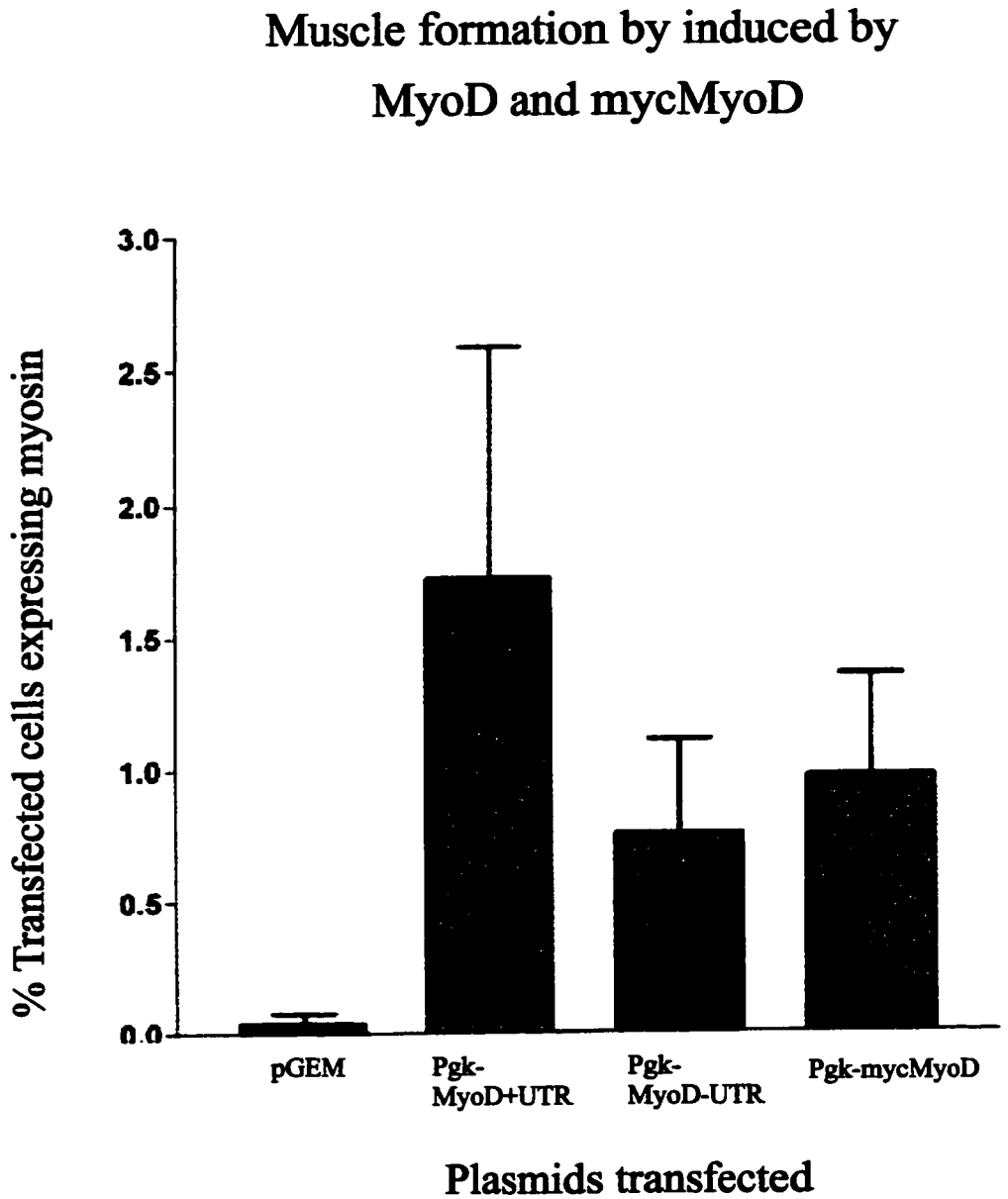
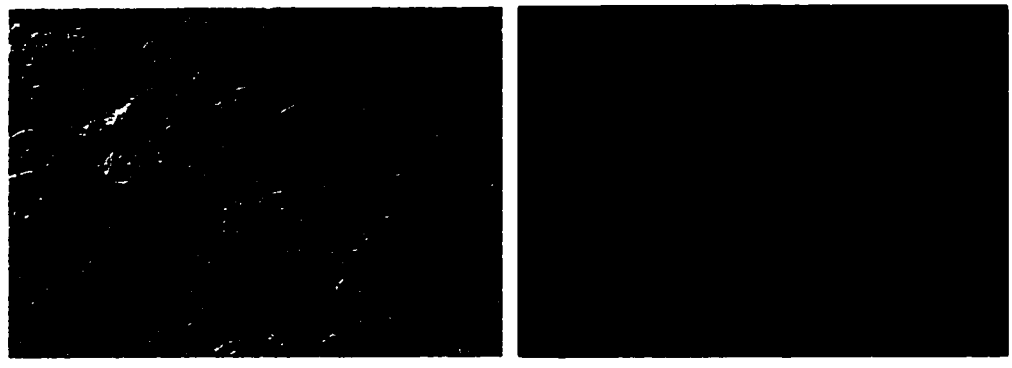

Muscle formation by induced by
MyoD and mycMyoD
% Transfected cells expressing myosin
3.0
2.5
2.0
1.5
1.0
0.5
0.0
pGEM
Pgk-
MyoD+UTR
Pgk-
MyoD-UTR
Pgk-mycMyoD
Plasmids transfected

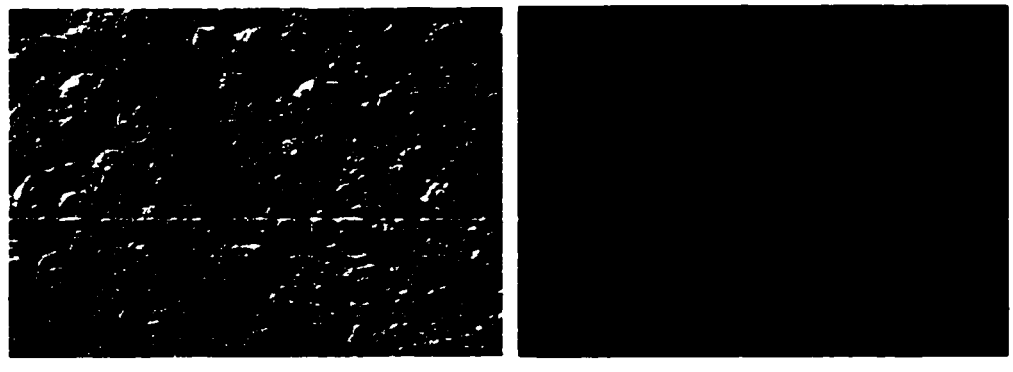
Fig. 6C **MycMyoD induces myogenesis in aggregated P19 cells**
Cell lines stably expressing mycMyoD following co-transfection with Pgk-puro and B17 were generated and cultured as monolayers (non-aggregated) or aggregated for five days. Control cultures were transfected but without the mycMyoD expression vector. Immunofluorescence of both control and [mycMyoD]P19 cells to detect myosin heavy chain was performed for non-aggregated and aggregated cells. Differential interference contrast shows the same field of view as seen under immunofluorescence.

α myosin heavy chain

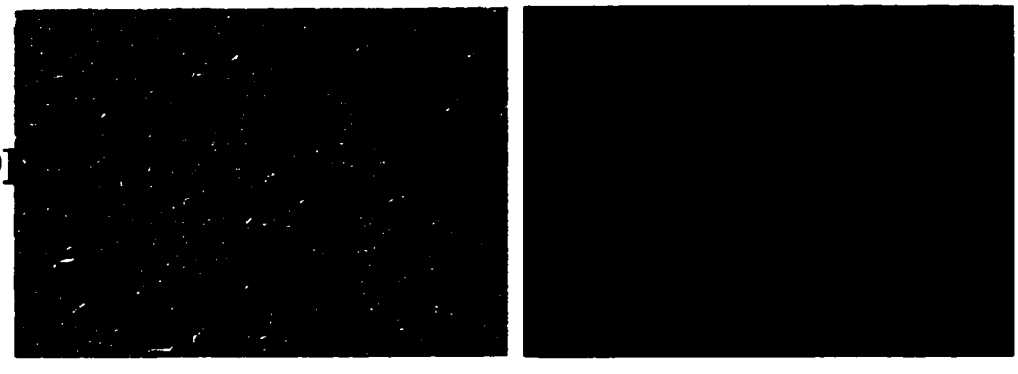
NAgg
P19



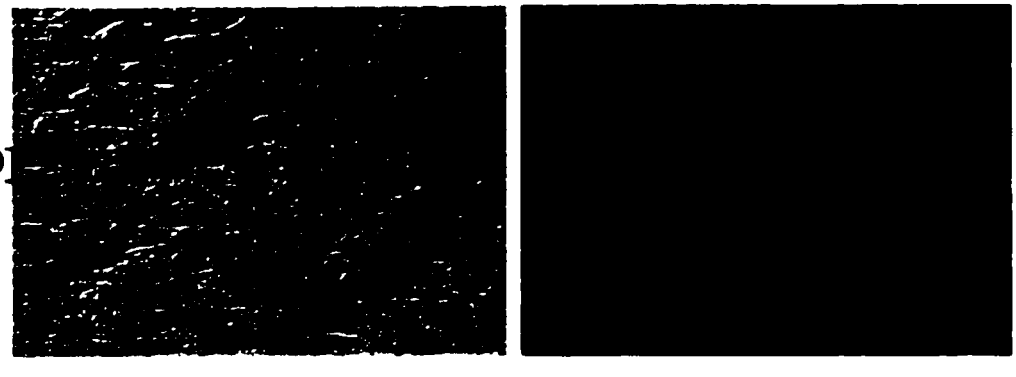
Agg
P19



NAgg
[mycMyoD]
P19



Agg
[mycMyoD]
P19

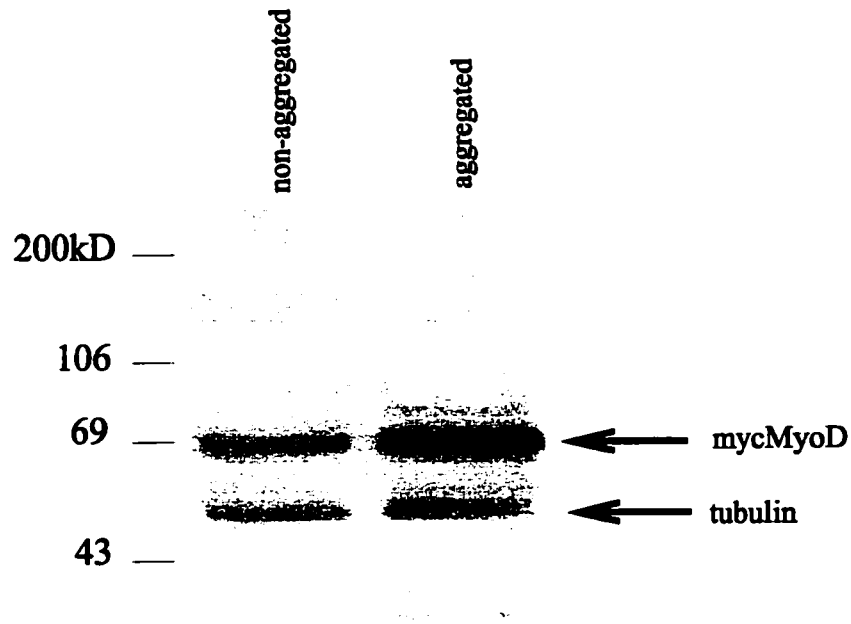


3.5 **Instability of mycMyoD expression and subsequent creation of pCA4**

In the course of verifying the levels of expression of the mycMyoD protein before and after aggregation it was observed that mycMyoD protein expression was sometimes equal but often lost or reduced in the non-aggregated cultures (Fig. 7). After many repeated experiments and further investigation it became apparent that the cause of the inconsistency was unstable expression of the Pgk driven *MyoD* or *mycMyoD* construct. Unstable expression of Pgk-lacZ transfected genes has been previously reported (McBurney et al., 1994). All *MyoD* expression constructs behaved in this manner. The loss of expression was consistent with inactivation of *mycMyoD* gene expression or with complete loss of the gene but without loss of puromycin expression. A variety of methods were tested to prevent the loss of *mycMyoD* expression. One method placed the selectable gene, puromycin, under the control of the CA promoter which would be transactivated only in cells co-expressing mycMyoD, thus forcing drug resistant cells to maintain *mycMyoD* expression (Skerjanc et al., 1994). Numerous attempts were made to quickly aggregate transfected and selected pooled colonies of [mycMyoD]P19 cells in an attempt to gather data before *mycMyoD* expression was lost. Even during the course of an experiment completed 12 days following transfection, *mycMyoD* expression was often reduced or lost. In all cases, inconsistent patterns of expression were observed.

In order to circumvent this problem, the vector pCA4 was constructed. This construct was similar to pCA2 in that the Pgk promoter was used to drive expression of *mycMyoD*, however, downstream of *mycMyoD*, pCA4 included an internal ribosome entry site (IRES) followed by the *β-geo* fusion gene (Fig. 2C)(Mountford et al., 1994).

Fig. 7. **Instability of mycMyoD expression**
P19 cell lines were co-transfected with Pgk-mycMyoD (pCA2) and Pgk-puromycin followed by selection in puromycin. Western blot analysis was performed with the anti-myc antibody. Non-aggregated and aggregated samples are as indicated. Tubulin was used as a loading control. Size markers are shown in kilodaltons.


non-aggregated
aggregated
200kD
106
69
43
mycMyoD
tubulin

The consequence of using an IRES sequence is that a bi-cistronic message is transcribed from which both the *mycMyoD* and the resistance gene, are translated. Such a construct should prevent the loss of *mycMyoD* since this would result in a concomitant loss of the resistance gene.

3.6 pCA4 expresses mycMyoD which induces muscle formation after aggregation

When used to generate stable [mycMyoD] P19 cells, pCA4 expressed mycMyoD which was detected by Western blot in both non-aggregated and aggregated cells (Fig 8A). The mycMyoD protein expressed by both pCA4 and the parental plasmid (pCA2) show the identical set of bands on Western blot in both the non-aggregated or aggregated samples (Fig 8A). The 9E10 antibody does not recognize any proteins from the control [CAT]P19 cells but does recognize an additional myc epitope tagged protein, myc-puromycin, which was co-transfected as a control for myc epitope detection (Fig. 8A). The resolution of the gel shown in Fig. 8A was sufficient to reveal that mycMyoD runs as a doublet as does MyoD. The doublet likely represents the hypophosphorylated (faster migrating) and hyperphosphorylated (slower migrating) forms of MyoD (Lassar et al., 1991; Tapscott et al., 1988). This doublet is only apparent in Western blot assays with sufficient resolution.

As in other *MyoD* expressing lines, the aggregated [mycMyoD]P19 cells generated from pCA4 differentiated into skeletal muscle and expressed myosin heavy chain whereas the non-aggregated cells showed little of this muscle specific marker (Fig 8B). The pCA4 plasmid was designed to co-express mycMyoD protein with the β-geo fusion protein. Lysates of pCA4 stable lines examined by Western blot with an anti-β-

galactosidase antibody showed co-expression of mycMyoD and β-geo (Fig. 9.). Both the parental plasmid, Pgk-IRES-βgeo, and pCA4 expressed β-geo while only pCA4 expressed mycMyoD (Fig. 9.).

Despite the use of pCA4, we found that some cells failed to express mycMyoD protein. It is unknown how this occurs but may arise from a deletion or mutation of the *mycMyoD* gene. The colonies that did express *mycMyoD* made muscle at varying efficiencies, some clones made very little, while others induced myogenesis at a much greater efficiency (50-70%) than the 30% originally documented (Skerjanc et al., 1994). The colonies which expressed mycMyoD protein after stable transfection with pCA4 expressed mycMyoD protein for longer periods of time than cultures transfected with pCA2, although they eventually lost mycMyoDexpression after extended culturing periods despite continued selection in G418. Duration of expression and expression stability was further enhanced by co-transfection of pCA2 and Pgk-puromycin into [mycMyoD]P19 cells. These cells were used for experiments described in section 3.9 and subsequent sections.

Immunofluorescence of stable [mycMyoD]P19 cells showed that β-galactosidase (β-gal) positive cells formed myocytes, as detected by myosin heavy chain (MHC) staining (Fig. 10 - empty arrows). Both β-geo and MHC proteins were detected in the cytoplasm. While the majority of cells expressed β-geo a few did not (Fig 10 - filled arrows). These may be cells which have enough β-geo to be resistant to G418, but not protein enough for β-gal detection. In addition, not all of the β-gal positive cells in aggregates stained positively for myosin heavy chain (MHC) (Fig. 10 - hatched arrows), although all of the cells that did stain positive for MHC were also β-gal positive.

Fig. 8 **pCA4 expresses mycMyoD protein which induces muscle formation following aggregation**

Western blot analysis was used to examine the mycMyoD expression from both pCA2 and pCA4 stable cultures. The control cultures were generated by co-transfecting Pgk-CAT and Pgk-neo and selecting for resistant cells. pCA2 stable lines were also co-transfected with Pgk-neo and likewise selected. pCA4 stable lines were made to express pCA4 alone or pCA4 and Pgk-mycpuro as a internal control for myc epitope detection. Non-aggregated (N) or aggregated (A) cultured cells were harvested from all four cell lines and examined using the anti-myc antibody (Panel A) or an anti-myosin heavy chain antibody (Panel B). The anti-myosin heavy chain appears lower than the actual molecular weight, probably due to protein degradation. Tubulin was used as a loading control. Size markers are as shown in kilodaltons.

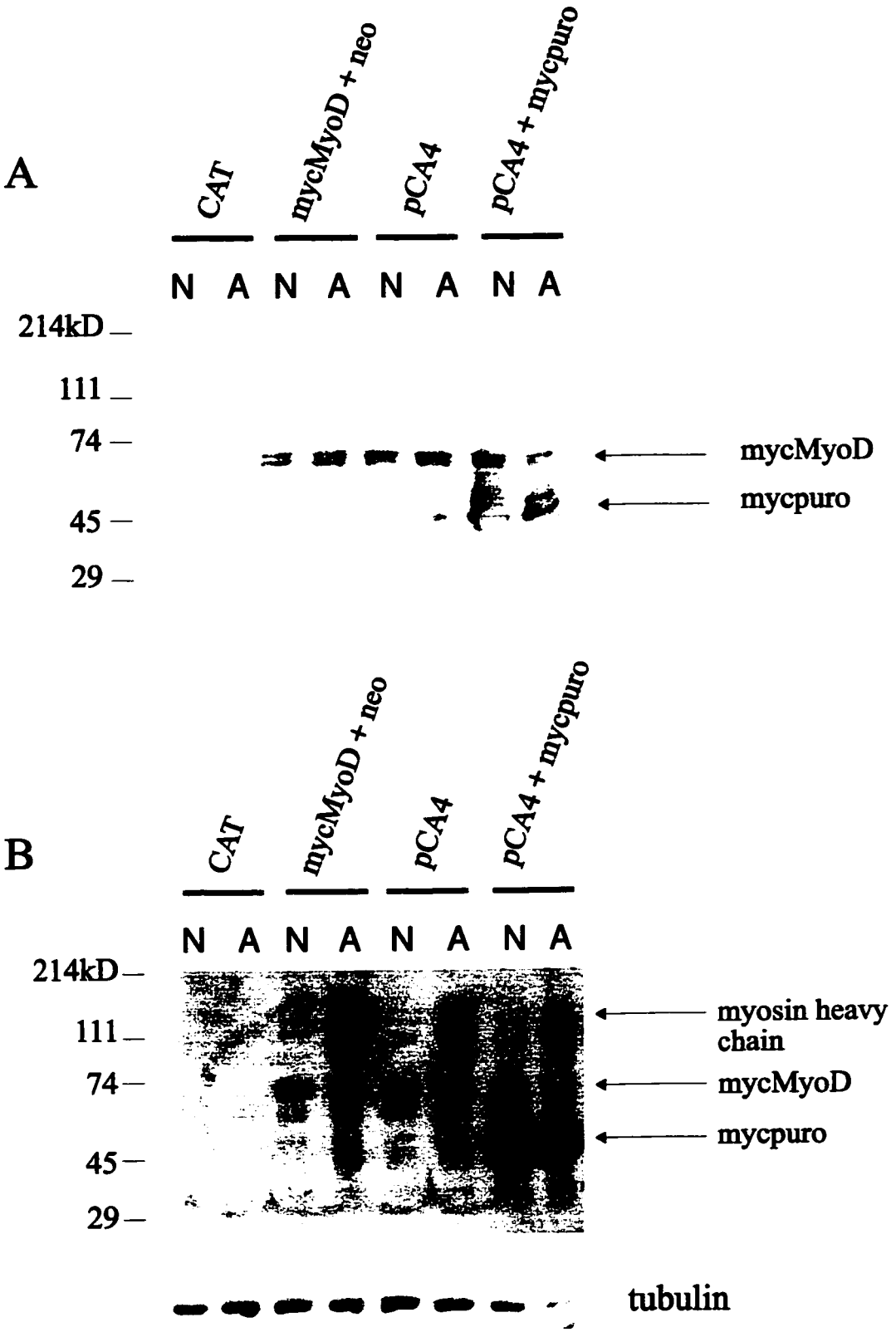

A
CAT
mycMyoD + neo
pCA4
pCA4 + mycpuro
N A N A N A N A
214kD
111
74
45
29
mycMyoD
mycpuro
B
CAT
mycMyoD + neo
pCA4
pCA4 + mycpuro
N A N A N A N A
214kD
111
74
45
29
myosin heavy chain
mycMyoD
mycpuro
tubulin

Fig. 9 **pCA4 co-expresses mycMyoD and β-geo proteins**
Western blot analysis was used to examine the expression of mycMyoD and β-geo from pCA4 and the parental plasmid, Pgk-IRES-βgeo. Lysates of non-aggregated and aggregated cells stably expressing either pCA4 or Pgk-IRES-βgeo were loaded in each lane as labelled. Blots were first analysed with an anti-β-galactosidase antibody, then with the anti-myc 9E10 and finally with an anti-tubulin. Markers are as shown in kilodaltons.

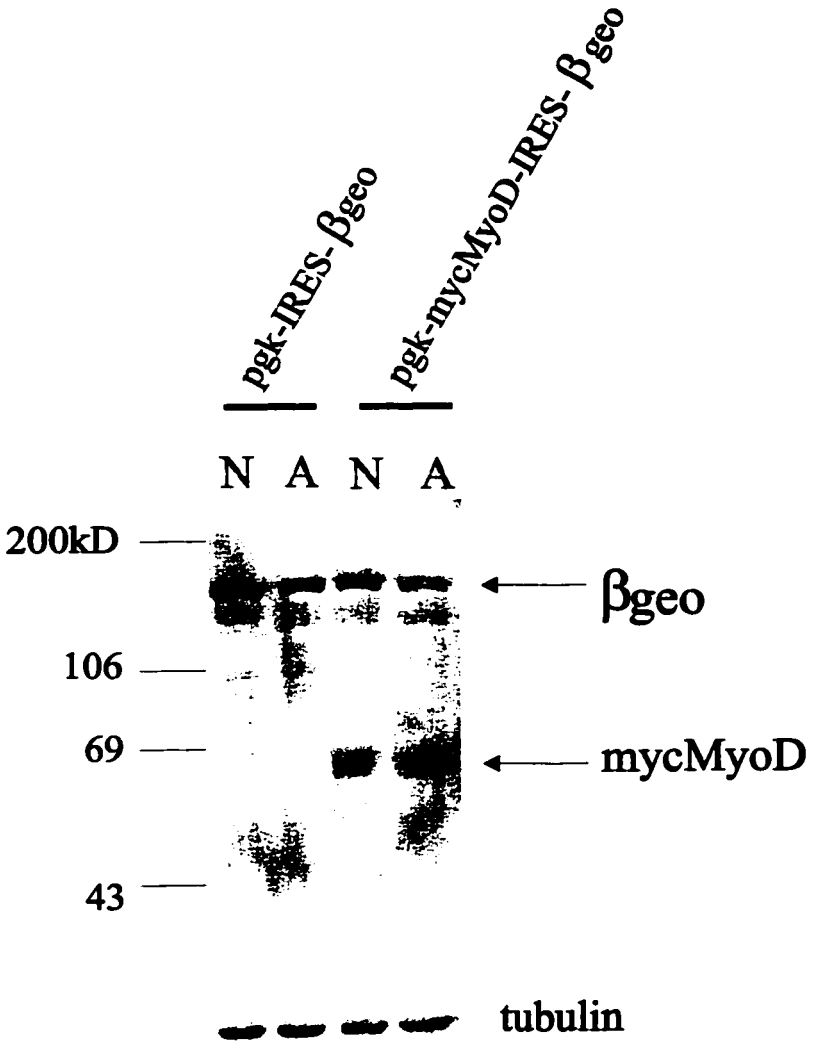
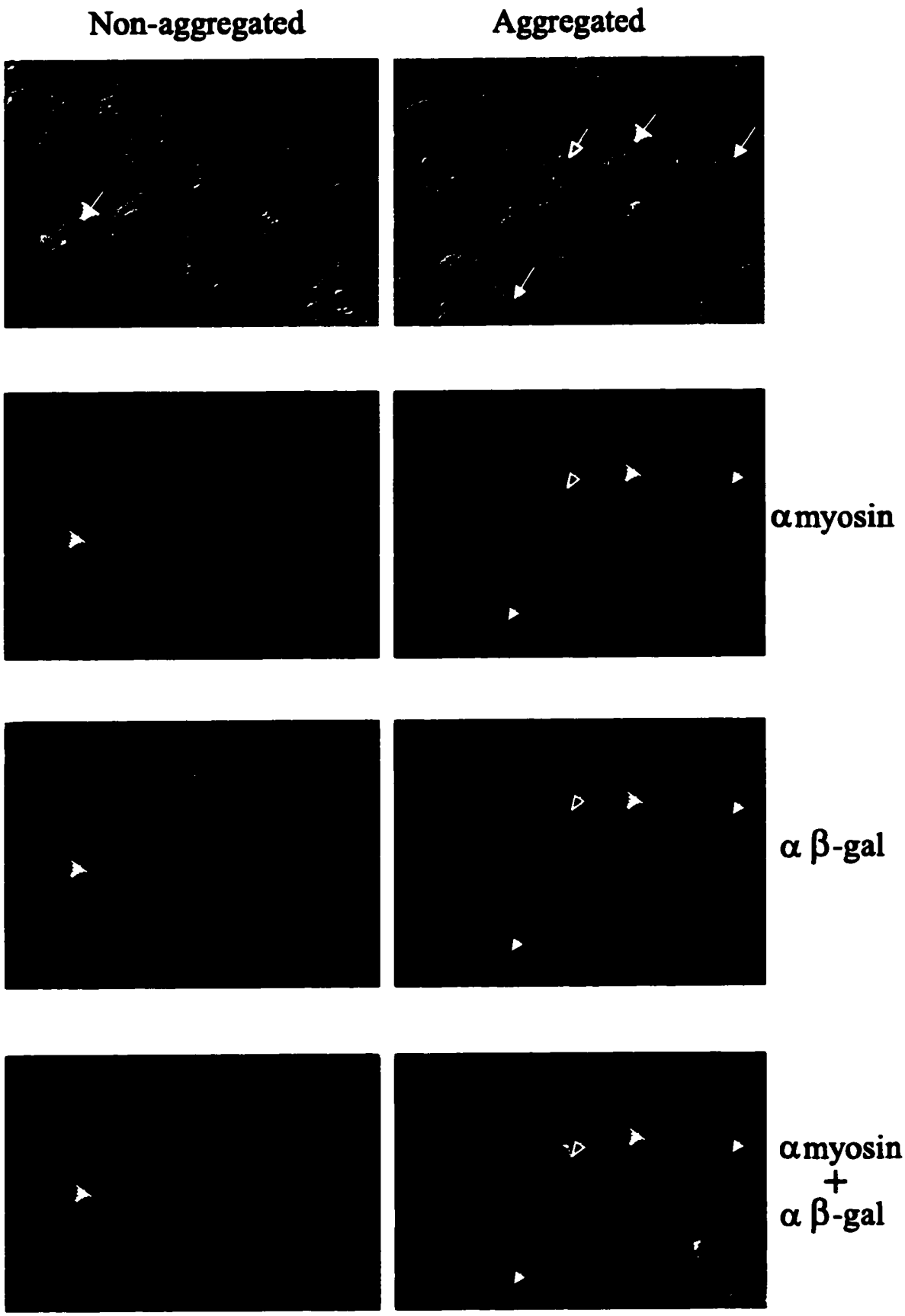

pgk-IRES-βgeo
pgk-mycMyoD-IRES-βgeo
N A N A
200kD
106
69
43
βgeo
mycMyoD
tubulin

Fig. 10 **Co-expression of myosin heavy chain and β-geo**

[mycMyoD]P19 cells were stained for myosin heavy chain and β-galactosidase. Panels are as labelled. Both non-aggregated and aggregated cells can be seen to express β-geo. Myosin heavy chain (MHC) positive cells are seen in aggregated cells. Double labelling shows those cells which are positive for both myosin heavy chain and β-geo. Solid arrows indicate cells which do not stain for β-geo or MHC. Empty arrows indicate those cells which stain positive for both β-geo and MHC and hatched arrows indicate those cells which stain positive only for β-geo. Differential interference contrast shows the same cells as viewed by immunofluorescence. Bar = 40μm.


Non-aggregated
Aggregated
αmyosin
α β-gal
αmyosin
+
α β-gal

3.7 MycMyoD protein levels do not increase after aggregation

Since an increase in protein levels after aggregation could lead to enhanced muscle formation, the level of mycMyoD protein was investigated. The level of protein did not increase after aggregation (Fig. 11). Indeed, the amount of mycMyoD protein remained relatively stable during the five day course in either the non-aggregated or aggregated cells, indicating that an increase in protein was not responsible for myogenesis following aggregation.

3.8 MycMyoD is localized in the nucleus

Although mycMyoD protein levels remained constant, the activity of MyoD can be regulated by localization of the protein in the cells. For example, in Xenopus embryos, XMyoD is specifically retained in the cytoplasm (Rupp et al., 1994). To determine whether such regulation inhibits myogenesis in the non-aggregated [MycMyoD]P19 cells, immunofluorescence studies of mycMyoD localization before and after aggregation were performed. In untransfected control P19 cells, only background signal was detected using the anti-myc 9E10 antibody to detect mycMyoD or an anti-β-gal antibody for β-geo detection (Fig. 12A). Cells which stably expressed mycMyoD, however, showed myc staining in the nuclei of both non-aggregated and aggregated cells (Fig. 12B). In these cells, β-geo staining was also apparent and appeared in the cytoplasm. These results indicated that in P19 cells, mycMyoD is not regulated by differential localization. It is of interest to note that in both the non-aggregated and aggregated culture conditions cells were found which were β-geo positive but did not exhibit detectable levels of mycMyoD protein (Fig. 12C).

Fig. 11 **The level of mycMyoD protein does not increase following aggregation** Western blot analysis of non-aggregated and aggregated [mycMyoD]P19 cells. Cells were harvested at the days shown and mycMyoD was detected using the anti-myc 9E10 antibody. An anti-tubulin antibody was used to detect tubulin to gauge relative amounts of protein loaded. Protein size markers are as shown and are in kilodaltons.

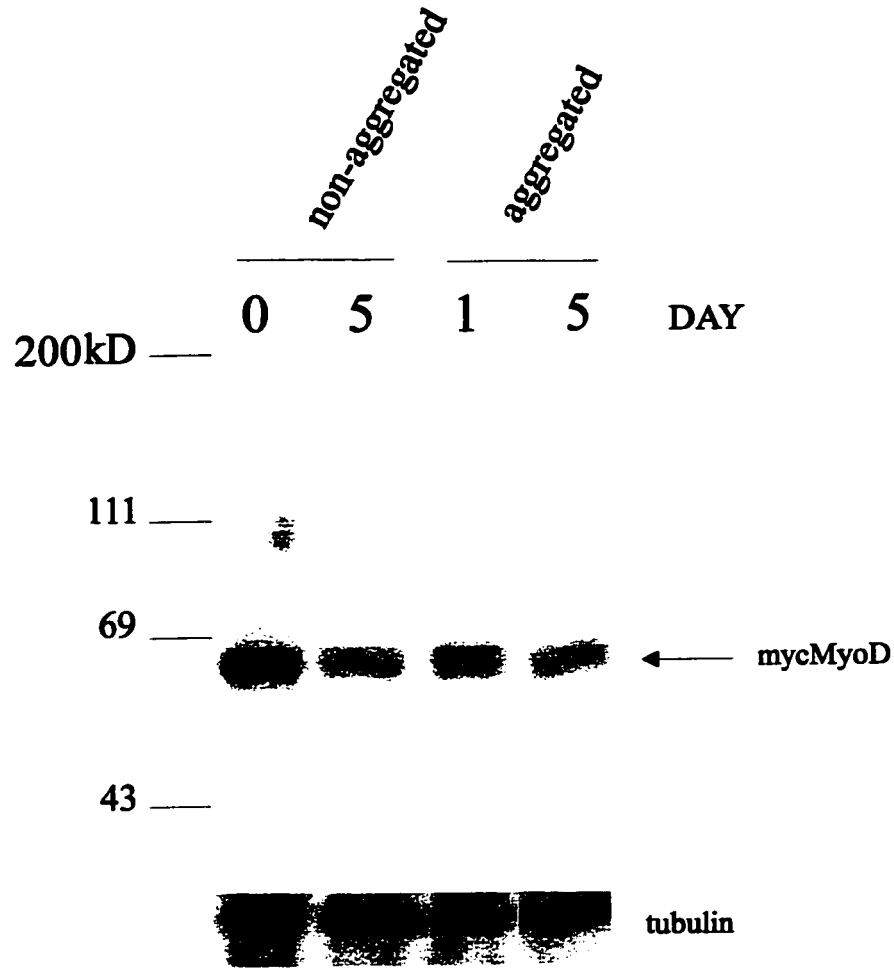
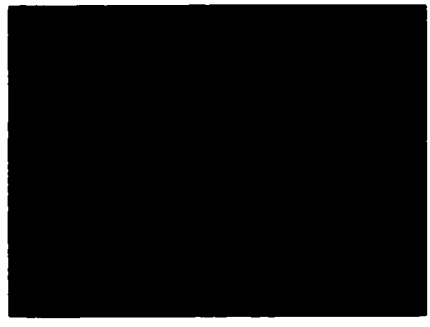

non-aggregated
aggregated
0
5
1
5
DAY
200kD
111
69
43
mycMyoD
tubulin

Fig. 12 **MycMyoD is located in the nucleus before and after aggregation**
Immunofluorescent detection of mycMyoD and β-geo in control P19 cells and in [mycMyoD]P19 cells. Panel A shows untransfected P19 cells stained for the myc epitope and β-geo. Panel B and C show the same staining of pCA4 transfected cells. Differential interference contrast microscopy in each panel shows the same cells as viewed by immunofluorescence. Bar = 20μm.

A

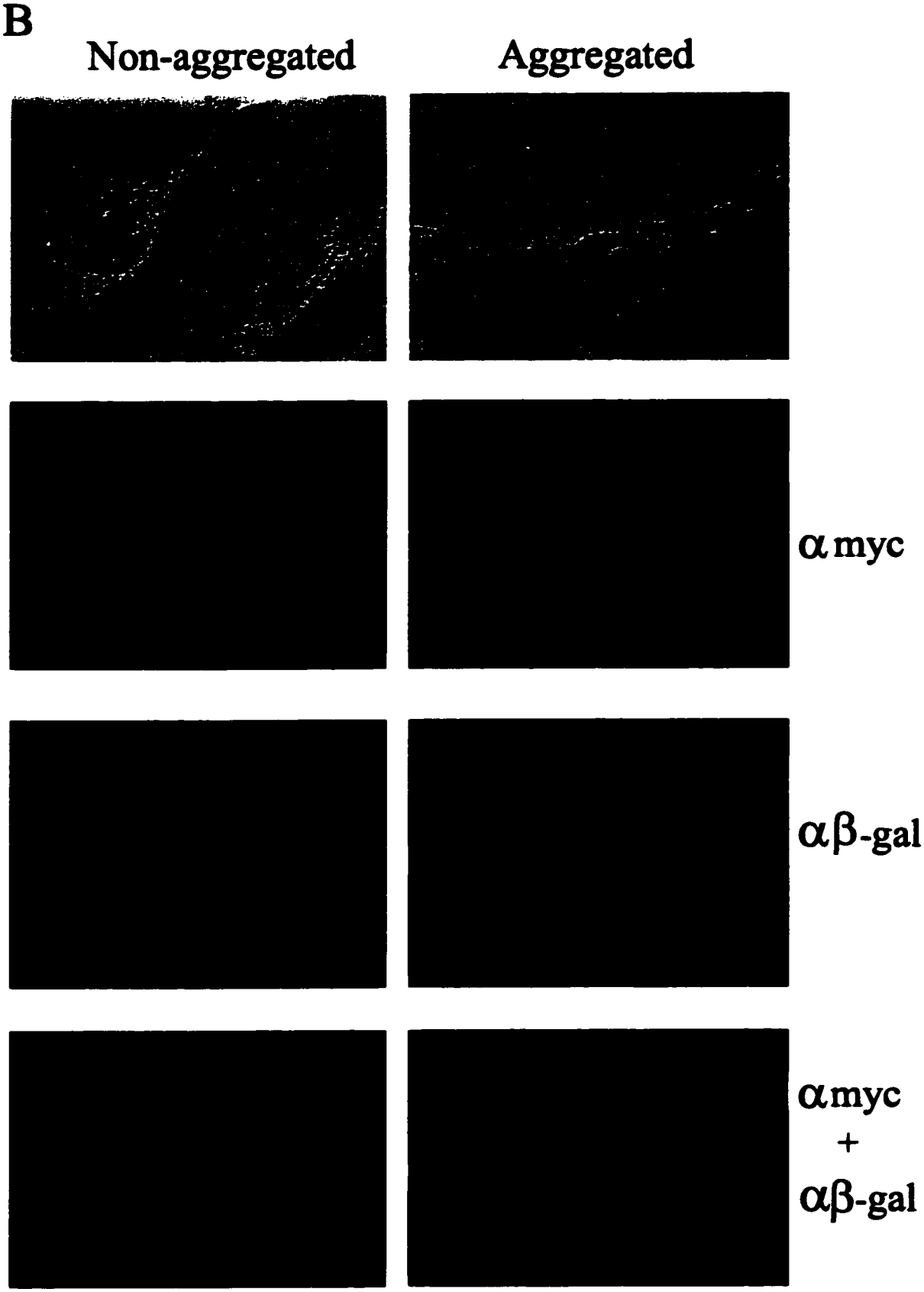
α myosin



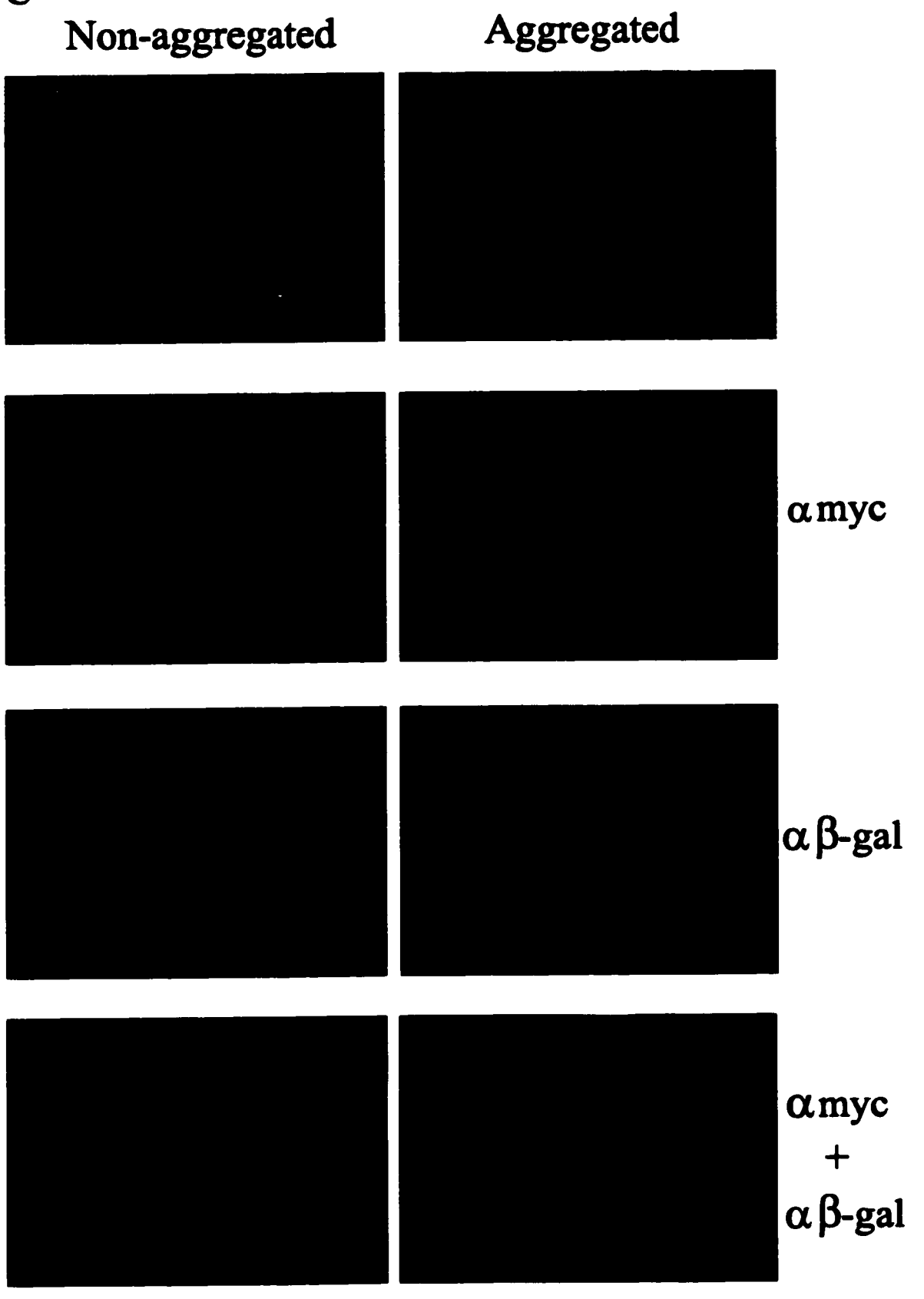
α β-gal

α myosin
+
α β-gal

B



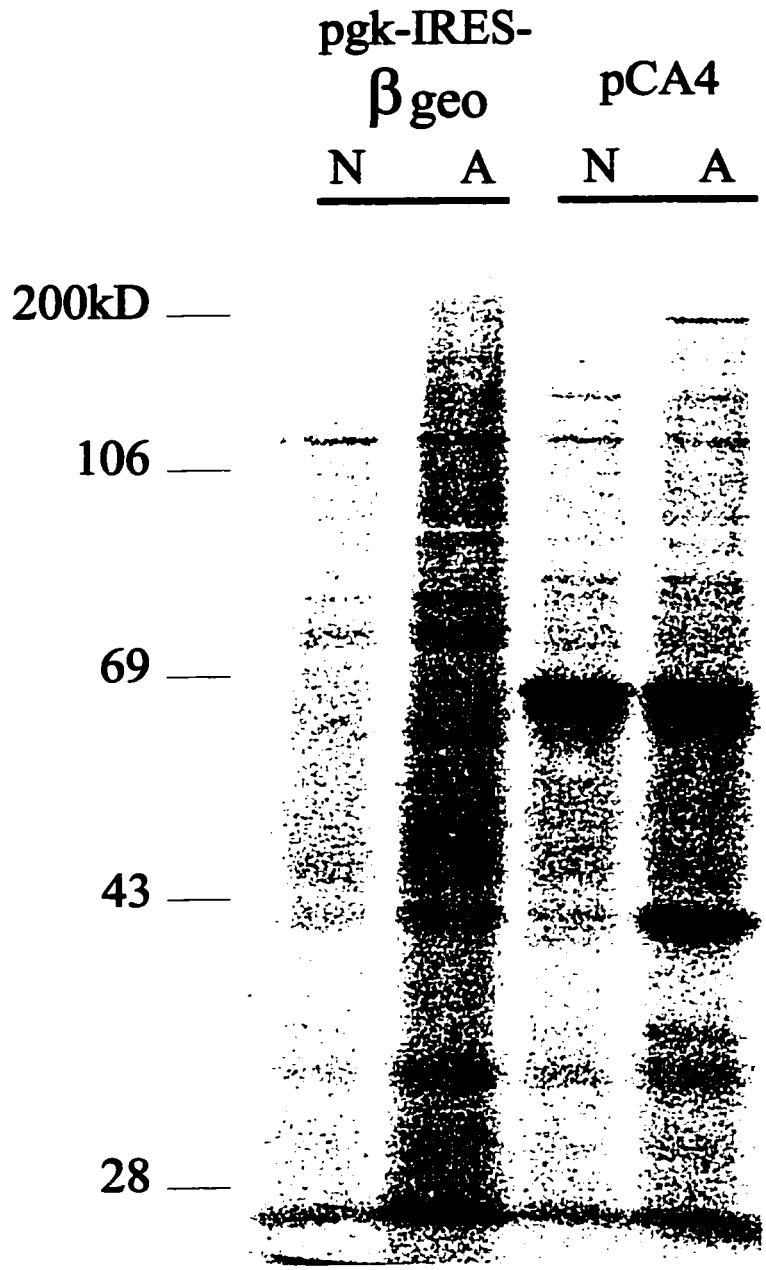
C



3.9 ^{35}S Met *in vivo* labelling and immunoprecipitation shows no obvious changes in binding partners

Next investigated was the possibility that MyoD activity was being regulated by a change in binding partners following aggregation. A number of proteins have been demonstrated to regulate the activity of MyoD following binding, including E-proteins (Lassar et al., 1991), Mos (Lenormand et al., 1997), members of the MEF2 family (reviewed in Olson, 1992.), pRb (Gu et al., 1993) and p300 (Sartorelli et al., 1997). In order to examine binding partners of MyoD, control cells stably transfected with Pgk-IRES-geo and cells transfected with pCA4 were *in vivo* labelled with [^{35}S] methionine. Lysates from both non-aggregated and aggregated control and mycMyoD expressing cells were immunoprecipitated with the anti-myc antibody and separated by SDS PAGE (Fig. 13). The mycMyoD protein was detected as a doublet in the pCA4 transfected cells. In the non-aggregated [mycMyoD]P19 cells, no clear co-precipitated proteins could be identified. Upon aggregation, two novel bands of approximately 43kD and 200kD appeared. The approximate sizes of these two bands correspond to the sizes of the muscle structural proteins, actin and myosin (respectively). It is believed that these proteins, which are in abundance in muscle cells, are insoluble contaminants that become trapped in the immunoprecipitate. The sizes of some of the proteins which are known MyoD partners include: E-proteins ≈73kD; p300 ≈ 300kD, pRb ≈ 109kD, MEF2 family members ≈ 40 - 60kD. No obvious bands appeared near these sizes. Thus, no evidence for changing binding partners was seen, although the inability to definitively identify proteins produced inconclusive results.

Fig. 13. ***In vivo* labelling indicates no obvious changes in binding partners**
Control cells or [mycMyoD]P19 cells were cultured either in monolayers or as aggregates for five days and were labelled in vivo with ^{35}S-methionine. Following labelling, total cell extracts were immunoprecipitated with the anti-myc 9E10 antibody. Immunoprecipitates were separated by SDS-PAGE the gel dried and exposed to a phosphorimager for visualization. N = non-aggregated A= aggregated. Protein size markers are as indicated and are in kilodaltons.


pgk-IRES-
βgeo
pCA4
N
A
N
A
200kD
106
69
43
28

3.10 **The amount of E2A proteins does not increase upon aggregation**

In an attempt to address the question of binding partners in a more specific fashion, the level of E2A proteins before and after aggregation was examined. Both E12 and E47, the protein products of the *E2A* gene, run at approximately 73kD on an SDS-PAGE gel (Vierra et al., 1994; Aronheim et al., 1993), making their separation difficult. The levels of E2A protein expression remained unchanged in P19 control or [mycMyoD]P19 cells, regardless of whether cells were non-aggregated or aggregated (Fig. 14).

3.11 **MycMyoD is bound to E2A proteins before and after aggregation**

As previously mentioned the binding of certain proteins can regulate the activity of MyoD. Specifically, the ability of E2A proteins to bind MyoD is modulated by such proteins as the Mos kinase (Lenormand et al., 1997), Id (Benezra et al., 1990), and Mtwist (Spicer et al., 1997). Since the binding of the E2A proteins to MyoD is required for transcription of muscle specific genes, the heterodimerization state of these proteins was investigated. To determine whether mycMyoD was bound to either E12/E47, the E-proteins were immunoprecipitated with an anti-E protein antibody. These immunoprecipitates were then analysed by Western blot to detect whether mycMyoD was complexed with E12/E47. In fact, mycMyoD was detected in immunoprecipitates from both non-aggregated and aggregated cells (Fig. 15). No corresponding band was visible in the corresponding P19 control lane nor was any mycMyoD band apparent in the mock immunoprecipitate. Likewise an immunoprecipitation with an anti-Bcl2 antibody showed no mycMyoD band indicating that the immunoprecipitation of

mycMyoD with E proteins represented a true co-precipitation. The same amount of lysate was loaded into the lanes containing total protein as was used in the immunoprecipitation thus allowing an approximate determination of the fraction of mycMyoD which was able to bind E12/E47 proteins. Comparison of these lanes indicates that less than half, perhaps as little as one tenth of the total mycMyoD was bound to E2A proteins. Since the E2A protein antibody also recognizes ITF-1, which is approximately the same size as E12/E47, it is possible that a fraction of the mycMyoD detected may have resided in complexes with this factor.

Fig. 14 **The level of E2A proteins does not increase during aggregation or differentiation**

Western blot analysis was performed on cell lysates from control P19 cells or [mycMyoD]P19 cells. An anti-E-protein antibody was used which recognizes both E12 and E47 as well as ITF-1.Tubulin was detected with an anti-tubulin antibody following the E-protein detection to allow comparison of protein loading. N= non-aggregated A= aggregated. Protein size markers are as shown and are given in kilodaltons.

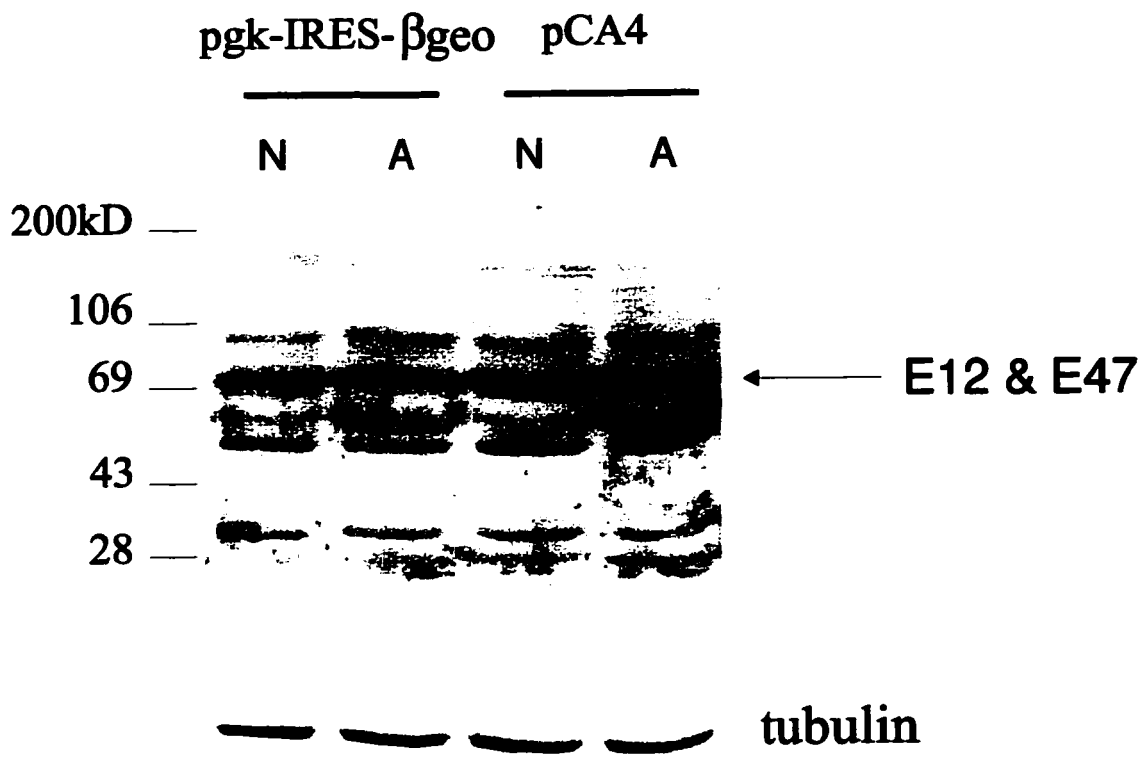
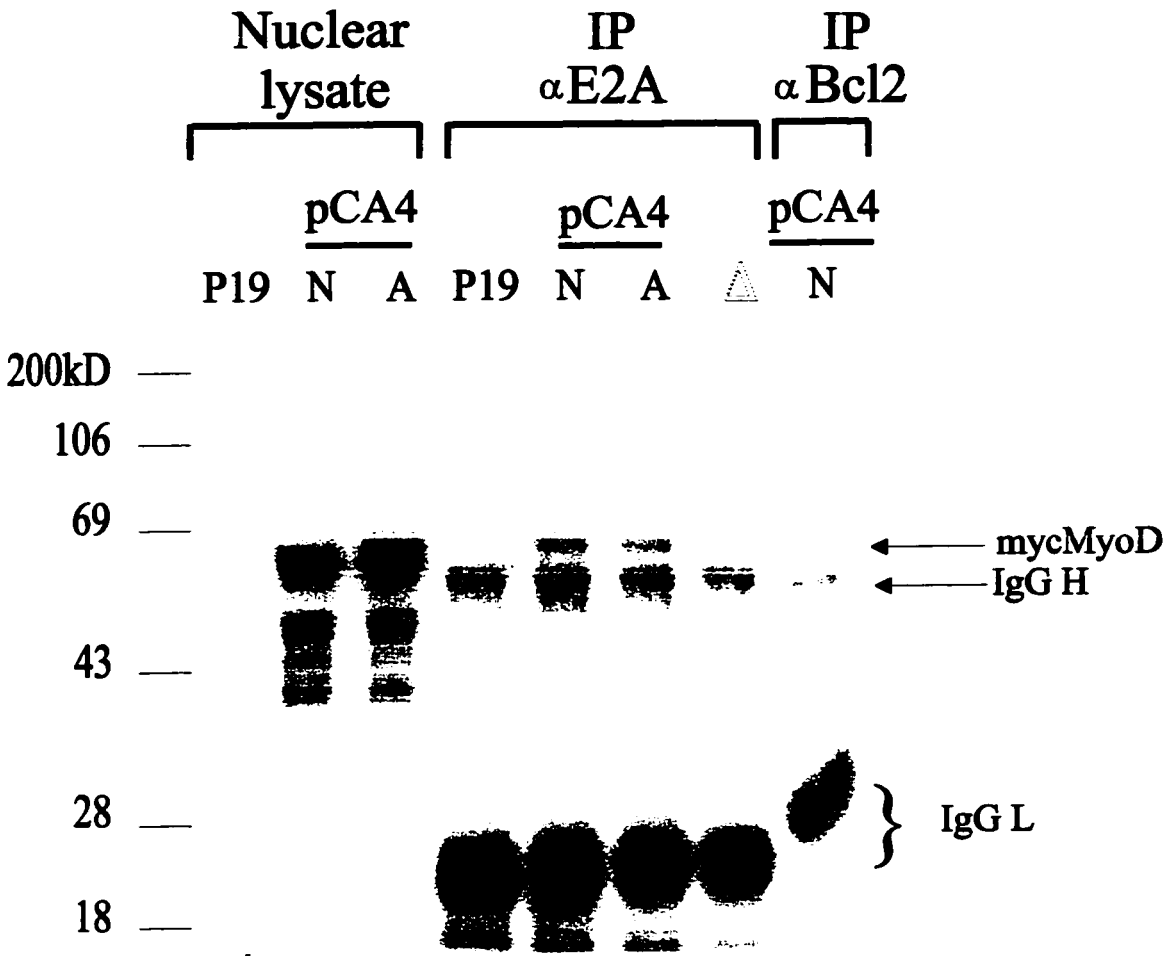

pgk-IRES-βgeo
pCA4
N
A
N
A
200kD
106
69
43
28
E12 & E47
tubulin

Fig. 15 **E2A proteins are bound to mycMyoD in both non-aggregated and aggregated P19 cells**

Immunoprecipitation of E-proteins followed by Western blot analysis. Nuclear lysates from P19 cells or [mycMyoD]P19 cells were used in an immunoprecipitation of E-proteins with an anti-E protein antibody. Control immunoprecipitations were carried out which used either no lysate or used an anti-Bcl2 antibody . Immunoprecipitates and nuclear lysate (the same amount used in the immunoprecipitation) were analysed by Western blot. Immunodetection of mycMyoD was done with the anti-myc 9E10 antibody. The light and heavy chains of the anti-E protein antibody are also apparent. N= non-aggregated A=aggregated Δ = no lysate. Protein markers are as shown and are given in kilodaltons.


Nuclear lysate
IP αE2A
IP αBcl2
pCA4
pCA4
pCA4
P19 N A P19 N A △ N
200kD
106
69
43
28
18
mycMyoD
IgG H
IgG L

3.12 MycMyoD from both non-aggregated and aggregated cells is able to bind DNA

Following heterodimerization with an E2A protein, MyoD heterodimers must bind DNA to mediate transcription of muscle specific genes. This step is highly regulated. PKA/PKC both inhibit DNA binding by MRF4, although this inhibition occurs indirectly (Hardy et al., 1993). FGF inhibits myogenin's ability to bind DNA through a conserved PKC site in its DNA binding domain (Li et al., 1992b). The growth factors TGF-β and bFGF, inhibit MyoD from remodelling chromatin at its binding sites, effectively preventing MyoD from binding DNA (Gerber et al., 1997). Thus, it was of interest to determine whether mycMyoD was prevented from binding DNA in the non-aggregated cells. To address this, electrophoretic mobility shift assays (EMSA) were performed using an E-box containing sequence. The radio-labelled oligonucleotide used in the mobility shift assays was the MEF-1 oligonucleotide, a 25 base pair oligonucleotide containing a high affinity E-box (Buskin and Hauschka, 1989). This sequence is known to serve as a MyoD binding site (Lassar et al., 1989). Mobility shift binding assays with this oligonucleotide did not show any binding complexes in P19 lysates, non-aggregated or aggregated (Fig. 16A). The sample containing wild-type labelled oligo and lysates from aggregated [mycMyoD]P19 exhibited a single band of retarded mobility indicating a DNA-protein complex. The protein binding to the oligo appeared to be binding to the E-box in a specific fashion since excess unlabelled wild-type oligo abolished binding while the complex did not form on a mutant oligo nor was an excess of mutant oligo able to abolish binding to labelled wild-type oligo (Fig. 16A). Once it was determined that a

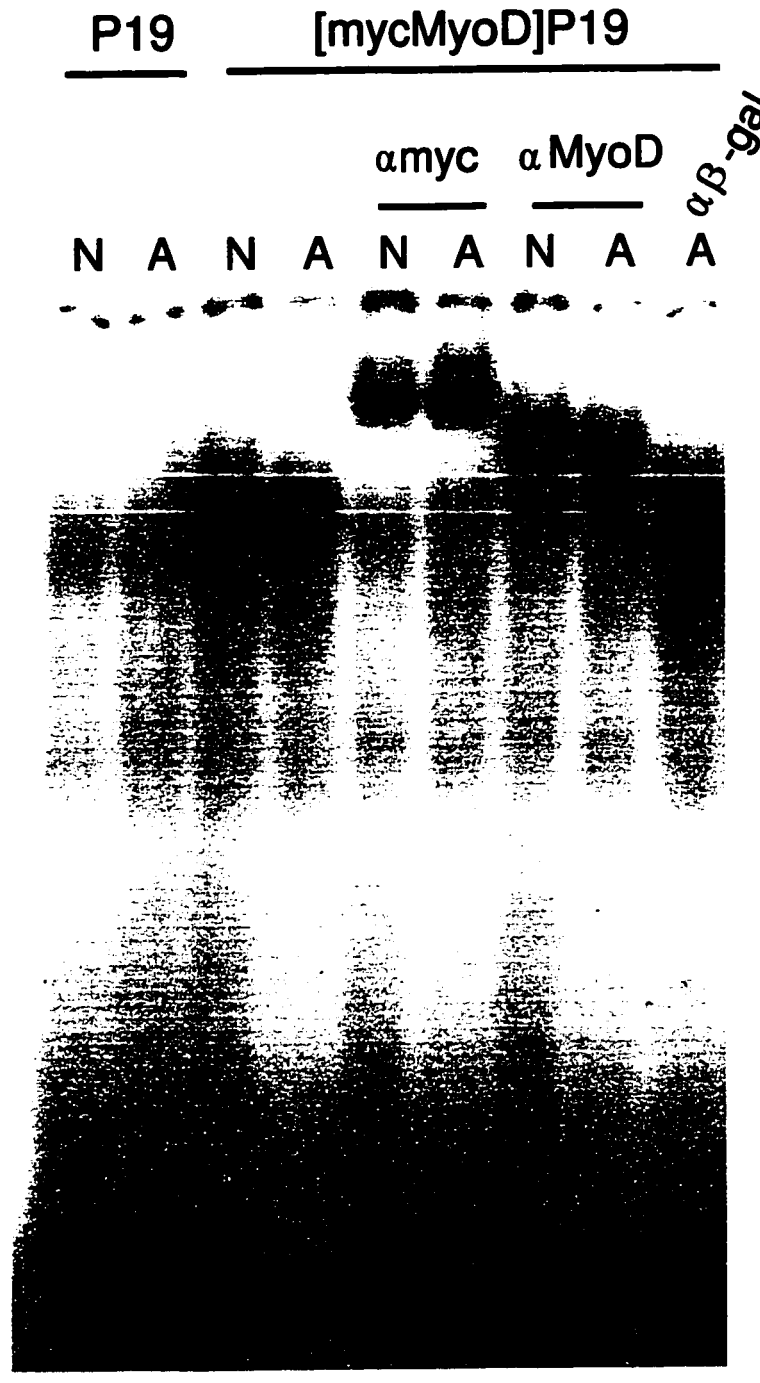
MEF-1 binding complex was present in aggregated [mycMyoD]P19 cells, further mobility shift assays were performed to determine whether a similar complex was found in non-aggregated [mycMyoD]P19 cells and whether the complex contained MyoD. As seen in Fig. 16B, the same complex appeared in samples prepared from extracts of non-aggregated [mycMyoD]P19 cells. This complex was supershifted in both the non-aggregated and aggregated samples with the addition of the anti-myc 9E10 antibody. Similarly, the MyoD 5.8A antibody also shifted the complex while a β-galactosidase antibody did not. Thus, the complex in both the non-aggregated and aggregated samples contains mycMyoD. This suggested that mycMyoD is able to bind to E-box containing sequences *in vitro* in both non-aggregated and aggregated cells and that the level of regulation was not at the level of mycMyoD's ability to bind DNA.

Fig. 16A **A complex from aggregated [mycMyoD]P19 cells binds specifically to the E-box of the MEF-1 oligo.**

Nuclear lysates from either non-aggregated or aggregated control P19 cells or nuclear lysate from aggregated [mycMyoD] cells were incubated with a 25 bp ^{32}P end-labelled wild-type (hot wt) MEF-1 oligo. The MEF-1 oligonucleotide contains a high affinity E-box. A ^{32}P labelled mutant version (hot mutant) of the MEF-1 oligonucleotide was also used and it has the same sequence except with six mutated base pairs spanning the E-box binding site. Specificity of binding was analysed by mixing in ten fold excess unlabelled wild-type MEF-1 (10X cold wt) or ten fold excess unlabelled mutant MEF-1 (10X cold mutant) along with the labelled wild-type MEF-1 oligonucleotide. Following incubation in the appropriate binding mix (containing 1μg poly (dI-dC)) with relevant oligonucleotides, DNA-protein complexes were resolved on a 5% poly-acrylamide gel and visualised using a phosphorimager. Unbound labelled oligonucleotide was run off the bottom of the gel.


P19
[mycMyoD]P19
N
A
hot wt
hot wt + 10X cold wt
hot wt + 10X cold mutant
hot mutant

Fig. 16B **A mycMyoD containing complex in both non-aggregated and aggregated [mycMyoD]P19 cells binds to the MEF-1 oligonucleotide.** A mobility shift of the MEF-1 oligonucleotide with a complex from non-aggregated and aggregated [mycMyoD]P19 cells. Nuclear lysates from either non-aggregated (N) or aggregated (A) control P19 cells or nuclear lysate from non-aggregated (N) or aggregated (A) [mycMyoD] cells were incubated with a 25 nucleotide ^{32}P- end-labelled wild-type MEF-1 oligo. The complex was supershifted with the addition of either the anti-myc 9E10 antibody or the 5.8A anti-MyoD antibody. The control antibody was an anti-βgalactosidase antibody. Unbound labelled oligonucleotide was run off the bottom of the gel.


P19
[mycMyoD]P19
αmyc
αMyoD
αβ-gal
N A N A N A N A A

Chapter Four

Discussion

4.1 Summary of findings

This thesis was aimed at studying the mechanism whereby aggregation induces myogenesis in [MyoD]P19 or [mycMyoD]P19 cells. The results obtained in this thesis can be summarized as follows. 1) The amount of MycMyoD protein does not increase following aggregation. 2) MycMyoD is found in the nucleus both before and after aggregation. 3) In both non-aggregated and aggregated cells, mycMyoD is bound to an E2A protein. 4) MycMyoD is able to bind to an E-box containing oligonucleotide in both cell states. These results indicate that in P19 cells, mycMyoD is likely regulated at the point of DNA binding or at the point of initiating transcription once bound to DNA.

4.2 Use of MycMyoD and pCA4

Initial observations of MyoD induced myogenesis in P19 cells demonstrated that non-aggregated [MyoD]P19 cells possessed characteristics of undifferentiated stem cells and myoblasts, while after aggregation these cells exhibited characteristics of skeletal muscle (Skerjanc et al., 1994). Preliminary results suggested that MyoD protein levels increased during aggregation; however, it became apparent that MyoD expression was rapidly lost in cultures of transfected cells. These difficulties made it essential that MyoD be expressed well in all cells during the course of each experiment and that the protein be readily detected. To assist in the detection of MyoD, a N-terminal fusion with six myc tags was made. Construction and use of mycMyoD proved to be advantageous

since the myc epitope tag allowed the detection of the fusion protein with use of the 9E10 monoclonal antibody which is relatively plentiful, inexpensive and reliable. An important concern with such epitope tags is that the modified protein may behave differently from the wild-type. The conformation of the tagged protein may become altered rendering it inactive. Alternatively, the epitope tag may sterically inhibit normal interactions with other molecules. However, evidence presented suggests that no such problems arose since the mycMyoD had little apparent difference in activity from the untagged protein. This was fortunate considering that the myc tag represents a length of amino acids roughly 1/3 of the original protein.

Expression of mycMyoD in P19 cells was unstable and only through the use of a bicistronic mRNA carrying the IRES sequence and βgeo fusion protein did expression of mycMyoD become consistent. The IRES is a conserved sequence found in a number of viruses (Jang et al., 1990) and is exploited in expression vectors to link the expression of two genes through the formation of a bicistronic message. The benefit of the IRES sequence followed by the βgeo fusion gene was two fold. A higher proportion of cells was found to express mycMyoD than with a co-transfection strategy which utilized a separate drug selection plasmid. Previously, Skerjanc et al (1994) reported myogenic conversion of 30% of [MyoD]P19 cells, while the remaining 70% were assumed to be refractory to this differentiation pathway. However, the higher conversion to muscle by pCA4 suggests that the cells that failed to differentiate also failed to express MyoD. A second significant advantage of the pCA4 vector was the concomitant expression of the

β-geo protein with mycMyoD which allowed direct visualization of cells which were resistant to selection and likely to be expressing mycMyoD.

While improved consistency of expression was obtained with pCA4, the IRES sequence in pCA4 did not ensure complete expression of mycMyoD in all cells since some cells which stained positively for β-geo did not stain positive for the mycMyoD protein. The reason for this is unknown, but may have been the consequence of recombinational events such that the mycMyoD gene was lost or perhaps splicing of the transcript such that the mycMyoD coding region was removed.

4.3 Regulation of MyoD in P19 cells

4.3.1 MycMyoD protein levels remain constant

Removal of the untranslated region was found to have little effect upon MyoD's ability to transactivate the cardiac actin promoter, to form muscle in a transient assay, or to induce myogenesis in [MyoD-UTR]P19 cells. In any event, once stable expression of mycMyoD was achieved using pCA4, it became apparent that the levels of mycMyoD protein did not change during aggregation. This ruled out translational control or changes in mycMyoD stability as mechanisms of downregulating mycMyoD activity in non-aggregated cells.

The amount of E2A protein may also have been regulated by the ubiquitin-proteosome pathway as previously reported (Kho et al., 1997). It is unlikely that such a method is used in regulating myogenesis of [mycMyoD] P19 cells since no increase in mycMyoD or E2A protein levels was observed after aggregation. Thus, regulation of

MyoD in this system is then unlikely to be a consequence of translational regulation or changes in the amount of MyoD or E2A proteins.

4.3.2 MycMyoD is nuclear

In both the non-aggregated and aggregated [mycMyoD]P19 cells, mycMyoD was present in the nucleus. Although MyoD has been documented to be regulated by differential localization by mechanisms including masking of a nuclear localization signal by I-mf (Chen et al., 1996) or by a PKA dependent nuclear import (Vandromme et al., 1994), such mechanisms do not appear to be involved in regulating mycMyoD mediated myogenesis in P19 cells.

4.3.3. MycMyoD binds E2A proteins before and after aggregation

Numerous proteins, such as Id (Benezra et al., 1990), Mtwist (Spicer et al., 1997), pRb (Gu et al., 1993), p300 (Sartorelli et al., 1997) and MEF2A (Molkentin et al., 1995) among others, can bind to MyoD and modulate its activity in either a positive or negative manner. Similarly, phosphorylation of MyoD by Mos, encourages the formation of MyoD:E2A heterodimers (Lenormand et al., 1997).

Immunoprecipitation of E2A proteins followed by Western blot detection of mycMyoD indicated that MyoD formed heterodimers in the non-aggregated cells. Thus, the regulation of heterodimerization with the E2A proteins through factors such as Id or Mtwist, which sequester the E2A proteins from MyoD (Spicer et al., 1997; Benezra et al., 1990), is not responsible for enhanced myogenesis following aggregation. It is of interest to note that only a fraction of the mycMyoD in non-aggregated and aggregated cells was immunoprecipitated, suggesting either that the amount of mycMyoD protein was greater

than the amount of E2A or that only a fraction of the mycMyoD was able to heterodimerize with the E2A proteins.

4.3.4 MycMyoD can bind to an E-box in both non-aggregated and aggregated cells

Having eliminated other possible types of MyoD regulation which may have occurred, the ability of mycMyoD to bind DNA was examined. MycMyoD from nuclear lysates of both non-aggregated and aggregated cells were able bind specifically to an E-box containing oligo. It is likely that the mycMyoD binding to this oligo was heterodimerized with an E2A protein since these heterodimers were present in the nuclear lysates and heterodimers have a significantly higher affinity for the E-box than do homodimers (Murre et al., 1989b).

4.4 Models for aggregation induced myogenesis mediated by MyoD

The findings described above indicate that mycMyoD was stably expressed and heterodimerized with E2A proteins on both the non-aggregated and aggregated cells. Downstream of these events, MyoD must bind to E-boxes in muscle specific promoters, which may require chromatin remodelling, and it must also activate transcription. It is possible that one of these two steps may be regulated by aggregation.

MyoD has been demonstrated to remodel chromatin in muscle specific enhancers at previously silent loci (Gerber et al., 1997). Such chromatin rearrangement may involve recruitment of remodelling complexes such as the muscle specific version of the SWI/SNF complex (Wang et al., 1996), although there is no evidence that this complex binds MyoD. The growth factors TGF-β, and basic FGF as well as sodium

butyrate were found to block this MyoD-mediated chromatin rearrangement. Although mycMyoD was able to bind to an oligonucleotide, its ability to access endogenous E-box sites in P19 cells is unknown. It is possible that in non-aggregated cells, a mechanism which acts through the same pathway as TGF-β and basic FGF prevents MyoD from remodelling the chromatin (Gerber et al., 1997). Following aggregation, the repression would be relieved allowing MyoD to remodel chromatin, bind to its E-box and initiate transcription of muscle specific genes leading to myogenesis (Fig. 17A).

The second possible type of regulation may be one which acts at the point of transcriptional activation. Binding of additional co-factors or modification of the MyoD heterodimer may be necessary for transcriptional activation. This model would predict that an active co-factor required for transcriptional activation by MyoD is absent in non-aggregated cells. The process of aggregation would result in the induction of the active co-factor, either by increased expression or modification, leading to transactivation of muscle gene expression by MyoD. MyoD mediated transcription would only proceed in the aggregated cells (Fig 17B).

Examples of co-factors which may serve to regulate transcriptional activation, could include p300 (Sartorelli et al., 1997), members of the MEF2 family or some of the cell cycle regulated proteins such as members of the pRb family whose interaction with MyoD has been shown to be required both for transcription of muscle specific genes and withdrawal from the cell cycle (Schneider et al., 1994; Gu et al., 1993).

Phosphorylation of a co-factor may be necessary for transcriptional activation.

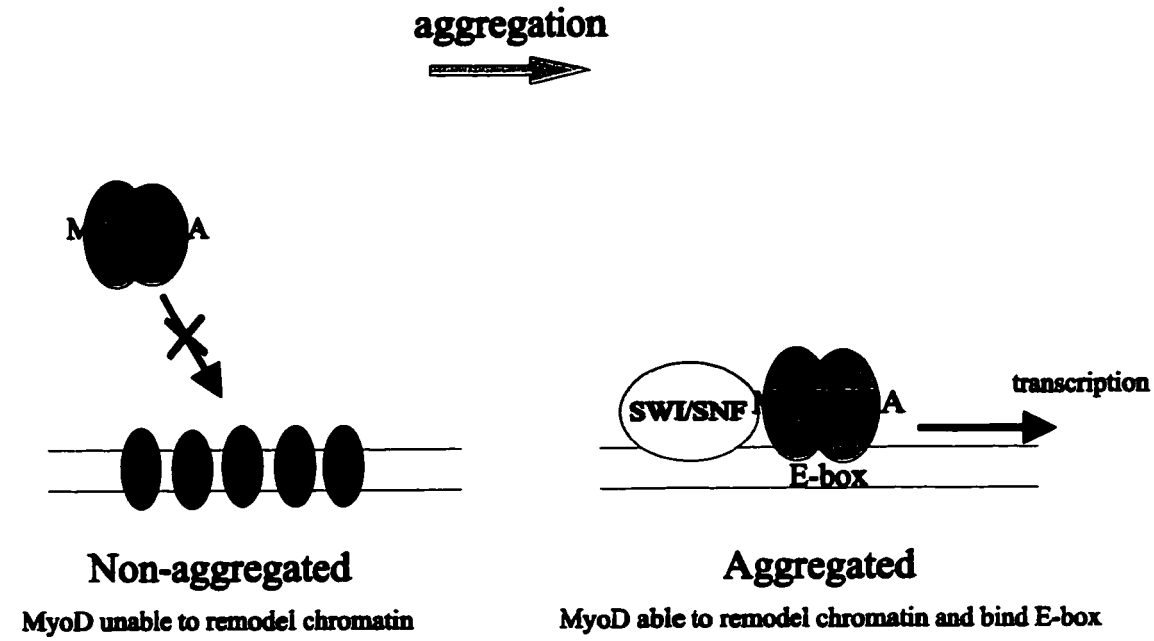
PKA is known to inhibit myogenic differentiation by MyoD at a point following DNA binding which does not involve phosphorylation of MyoD (Winter et al., 1993; Li et al., 1992). It may be that the target of PKA is this potential co-factor. PKA subunit expression, however, does not change during differentiation of P19 cells into muscle (Gael Vidricaire, M.Sc. thesis).

Fig. 17. Two models of MyoD regulation during aggregation

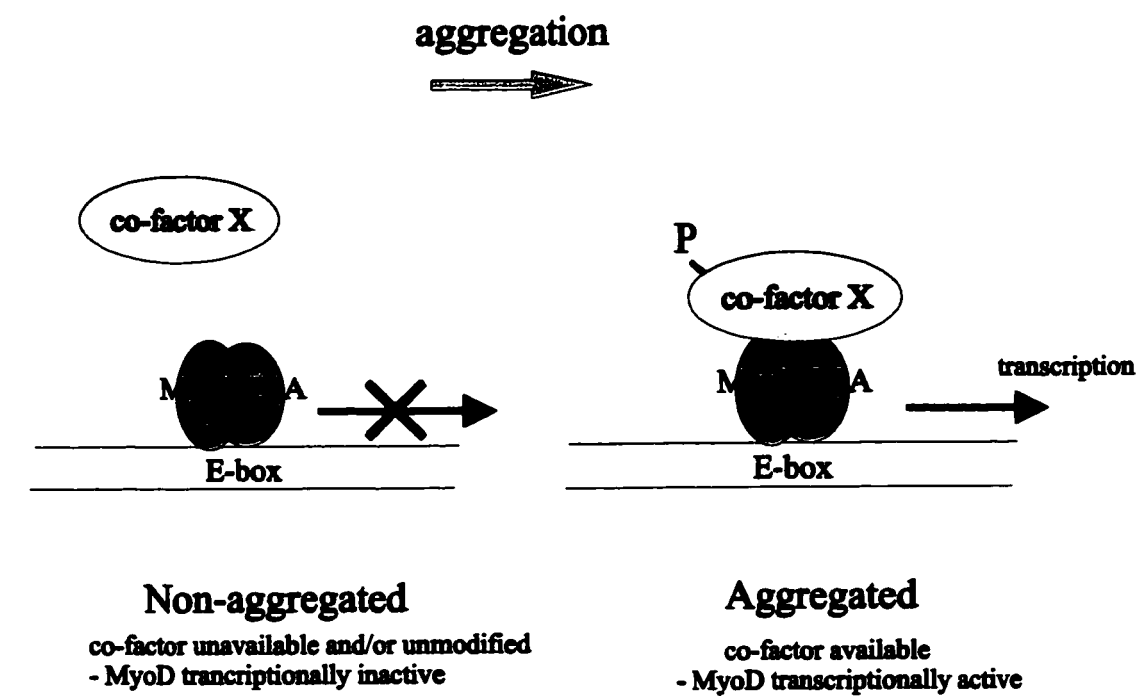
A. Model 1:
In non-aggregated cells, nuclear MyoD/E2A heterodimers are unable to access E-boxes in muscle specific promoters. The 'X' indicates the block upon MyoD's ability to remodel chromatin. The inhibitory signal which acts to block remodelling of chromatin by MyoD in non-aggregated cells may be one that has TGFβ/bFGF like signalling activities. These two growth factors are known to inhibit MyoD in this fashion. Following aggregation these growth factors would no longer inhibit MyoD from remodelling chromatin. Alternatively, a Wnt or cadherin signal may be required for remodelling to occur. Such signalling would be absent in the non-aggregated cells and would be active following aggregation. Remodelling may involve the recruitment of a muscle specific SWI/SNF complex.

B. Model 2:
In non-aggregated cells, nuclear MyoD/E2A heterodimers are bound to their DNA binding sites, however, a co-factor necessary for transcription is unavailable or unmodified. A signal received as a result of aggregation, perhaps a Wnt or cadherin signal both of which act through β-catenin, may allow a co-factor to bind and MyoD and activate transcription.

A


aggregation
M
A
SWI/SNF
A
transcription
E-box
Non-aggregated
MyoD unable to remodel chromatin
Aggregated
MyoD able to remodel chromatin and bind E-box

B


aggregation
co-factor X
P
co-factor X
M
A
M
A
transcription
E-box
E-box
Non-aggregated
co-factor unavailable and/or unmodified
- MyoD trancriptionally inactive
Aggregated
co-factor available
- MyoD transcriptionally active

4.5 Role of aggregation: possible signals involved in inducing myogenesis

A required component for efficient differentiation in P19 cells is aggregation, although aggregation alone is insufficient to induce differentiation. Efficient induction of P19 cells into skeletal muscle by MyoD, into neurons using retinoic acid and into cardiac muscle using DMSO, all require aggregation (McBurney et al., 1982; Jones-Villeneuve et al., 1983; Skerjanc et al., 1994). The signals conveyed by aggregation may or may not be the same for each type of differentiation. Two possible types of pathways which may be involved in transmitting a signal to the interior of the cell following aggregation, are discussed below.

In vivo, myogenesis occurs as a result of numerous inductive signals from surrounding cells and tissues (reviewed in Yun and Wold, 1996; Rawls and Olson 1997). Many of these signals are position-dependent to ensure myogenesis of a restricted population of precursor cells. Some of the mediators of these signals include members of the Wnt family, and sonic hedgehog (Shh), which act to induce members of the Pax family, which in turn induce MyoD and myogenin. Wnt signalling is transduced to the nucleus by a number of proteins, ending with β-catenin (reviewed in Kuhl, 1997). BMP-4, another of these position dependent signals, works in a negative fashion by inhibiting myogenesis. It is unknown how these signals control the onset of myogenesis and indeed what other signals may be involved. However, it is clear that myoblasts arise from such position dependent signals. These signals may be responsible for the induction of differentiation in aggregated P19 cells. Various members of the Wnt family have been detected in P19 cells; some members are detected in undifferentiated P19 cells (Smolich

and Papkoff, 1994) while the majority have been demonstrated to be up-regulated upon aggregation and RA induced differentiation (Smolich and Papkoff, 1994; St-Arnaud and Moir, 1993; Papkoff, 1994; Smolich et al., 1993). The only signalling molecule mentioned above which has been reported to act in a manner consistent with the results found in this thesis, is a member of the BMP family. BMP-2 inhibits the activity of MyoD through an unknown mechanism, but this inhibition does not affect MyoD's ability to bind to an E-box containing oligonucleotide (Katagiri et al., 1997). BMP-2 could either inhibit MyoD from remodelling chromatin or from initiating transcription. The expression patterns of this factor during aggregation of P19 cells is not known.

Other growth factors such as the TGF-β and bFGF may also be involved in the aggregation phenomenon. These growth factors are present in developing limb buds and somites (Hannon et al., 1992; Li et al., 1992). To correspond with their known ability to inhibit remodelling of chromatin by MyoD (Gerber et al., 1997), and to fit the results reported in this thesis, these two growth factors would have to be present and active in non-aggregated [mycMyoD] P19 cells and inactive or down-regulated in the aggregated cells. The expression patterns of TGF-β and bFGF are unknown, although TGF-β2 is not expressed in undifferentiated P19 cells and is expressed following RA induced differentiation (Mummery et al., 1990) .

Another type of signalling that may be involved in modulating the effects of aggregation in P19 cells is that conveyed by cell adhesion molecules. Cadherin-catenin complexes have been implicated in the formation of mesoderm. In *Xenopus*, cadherin

mediated cell interactions are necessary for the activation of MyoD (Holt et al., 1994). A balance of these factors is likely required, since overexpression of cadherins or underexpression of β-catenin in *Xenopus* inhibits the formation of mesoderm and the induction of MyoD (Heasman et al., 1994). It has not been reported that β-catenin affects MyoD's ability to remodel chromatin or initiate transcription; it has only been demonstrated to influence the level of MyoD expression (Heasman et al., 1994). Until all the pathways described above are further elucidated and their status determined in the P19 cell model, it would present a formidable challenge to identify which factors act to repress/activate MyoD function.

4.6 Future directions

The [mycMyoD]P19 model system should be useful for further investigations into the role of aggregation in myogenesis. One definitive experiment which may include or exclude some of the models presented would be *in vivo* footprinting on the mouse muscle creatine kinase promoter. Such an experiment would determine whether MyoD complexes can bind to or occupy E-boxes present in endogenous genes. This may help differentiate between chromatin remodelling or transactivation as the point of regulation in aggregating cells.

A further experiment which may address the signalling pathway used by aggregation would be the overexpression of β-catenin. Should β-catenin be involved in mediating the signal induced by aggregation, overexpression in non-aggregated cells may result in enhanced myogenesis.

Ultimately, by understanding the signals induced upon cell aggregation which render [MyoD]P19 cells permissive for myogenesis, it is hoped to have identified a component of myogenic control that may be exerted in deciding the fate of early mesodermal cells.

Appendix A

p300 transcriptionally activates mycMyoD in P19 cells but is not upregulated during myogenesis

A.1 Introduction

Transcriptional adapters are proteins which are thought to bind to transcription factors and enhance transcription by facilitating recruitment of the polymerase II holoenzyme to the transcription start site (Lassar and Munsterberg, 1994; Thayer and Weintraub, 1993). Two proteins which exhibit characteristics of transcriptional adapters include p300 and CBP (Kwok et al., 1994; Arany et al., 1994; Eckner et al., 1994) These two proteins have been demonstrated to co-activate both MyoD and members of the MEF2 family *in vitro* and *in vivo* (Yuan et al., 1997; Sartorelli et al., 1997). This coactivation occurs as a result of direct interaction of p300 with MyoD and/or a MEF2 member. The interaction of p300 with MyoD and/or MEF2C is mediated by the amino terminal domain of MyoD, the MADS domain of MEF2C, and both N and C terminal domains of p300 (Sartorelli et al., 1997). A dominant negative p300 mutant inhibits transactivation by MyoD, implying that p300 is required for myogenesis.

P300 is expressed in the C2C12 myoblast cell line, at approximately equal levels before and after differentiation from myoblasts to myotubes (Sartorelli et al., 1997). Transfected p300 increased the activity of a myogenic specific reporter in C2C12 cells maintained in growth medium [undifferentiated](Sartorelli et al., 1997). When these transfected cells were cultured in differentiation media, the activity of the reporter was

further enhanced.

A. 2 Results

To determine whether p300 is able to coactivate mycMyoD in P19 cells, transient transfections were performed. The reporter construct, CA-CAT was transfected with a combination of p300 and mycMyoD expression vectors as well as an internal control which allows normalization of transfection efficiencies. The p300 containing transfections were performed only once, while the others were repeated three times. As seen in Figure 18A, mycMyoD was able to transactivate the CA promoter as previously shown. P300 alone also appeared to mediate increased transcription from the CA promoter. The co-transfection of both p300 and mycMyoD increased the rate of transcription approximately ten-fold over that achieved by MyoD alone.

We next investigated whether p300 is expressed in P19 cells and examined its expression patterns during differentiation to determine whether changes in the amount of p300 might be responsible for the aggregation dependent activation of MyoD. Northern blot analysis of P19 cells alone and P19 cells differentiated in two different concentrations of DMSO or in RA are shown in Fig. 18B. As reported for C2C12 cells (Sartorelli et al., 1997), only a low level of p300 was detected and the level of p300 did not appear to change during differentiation into muscle (Fig. 18B). However, an increase in p300 mRNA was detected in day 8 and day 10 of RA differentiated P19 cells.

A.3 Discussion

As reported for 3T3 cells transfected with MyoD (Sartorelli et al., 1997), p300 was able to co-activate mycMyoD in P19 cells. It is likely that p300 exerts this coactivation by binding mycMyoD as was reported for MyoD (Sartorelli et al., 1997), although definitive proof for the same interactions in P19 cells would require an immunoprecipitation experiment. Since p300 coactivates mycMyoD, it may be the target of the effect mediated by aggregation which induces myogenesis in [mycMyoD]P19 cells. It is not surprising that p300 mRNA did not increase upon myogenesis in P19 cells considering that C2C12 cells likewise did not show an increase upon myotube formation from myoblasts (Sartorelli et al., 1997). It of interest to note that an increase was observed for the RA induced differentiation, indicating that p300 upregulation may play a role in neurogenesis.

Determining whether the activity of p300, and likewise the activity of mycMyoD, is regulated by aggregation would require further examination of the binding partners of p300 and phosphorylation state of p300 both before and after aggregation of [mycMyoD]P19 cells.

Fig. 18A **p300 coactivates with mycMyoD in P19 cells in a transient assay**
P19 cells were transiently transfected with reporter plasmid (CA-CAT), an internal control (Pgk-lacZ) to measure transfection efficiency, and a combination of mycMyoD and p300 expression constructs as shown. CAT activity was determined and normalized to β-galactosidase activity. The control transfection, shown as pDM2, contains only Pgk-lacZ and CA-CAT.

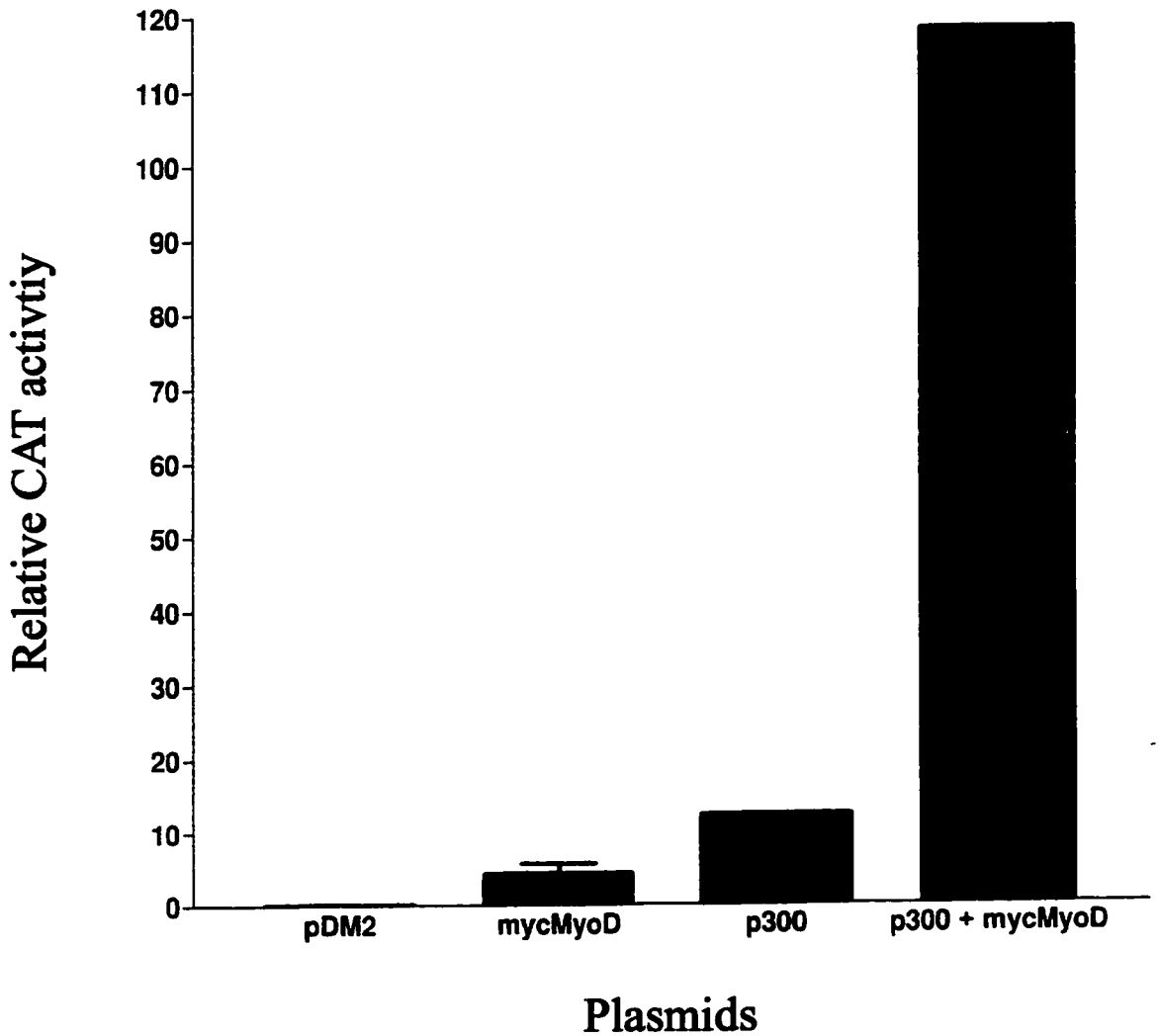
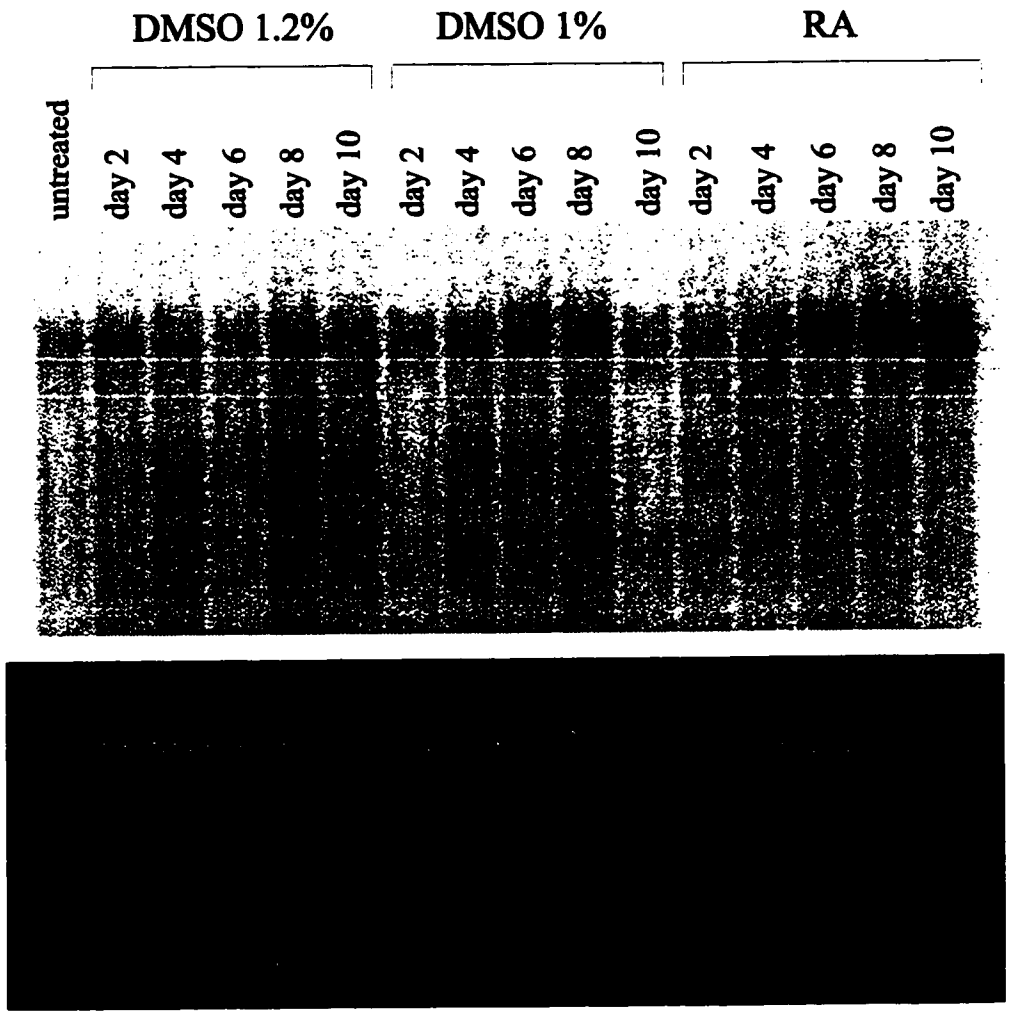

Relative CAT activtiy
120
110
100
90
80
70
60
50
40
30
20
10
0
pDM2
mycMyoD
p300
p300 + mycMyoD
Plasmids

Fig. 18B. **p300 expression during differentiation of P19 cells**

Total RNA from untreated P19 cells or P19 cells aggregated and treated with DMSO or RA for the days indicated, was harvested [courtesy of Karen Jardine] and analysed by Northern blot. The blot was hybridized with a p300 probe. To gauge loading efficiency the ethidium bromide stained gel is shown, with the 28S and 18S rRNAs as indicated. The 28s rRNA is 4712bp.


DMSO 1.2%
DMSO 1%
RA
untreated
day 2
day 4
day 6
day 8
day 10
day 2
day 4
day 6
day 8
day 10
day 2
day 4
day 6
day 8
day 10

Appendix B

E1A inhibits mycMyoD mediated myogenesis in P19 cells

B1 Introduction

The adenovirus E1A gene products are known to inhibit transcription of muscle specific genes and consequently inhibit myogenesis (Webster et al., 1988). The effect of E1A upon MyoD during myogenesis is twofold; E1A inhibits both the expression of MyoD and its ability to transcribe muscle specific genes (Caruso et al., 1993). E1A binds a number of cellular proteins including p300, pRb, and p107 (Whyte et al., 1989). It believed to be through inactivation or alteration of these proteins that E1A inhibits the transcriptional ability of MyoD.

A P19 cell line stably expressing E1A, termed M3, has been generated in our laboratory. The E1A expressed in these cells carries a deletion in the CR1 region, which abolishes p300 binding. These M3 cells are unable to differentiate upon induction with either RA or DMSO (Slack et al., 1995).

B.2 Results

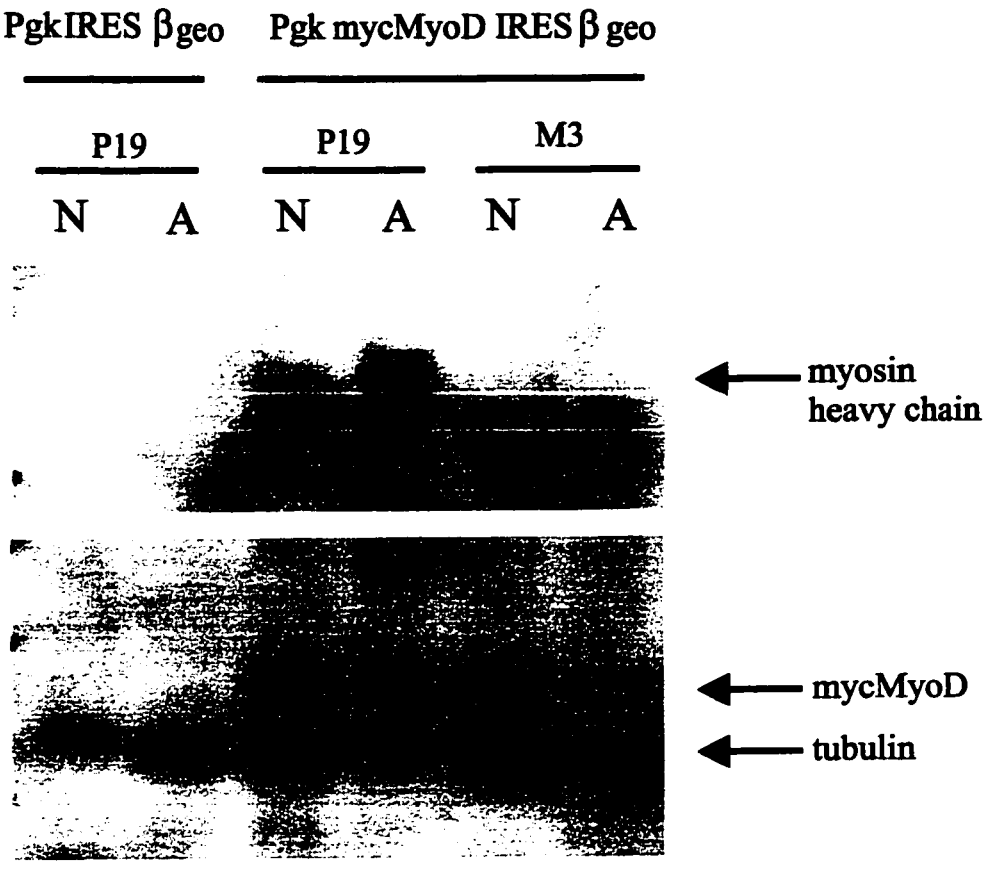
To examine whether the presence of E1A inhibits myogenesis of mycMyoD differentiated P19 cells, mycMyoD was stably expressed by transfection of M3 cells with pCA4 followed by the selection of drug resistant clones. The [mycMyoD]M3 cells, as well as [mycMyoD]P19 cells and control lines were either grown in monolayers or aggregated for five days. Cells were then harvested and their proteins analysed by Western blot (Fig. 19). The lanes representing [mycMyoD]P19 cells show the presence

of myosin heavy chain indicating muscle, whereas [mycMyoD]M3 cells do not, despite the maintained expression of mycMyoD both before and after aggregation.

B3 Discussion

The inability of M3 cells to differentiate into skeletal muscle following mycMyoD expression and aggregation indicated that E1A interferes with a protein, perhaps pRb, which is essential to myogenesis.

Fig. 19. **[mycMyoD]M3 cells do not form skeletal muscle**
Western blot analysis of non-aggregated (N) and aggregated (A) [mycmyoD]M3 cells. Cells were harvested at the days of aggregation shown. MycMyoD was detected with the anti-myc 9E10 monoclonal antibody, and myosin heavy chain with a anti-myosin heavy chain monoclonal antibody. An anti-tubulin antibody was used to detect tubulin to gauge relative amounts of protein loaded. Protein size markers are as indicated and are in kilodaltons.


PgkIRES βgeo
Pgk mycMyoD IRES β geo
P19
P19
M3
N
A
N
A
N
A
myosin heavy chain
mycMyoD
tubulin

References

Adra, C.N., Boer, P.H., and McBurney, M.W. (1987). Cloning and expression of the mouse pgk-1 gene and the nucleotide sequence of its promoter. Gene *60*, 65-74.

Arany, Z., Sellers, W.R., Livingston, D.M., and Eckner, R. (1994). E1A-associated p300 and CREB-associated CBP belong to a conserved family of coactivators [letter]. Cell *77*, 799-800.

Aronheim, A., Shiran, R., Rosen, A., and Walker, M.D. (1993). Cell-specific expression of helix-loop-helix transcription factors encoded by the E2A gene. Nucleic. Acids. Res. *21*, 1601-1606.

Bader, D., Masaki, T., and Fischman, D.A. (1982). Immunochemical analysis of myosin heavy chain during avian myogenesis in vivo and in vitro. J. Cell Biol. *95*, 763-770.

Benezra, R., Davis, R.L., Lockshon, D., Turner, D.L., and Weintraub, H. (1990). The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell *61*, 49-59.

Benezra, R. (1994). An intermoldcular disulfide bond stabilizes E2A homodimers and is required for DNA binding at physiological temperatures. Cell *79*, 1057-1067.

Berg, R.W. and McBurney, M.W. (1990). Cell density and cell cycle effects on retinoic acid-induced embryonal carcinoma cell differentiation. Dev. Biol. *138*, 123-135.

Birnboim, H.C. (1983). A rapid alkaline extraction method for the isolation of plasmid DNA. Methods Enzymol. *100:243-55*, 243-255.

Blackwell, T.K. and Weintraub, H. (1990). Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection. Science *250*, 1104-1110.

Braun, T., Bober, E., Buschhausen-Denker, G., Kohtz, S., Grzeschik, K.H., Arnold, H.H., and Kotz, S. (1989a). Differential expression of myogenic determination genes in muscle cells: possible autoactivation by the Myf gene products [published erratum appears in EMBO J 1989 Dec;8(13):4358]. EMBO J. *8*, 3617-3625.

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

Braun, T., Bober, E., Winter, B., Rosenthal, N., and Arnold, H.H. (1990). Myf-6, a new member of the human gene family of myogenic determination factors: evidence for a gene cluster on chromosome 12. EMBO J. *9*, 821-831.

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

Brennan, T.J., Chakraborty, T., and Olson, E.N. (1991). Mutagenesis of the myogenin basic region identifies an ancient protein motif critical for activation of myogenesis. Proc. Natl. Acad. Sci. U. S. A. *88*, 5675-5679.

Buskin, J.N. and Hauschka, S.D. (1989). Identification of a myocyte nuclear factor that binds to the muscle- specific enhancer of the mouse muscle creatine kinase gene. Mol. Cell Biol. *9*, 2627-2640.

Caruso, M., Martelli, F., Giordano, A., and Felsani, A. (1993). Regulation of MyoD gene transcription and protein function by the transforming domains of the adenovirus E1A oncoprotein. Oncogene *8*, 267-278.

Chan, S.Y., Evan, G.I., Ritson, A., Watson, J., Wraight, P., and Sikora, K. (1986). Localisation of lung cancer by a radiolabelled monoclonal antibody against the c-myc oncogene product. Br. J. Cancer *54*, 761-769.

Chen, C. and Okayama, H. (1987). High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell Biol. *7*, 2745-2752.

Chen, C.M., Kraut, N., Groudine, M., and Weintraub, H. (1996). I-mf, a novel myogenic repressor, interacts with members of the MyoD family. Cell *86*, 731-741.

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

Cusella-De Angelis, M.G., Lyons, G., Sonnino, C., De Angelis, L., Vivarelli, E., Farmer, K., Wright, W.E., Molinaro, M., Bouche, M., and Buckingham, M. (1992). MyoD, myogenin independent differentiation of primordial myoblasts in mouse somites. J. Cell Biol. *116*, 1243-1255.

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

Davis, R.L., Cheng, P.F., Lassar, A.B., and Weintraub, H. (1990). The MyoD DNA binding domain contains a recognition code for muscle- specific gene activation. Cell *60*, 733-746.

Davis, R.L. and Weintraub, H. (1992). Acquisition of myogenic specificity by replacement of three amino acid residues from MyoD into E12. Science *256*, 1027-1030.

Dias, P., Parham, D.M., Shapiro, D.N., Tapscott, S.J., and Houghton, P.J. (1992). Monoclonal antibodies to the myogenic regulatory protein MyoD1: epitope mapping and diagnostic utility. Cancer Res. *52*, 6431-6439.

Dias, P., Dilling, M., and Houghton, P. (1994). The molecular basis of skeletal muscle differentiation. Semin. Diagn. Pathol. *11*, 3-14.

Eckner, R., Arany, Z., Ewen, M., Sellers, W., and Livingston, D.M. (1994a). The adenovirus E1A-associated 300-kD protein exhibits properties of a transcriptional coactivator and belongs to an evolutionarily conserved family. Cold Spring Harb. Symp. Quant. Biol. *59:85-95*, 85-95.

Eckner, R., Ewen, M.E., Newsome, D., Gerdes, M., DeCaprio, J.A., Lawrence, J.B., and Livingston, D.M. (1994b). Molecular cloning and functional analysis of the adenovirus E1A-associated 300-kD protein (p300) reveals a protein with properties of a transcriptional adaptor. Genes Dev. *8*, 869-884.

Edmondson, D.G. and Olson, E.N. (1989). A gene with homology to the myc similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program [published erratum appears in Genes Dev 1990 Aug;4(8):1450]. Genes Dev. *3*, 628-640.

Edwards, M.K., Harris, J.F., and McBurney, M.W. (1983). Induced muscle differentiation in an embryonal carcinoma cell line. Mol. Cell Biol. *3*, 2280-2286.

Ephrussi, A., Church, G.M., Tonegawa, S., and Gilbert, W. (1985). B lineage--specific interactions of an immunoglobulin enhancer with cellular factors in vivo. Science *227*, 134-140.

Gerber, A.N., Klesert, T.R., Bergstrom, D.A., and Tapscott, S.J. (1997). Two domains of MyoD mediate transcriptional activation of genes in repressive chromatin: a mechanism for lineage determination in myogenesis. Genes Dev. *11*, 436-450.

Gu, W., Schneider, J.W., Condorelli, G., Kaushal, S., Mahdavi, V., and Nadal-Ginard, B. (1993). Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. Cell *72*, 309-324.

Halevy, O., Novitch, B.G., Spicer, D.B., Skapek, S.X., Rhee, J., Hannon, G.J., Beach, D., and Lassar, A.B. (1995). Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD [see comments]. Science *267*, 1018-1021.

Hannan, G.N., Lehnert S.A., MacAvoy E.S., Jennings P.A., Molloy P.L.(1993) An engineered *PGK* promoter and *lac* operator-repressor system for the regulation of gene expression in mammalian cells. Gene *130*, 233-239.

Hannon, K., Smith C.K., Bales K.R. and Santerre R.F. (1992) Temporal and qualitative analysis

of myogenic regulatory and growth factor gene expression in the developing mouse embryo. Dev Biol. *151*, 137-144.

Hardy, S., Kong, Y., and Konieczny, S.F. (1993). Fibroblast growth factor inhibits MRF4 activity independently of the phosphorylation status of a conserved threonine residue within the DNA- binding domain. Mol. Cell Biol. *13*, 5943-5956.

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

Heasman, J., Crawford, A., Goldstone, K., Garner-Hamrick, P., Gumbiner, B., McCrea, P., Kintner, C., Noro, C.Y., and Wylie, C. (1994). Overexpression of cadherins and underexpression of beta-catenin inhibit dorsal mesoderm induction in early Xenopus embryos. Cell *79*, 791-803.

Henthorn, P., Kiledjian, M., and Kadesch, T. (1990). Two distinct transcription factors that bind the immunoglobulin enhancer microE5/kappa 2 motif. Science *247*, 467-470.

Holt, C.E., Lemaire, P., and Gurdon, J.B. (1994). Cadherin-mediated cell interactions are necessary for the activation of MyoD in Xenopus mesoderm. Proc. Natl. Acad. Sci. U. S. A. *91*, 10844-10848.

Hoppler, S., Brown, J.D., and Moon, R.T. (1996). Expression of a dominant-negative Wnt blocks induction of MyoD in Xenopus embryos. Genes Dev. *10*, 2805-2817.

Huang, J., Blackwell, T.K., Kedes, L., and Weintraub, H. (1996). Differences between MyoD DNA binding and activation site requirements revealed by functional random sequence selection. Mol. Cell Biol. *16*, 3893-3900.

Jang, S.K., Pestova, T.V., Hellen, C.U., Witherell, G.W., and Wimmer, E. (1990). Cap-independent translation of picornavirus RNAs: structure and function of the internal ribosomal entry site. Enzyme *44*, 292-309.

Jen, Y., Weintraub, H., and Benezra, R. (1992). Overexpression of Id protein inhibits the muscle differentiation program: *in vivo* association of Id with E2A proteins. Genes Dev. *6*, 1466-1479.

Johnson, S.E., Wang, X., Hardy, S., Taparowsky, E.J., and Konieczny, S.F. (1997). Casein kinase II increases the transcriptional activities of MRF4 and MyoD independently of their direct phosphorylation. 1996. Apr;16(4):1604-1613. *16*, 1604-1613.

Jones-Villeneuve, E.M., Rudnicki, M.A., Harris, J.F., and McBurney, M.W. (1983). Retinoic acid-induced neural differentiation of embryonal carcinoma cells. Mol. Cell Biol. *3*, 2271-2279.

Katagiri, T., Akiyama, S., Namiki, M., Komaki, M., Yamaguchi, A., Rosen, V., Wozney, J.M.,

Fujisawa-Sehara, A., and Suda, T. (1997). Bone morphogenetic protein-2 inhibits terminal differentiation of myogenic cells by suppressing the transcriptional activity of MyoD and myogenin. Exp. Cell Res. *230*, 342-351.

Kho, C.J., Huggins, G.S., Endege, W.O., Hsieh, C.M., Lee, M.E., and Haber, E. (1997). Degradation of E2A proteins through a ubiquitin-conjugating enzyme, UbcE2A. J. Biol. Chem. *272*, 3845-3851.

Kuhl M. (1997) Wnt signalling goes nuclear. Bioessays *19(2)*, 104-107.

Kwok, R.P., Lundblad, J.R., Chrivia, J.C., Richards, J.P., Bachinger, H.P., Brennan, R.G., Roberts, S.G., Green, M.R., and Goodman, R.H. (1994). Nuclear protein CBP is a coactivator for the transcription factor CREB [see comments]. Nature *370*, 223-226.

Lassar, A. and Munsterberg, A. (1994). Wiring diagrams: regulatory circuits and the control of skeletal myogenesis. Curr. Opin. Cell Biol. *6*, 432-442.

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

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

Lenormand, J.L., Benayoun, B., Guillier, M., Vandromme, M., Leibovitch, M.P., and Leibovitch, S.A. (1997). Mos activates myogenic differentiation by promoting heterodimerization of MyoD and E12 proteins. Mol. Cell Biol. *17*, 584-593.

Leyns, L., Bouwmeester, T., Kim, S.H., Piccolo, S., and De Robertis, E.M. (1997). Frzb-1 is a secreted antagonist of Wnt signaling expressed in the Spemann organizer. Cell *88*, 747-756.

Li, L., Heller-Harrison, R., Czech, M., and Olson, E.N. (1992a). Cyclic AMP-dependent protein kinase inhibits the activity of myogenic helix-loop-helix proteins. Mol. Cell Biol. *12*, 4478-4485.

Li, L., Zhou, J., James, G., Heller-Harrison, R., Czech, M.P., and Olson, E.N. (1992b). FGF inactivates myogenic helix-loop-helix proteins through phosphorylation of a conserved protein kinase C site in their DNA-binding domains. Cell *71*, 1181-1194.

Ma, P.C., Rould, M.A., Weintraub, H., and Pabo, C.O. (1994). Crystal structure of MyoD bHLH domain-DNA complex: perspectives on DNA recognition and implications for transcriptional activation. Cell *77*, 451-459.

Maroto, M., Reshef, R., Munsterberg, A.E., Koester, S., Goulding, M., and Lassar, A.B. (1997).

Ectopic Pax-3 activates MyoD and Myf-5 expression in embryonic mesoderm and neural tissue. Cell *89*, 139-148.

Martelli, F., Cenciarelli, C., Santarelli, G., Polikar, B., Felsani, A., and Caruso, M. (1994). MyoD induces retinoblastoma gene expression during myogenic differentiation. Oncogene *9*, 3579-3590.

McBurney, M.W., Jones-Villeneuve, E.M., Edwards, M.K., and Anderson, P.J. (1982). Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line. Nature *299*, 165-167.

McBurney, M.W., Sutherland, L.C., Adra, C.N., Leclair, B., Rudnicki, M.A., and Jardine, K. (1991). The mouse Pgk-1 gene promoter contains an upstream activator sequence. Nucleic. Acids. Res. *19*, 5755-5761.

McBurney, M.W., Fournier, S., Jardine, K., and Sutherland, L. (1994). Intragenic regions of the murine Pgk-1 locus enhance integration of transfected DNAs into genomes of embryonal carcinoma cells. Somat. Cell Mol. Genet. *20*, 515-528.

McBurney, M.W. and Rogers, B.J. (1982). Isolation of male embryonal carcinoma cells and their chromosome replication patterns. Dev. Biol. *89*, 503-508.

Melefors, O. and Hentze, M.W. (1993). Translational regulation by mRNA/protein interactions in eukaryotic cells: ferritin and beyond. Bioessays *15*, 85-90.

Miner, J.H. and Wold, B. (1990). Herculin, a fourth member of the MyoD family of myogenic regulatory genes. Proc. Natl. Acad. Sci. U. S. A. *87*, 1089-1093.

Molkentin, J.D., Black, B.L., Martin, J.F., and Olson, E.N. (1995). Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. Cell *83*, 1125-1136.

Mountford, P., Zevnik, B., Duwel, A., Nichols, J., Li, M., Dani, C., Robertson, M., Chambers, I., and Smith, A. (1994). Dicistronic targeting constructs: reporters and modifiers of mammalian gene expression. Proc. Natl. Acad. Sci. U. S. A. *91*, 4303-4307.

Mummery C.L., Slager H., Kruijer W., Feijen A., Freund E., Koornneef I., van den Eijnden-van Raaij A.J. (1990) Expression of transforming growth factor beta 2 during the differentiation of muring embryonal carcinoma and embryonic stem cells. Dev Biol. *137(1)*, 161-170.

Murre, C., McCaw, P.S., and Baltimore, D. (1989a). A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. Cell *56*, 777-783.

Murre, C., McCaw, P.S., Vaessin, H., Caudy, M., Jan, L.Y., Jan, Y.N., Cabrera, C.V., Buskin, J.N., Hauschka, S.D., and Lassar, A.B. (1989b). Interactions between heterologous

helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell *58*, 537-544.

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

Olson, E.N. (1990). MyoD family: a paradigm for development? Genes Dev. *4*, 1454-1461.

Papkoff, J. (1994). Identification and biochemical characterization of secreted Wnt-1 protein from P19 embryonal carcinoma cells induced to differentiate along the neuroectodermal lineage. Oncogene *9*, 313-317.

Pari, G. (1990) Muscle specific gene expression in differentiated embryonal carcinoma cells. M.Sc thesis. Dept. of Biology. University of Ottawa.

Pari, G., Jardine, K., and McBurney, M.W. (1991). Multiple CArG boxes in the human cardiac actin gene promoter required for expression in embryonic cardiac muscle cells developing in vitro from embryonal carcinoma cells. Mol. Cell Biol. *11*, 4796-4803.

Parker, S.B., Eichele, G., Zhang, P., Rawls, A., Sands, A.T., Bradley, A., Olson, E.N., Harper, J.W., and Elledge, S.J. (1995). p53-independent expression of p21Cip1 in muscle and other terminally differentiating cells [see comments]. Science *267*, 1024-1027.

Peterson, C.A., Gordon, H., Hall, Z.W., Paterson, B.M., and Blau, H.M. (1990). Negative control of the helix-loop-helix family of myogenic regulators in the NFB mutant. Cell *62*, 493-502.

Rawls, A. and Olson, E.N. (1997). MyoD meets its maker. Cell *89*, 5-8.

Rhodes, S.J. and Konieczny, S.F. (1989). Identification of MRF4: a new member of the muscle regulatory factor gene family. Genes Dev. *3*, 2050-2061.

Riechmann, V., van Cruchten, I., and Sablitzky, F. (1994). The expression pattern of Id4, a novel dominant negative helix-loop- helix protein, is distinct from Id1, Id2 and Id3. Nucleic. Acids. Res. *22*, 749-755.

Rossant, J. and McBurney, M.W. (1982). The developmental potential of a euploid male teratocarcinoma cell line after blastocyst injection. J. Embryol. Exp. Morphol. *70:99-112*, 99-112.

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

Rudnicki, M.A. and McBurney, M.W. (1987). Cell culture methods and induction of differentiation of embryonal carcinoma cell lines. In Teratocarcinomas and embryonic stem cells: a practical approach. E.J. Robinson, ed. (Oxford: IRL Press), pp. 19-49.

Rupp, R.A., Snider, L., and Weintraub, H. (1994). Xenopus embryos regulate the nuclear localization of XMyoD. Genes Dev. *8*, 1311-1323.

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

Sarkar, S. (1984). Translational control involving a novel cytoplasmic RNA and ribonucleoprotein. Prog. Nucleic. Acid. Res. Mol. Biol. *31:267-93*, 267-293.

Sartorelli, V., Webster, K.A., and Kedes, L. (1990). Muscle-specific expression of the cardiac alpha-actin gene requires MyoD1, CArG-box binding factor, and Sp1. Genes Dev. *4*, 1811-1822.

Sartorelli, V., Hong, N.A., Bishopric, N.H., and Kedes, L. (1992). Myocardial activation of the human cardiac alpha-actin promoter by helix-loop-helix proteins. Proc. Natl. Acad. Sci. U. S. A. *89*, 4047-4051.

Sartorelli, V., Huang, J., Hamamori, Y., and Kedes, L. (1997). Molecular mechanisms of myogenic coactivation by p300: direct interaction with the activation domain of MyoD and with the MADS box of MEF2C. Mol. Cell Biol. *17*, 1010-1026.

Schneider, J.W., Gu, W., Zhu, L., Mahdavi, V., and Nadal-Ginard, B. (1994). Reversal of terminal differentiation mediated by p107 in Rb-/-muscle cells. Science *264*, 1467-1471.

Skapek, S.X., Rhee, J., Spicer, D.B., and Lassar, A.B. (1995). Inhibition of myogenic differentiation in proliferating myoblasts by cyclin D1-dependent kinase [see comments]. Science *267*, 1022-1024.

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

Skerjanc, I.S. and McBurney, M.W. (1994). The E box is essential for activity of the cardiac actin promoter in skeletal but not in cardiac muscle. Dev. Biol. *163*, 125-132.

Slack, R.S., Craig, J., Costa, S., and McBurney, M.W. (1995). Adenovirus 5 E1A induced differentiation of P19 embryonal carcinoma cells requires binding to p300. Oncogene *10*, 19-25.

Smolich, B.D. and Papkoff, J. (1994). Regulated expression of Wnt family members during neuroectodermal differentiation of P19 embryonal carcinoma cells: overexpression of Wnt- 1 perturbs normal differentiation-specific properties. Dev. Biol. *166*, 300-310.

Smolich, B.D., McMahon J.A., McMahon, A.P. and Papkoff, J. (1993) Wnt family members are secreted and associated with the cell surface. Mol. Cell Bio. 4*(12)*, 1267-1275.

Sorrentino, V., Pepperkok, R., Davis, R.L., Ansorge, W., and Philipson, L. (1990). Cell proliferation inhibited by MyoD1 independently of myogenic differentiation. Nature *345*, 813-815.

Spicer, D.B., Rhee, J., Cheung, W.L., and Lassar, A.B. (1997). Inhibition of myogenic bHLH and MEF2 transcription factors by the bHLH protein Twist. 1996. Jun. 7;272(5267):1476-1480. *272*, 1476-1480.

St-Arnaud, R. and Moir, J.M. (1993). Wnt-1-inducing factor-1: a novel G/C box-binding transcription factor regulating the expression of Wnt-1 during neuroectodermal differentiation. Mol. Cell Biol. *13*, 1590-1598.

Stone, J., de Lange, T., Ramsay, G., Jakobovits, E., Bishop, J.M., Varmus, H., and Lee, W. (1987). Definition of regions in human c-myc that are involved in transformation and nuclear localization. Mol. Cell Biol. *7*, 1697-1709.

Sun, X.H., Copeland, N.G., Jenkins, N.A., and Baltimore, D. (1991). Id proteins Id1 and Id2 selectively inhibit DNA binding by one class of helix-loop-helix proteins. Mol. Cell Biol. *11*, 5603-5611.

Sun, X.H. and Baltimore, D. (1991). An inhibitory domain of E12 transcription factor prevents DNA binding in E12 homodimers but not in E12 heterodimers [published erratum appears in Cell 1991 Aug 9;66(3):423]. Cell *64*, 459-470.

Tajbakhsh, S., Rocancourt, D., Cossu, G., and Buckingham, M. (1997). Redefining the genetic hierarchies controlling skeletal myogenesis: Pax- 3 and Myf-5 act upstream of MyoD. Cell *89*, 127-138.

Tapscott, S.J., Davis, R.L., Thayer, M.J., Cheng, P.F., Weintraub, H., and Lassar, A.B. (1988). MyoD1: a nuclear phosphoprotein requiring a Myc homology region to convert fibroblasts to myoblasts. Science *242*, 405-411.

Thayer, M.J., Tapscott, S.J., Davis, R.L., Wright, W.E., Lassar, A.B., and Weintraub, H. (1989). Positive autoregulation of the myogenic determination gene MyoD1. Cell *58*, 241-248.

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

Vaidya, T.B., Rhodes, S.J., Taparowsky, E.J., and Konieczny, S.F. (1989). Fibroblast growth factor and transforming growth factor beta repress transcription of the myogenic regulatory gene MyoD1. Mol. Cell Biol. *9*, 3576-3579.

Vandromme, M., Carnac, G., Gauthier-Rouviere, C., Fesquet, D., Lamb, N., and Fernandez, A. (1994). Nuclear import of the myogenic factor MyoD requires cAMP-dependent protein kinase activity but not the direct phosphorylation of MyoD. J. Cell Sci. *107*, 613-620.

Vandromme, M., Cavadore, J.C., Bonnieu, A., Froeschle, A., Lamb, N., and Fernandez, A. (1995). Two nuclear localization signals present in the basic-helix 1 domains of MyoD promote its active nuclear translocation and can function independently. Proc. Natl. Acad. Sci. U. S. A. *92*, 4646-4650.

Vara J.A., Portela A., Ortin J., Jimenez A. (1986) Expression in mammalian cells of a gene from *Streptomyces alboniger* conferring puromycin resistance. Nucleic Acids Res. *14*, 4617-4624.

Vidricaire G. (1993) Role of the Brachyury T gene in P19 embryonal carcinoma cell differentiation. M.Sc. Thesis. Dept. of Biochemistry. University of Ottawa.

Vidricaire, G., Jardine, K., and McBurney, M.W. (1994). Expression of the Brachyury gene during mesoderm development in differentiating embryonal carcinoma cell cultures. Development *120*, 115-122.

Vierra, C.A., Xin, X.Q., Jacobs, Y.T., Campbell, J.M., Shen, L.P., Rutter, W.J., and Nelson, C. (1994). Purification of E. coli-synthesized Pan proteins and development of a Pan-specific monoclonal antibody. Hybridoma *13*, 191-197.

Voronova, A. and Baltimore, D. (1990). Mutations that disrupt DNA binding and dimer formation in the E47 helix- loop-helix protein map to distinct domains. Proc. Natl. Acad. Sci. U. S. A. *87*, 4722-4726.

Wang, S., Krinks, M., Lin, K., Luyten, F.P., and Moos, M., Jr. (1997). Frzb, a secreted protein expressed in the Spemann organizer, binds and inhibits Wnt-8. Cell *88*, 757-766.

Wang, W., Xue, Y., Zhou, S., Kuo, A., Cairns, B.R., and Crabtree, G.R. (1996). Diversity and specialization of mammalian SWI/SNF complexes. Genes Dev. *10*, 2117-2130.

Wang, Y., Benezra, R., and Sassoon, D.A. (1992). Id expression during mouse development: a role in morphogenesis. Dev. Dyn. *194*, 222-230.

Webster, K.A., Muscat, G.E., and Kedes, L. (1988). Adenovirus E1A products suppress myogenic differentiation and inhibit transcription from muscle-specific promoters. Nature *332*, 553-557.

Weintraub, H., Dwarki, V.J., Verma, I., Davis, R., Hollenberg, S., Snider, L., Lassar, A., and Tapscott, S.J. (1991). Muscle-specific transcriptional activation by MyoD. Genes Dev. *5*, 1377-1386.

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

Weintraub, H., Genetta, T., and Kadesch, T. (1997). Tissue-specific gene activation by MyoD: determination of specificity by cis-acting repression elements. 1994. Sep. 15;8(18):2203-11. *8*, 2203-2211.

Whyte, P., Williamson, N.M., and Harlow, E. (1989). Cellular targets for transformation by the adenovirus E1A proteins. Cell *56*, 67-75.

Winter, B., Braun, T., and Arnold, H.H. (1993). cAMP-dependent protein kinase represses myogenic differentiation and the activity of the muscle-specific helix-loop-helix transcription factors Myf-5 and MyoD. J. Biol. Chem. *268*, 9869-9878.

Wolf, C., Thisse, C., Stoetzel, C., Thisse, B., Gerlinger, P., and Perrin-Schmitt, F. (1991). The M-twist gene of Mus is expressed in subsets of mesodermal cells and is closely related to the Xenopus X-twi and the Drosophila twist genes. Dev. Biol. *143*, 363-373.

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

Yuan, W., Condorelli, G., Caruso, M., Felsani, A., and Giordano, A. (1997). Human p300 protein is a coactivator for the transcription factor MyoD. 1996. Apr. 12;271(15):9009-9013. *271*, 9009-9013.

Yutzey, K.E. and Konieczny, S.F. (1992). Different E-box regulatory sequences are functionally distinct when placed within the context of the troponin I enhancer. Nucleic. Acids. Res. *20*, 5105-13I - 96069901.

Zhou, J. and Olson, E.N. (1994). Dimerization through the helix-loop-helix motif enhances phosphorylation of the transcription activation domains of myogenin. Mol. Cell Biol. *14*, 6232-6243.

Curriculum Vitae

Christine Marion Armour
278 Stonequarry
Ottawa, Ont.
K1K 3Y2
(613)749-2825

Date of Birth: Dec. 19, 1971.
Place of Birth: Ottawa, Ont.
Citizenship: Canadian

Education

Immaculata High School
Ottawa, Ont.
Senior Matriculation, 1990

Carleton University
Ottawa, Ont.
B.Sc. (Hons), Biology, 1994.

University of Ottawa
Ottawa, Ont.
M.Sc. Biochemistry, presently registered

Awards:

Canada Scholar, 1990- 1994
Carleton University Entrance Scholarship 1990
Ottawa Ladies' College Scholarship 1991
H.H.J. Nesbitt Scholarship in Biology 1992
H. H. Southam Scholarship 1992
Muscular Dystrophy Fellowship 1993
E.W.R. Steacie Scholarship 1993
H.H.J. Nesbitt Scholarship in Biology 1993
H.R.H. the Princess of Wales' Scholarship in the Biological Sciences 1993
Ottawa University Entrance Scholarship 1994
Networks of Centres of Excellence Fellowship 1994
Ottawa University Excellence Scholarship 1995

Experience:

Summer student 3 years, 1992 - 94 with Dr. M.W. McBurney,
Ottawa Cancer Research Group

Lab demonstrator - 3rd year Biochemistry
University of Ottawa 1995

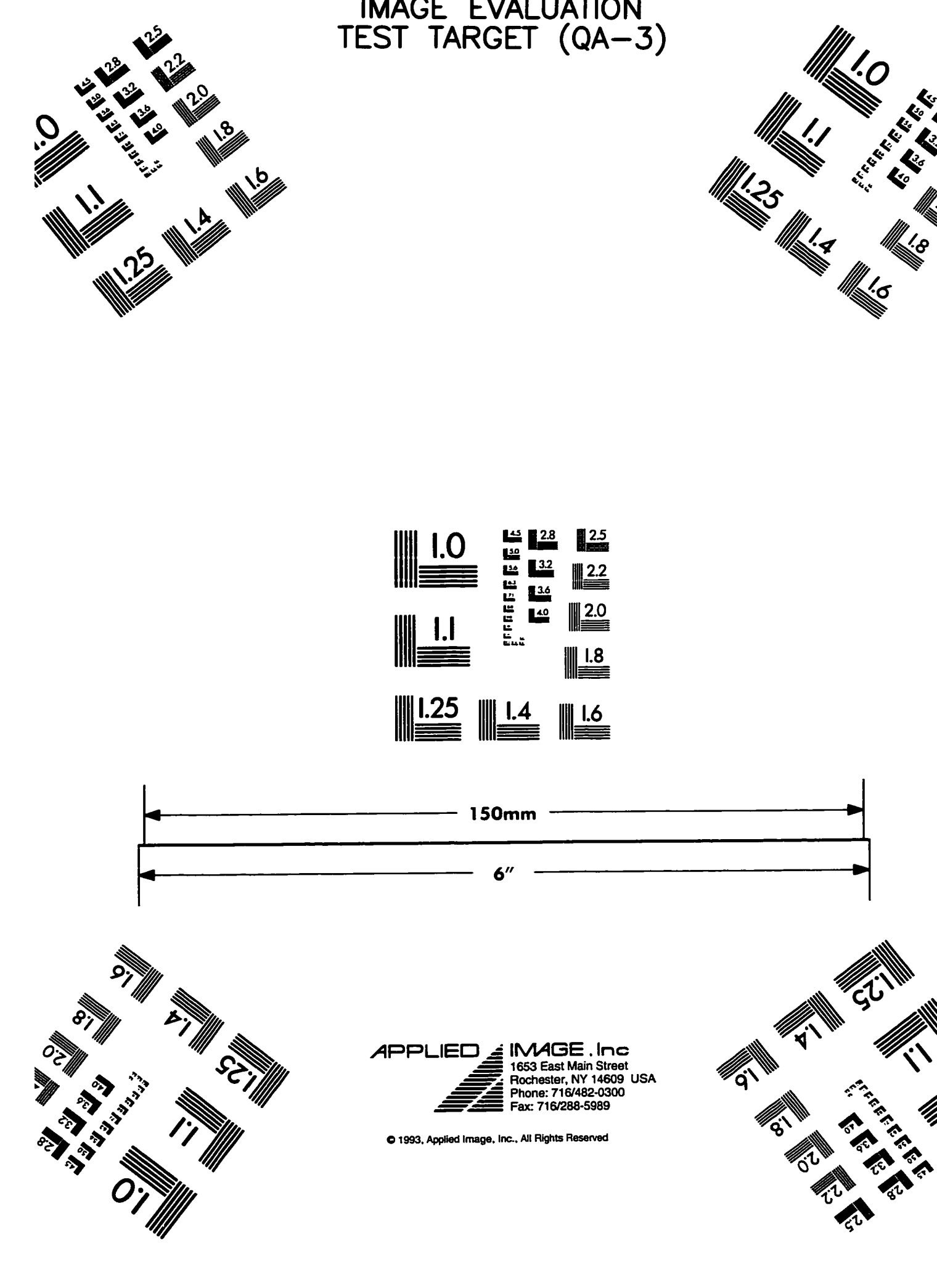


IMAGE EVALUATION
TEST TARGET (QA-3)
1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
150mm
6"
APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989
© 1993, Applied Image, Inc., All Rights Reserved