MYOGENIC BHLH TRANSCRIPTION FACTORS: THEIR OVERLAPPING FUNCTIONS AND DIRECT REGULATION OF *MEF2C* PROVIDE A REGULATORY NETWORK FOR THE MAINTENANCE AND AMPLIFICATION OF VERTEBRATE MYOGENESIS

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DEDICATION

This body of work is dedicated - in no uncertain terms - to my family. First and foremost, my parents, Reynaldo Garza Valdez Jr. and Sonia Vite Valdez, deserve the greatest measure of my gratitude and love for the innumerable gifts they have bestowed upon me. Not the least of these are the sense of indomitability they have instilled in me and the infinite support that they provide when this confidence waivers. My brother, Reynaldo Alexander Valdez III, is twelve years my junior and, in many ways, like a son to me. His capacity for love and his creativity astonishes me and gives me the courage to dream. Finally, in my new husband, David Lane Scott, I have finally found a person that I can love and trust and who, very importantly, helps me to keep things in perspective. I only hope that I will have the opportunity to provide him with the same immense support that he has given me in our short time together.

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by

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DISSERTATION

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The myogenic basic helix-loop-helix (bHLH) genes - *Myf5, MyoD, myogenin* and *MRF4* - exhibit distinct, but overlapping expression patterns during vertebrate myogenesis. Loss-of-function mutations in these genes have defined an *in vivo* model for myogenesis in which MyoD and Myf5 have redundant functions in myoblast specification, whereas myogenin acts to control myoblast differentiation. A role for MRF4 in differentiation has been suggested by various studies, but not defined. Through the analysis of *MyoD-/-MRF4-/-* and *myogenin-/-MRF4-/-* mutants, we show that MRF4 plays a role in differentiation which it shares with MyoD, but not myogenin, thereby defining a novel myogenin-independent differentiation pathway.

The functional redundancy of the myogenic bHLH factors demonstrated in these and other studies led us to investigate the ability of a single factor to direct the myogenic program in the absence of the other myogenic bHLH proteins. Analysis of *myogenin-/-MyoD-/-MRF4-/-* mutant animals showed that alone, Myf 5 was unable to bring about differentiation, although specification of myoblasts was not affected. These results suggest that these myogenic factors possess specialized functions. However, the remarkably low level of *Myf5* available in triple mutant neonatal muscle leaves open the possibility that it is the total level of myogenic bHLH transcription factors that is critical to the completion of muscle differentiation.

The auto- and cross-regulation that the myogenic bHLH factors provide for one another, combined with their functional redundancy, comprises a mechanism whereby myogenesis is induced and maintained. Members of the MEF2 family of transcription factors cooperate with the myogenic bHLH factors to control the expression of muscle-

specific genes, thereby contributing to the maintenance and amplification of muscle development. To determine the mechanisms that regulate the expression of *MEF2C*, the earliest of the MEF2 factors expressed in the myogenic lineage, the mouse *MEF2C* gene was analyzed for *cis*-regulatory elements that direct its expression in the skeletal muscle lineage *in vivo*. As described herein, such a control region was identified, characterized and shown to be a direct transcriptional target of myogenic bHLH and MEF2 proteins. These results further define the regulatory circuit that induces, amplifies and maintains myogenesis *in vivo*.

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%	percent
+/+	homozygous wild-type
+/-	heterozygous
+1	transcriptional start site
-/-	homozygous null
α-SkAct	alpha-skeletal actin
aa	amino acid
AChR-δ	acetylcholine receptor-delta
bFGF	basic fibroblast growth factor
bHLH	basic helix-loop-helix
bp	base pairs
C	celsius
cDNA	complimentary deoxyribonucleic acid
cpm	counts per minute
DAB	diaminobenzidine tetrahydrochloride
dCTP	deoxycytidine triphosphate
DMEM	Dulbecco's Modified Eagle's Medium
DML	dorsomedial lip
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside tri-phosphate
DTT	dithio-thrietol
E	embryonic day
eMHC	embryonic myosin heavy chain
EMSA	electrophoretic mobility shift assays
EST	expressed sequence tag
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

H&E	hematoxylin and eosin
hr	hour
HS	horse serum
hsp	heat shock promoter
IHC	immunohistochemistry
kb	kilobases
lacZ	beta galactosidase gene
MCK	muscle creatine kinase
MHC	myosin heavy chain
min	minute
mRNA	messenger ribonucleic acid
NCE	neural crest enhancer
NLS	nuclear localization signal
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PF	paraformaldehyde
PK	proteinase k
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
RT-PCR	reverse transcriptase - polymerase chain reaction
SDS	sodium dodecyl sulfate
sec	second
Taq	*taq* polymerase
U	units
UTR	untranslated region

CHAPTER I

INTRODUCTION

Life begins with the creation of a unicellular zygote through the process of fertilization. As time progresses, this single cell goes on to form a complex organism comprised of multiple interdependent organ systems. These systems are in turn composed of cells highly specialized in both structure and function. The primary focus of developmental research is to understand the mechanisms by which vastly different tissue types are derived from pluripotential precursor cells in the developing embryo. Through the analysis of the development of a single tissue type, a better understanding can be gained for the process of lineage determination. The development of skeletal muscle has been studied as a model for understanding the mechanisms that control cell specification and differentiation during embryogenesis. Through these studies it has been determined that muscle development is an intricate process driven by both cell autonomous and non-cell autonomous molecular signals. The intricacy of myogenesis is compounded by spatiotemporal circumstances in the developing embryo such that the these intrinsic and extrinsic influences are differently integrated by myogenic cells throughout the embryo, resulting in the complex patterning of skeletal muscle that is observed *in vivo*.

SKELETAL MUSCLE DEVELOPMENT FROM EMBRYONIC MESODERM

Myogenesis *in vivo* occurs by a two step mechanism. First, cells become committed to the myogenic lineage, although they express no markers of differentiated muscle. These committed myoblasts then proceed to differentiate into mature muscle fibers by fusion into multinucleate myotubes, expression of muscle structural genes and the assembly of these structural proteins, creating a functioning muscle fiber (Hauschka, 1994). It is well established that the majority of vertebrate skeletal muscle is derived from the paraxial mesoderm of the developing embryo (Gilbert, 1991; Hogan et al., 1994), while some head musculature, including the tongue and the extraocular muscles of the eye, is derived wholly or in part from the prechordal plate or cranial mesoderm (Hogan et al., 1994; Noden, 1983; Wachtler et al., 1984). Interestingly, there is also evidence to suggest that some skeletal

muscle, specifically that of the esophagus, is derived from the transdifferentiation of smooth muscle (Patapoutian et al., 1995a).

The paraxial mesoderm is composed of pluripotential mesodermal cells that are derived from the ectodermal cells that migrate through the primitive streak to form embryonic mesoderm during gastrulation (Gilbert, 1991; Hogan et al., 1994). Beginning at embryonic day 8.0 (E8.0) in the mouse embryo (Kaufman, 1992), the paraxial mesoderm, which is located longitudinally along either side of the neural tube and notochord, segments to form epithelialized spheres of cells called somites. These somites pinch off from the paraxial mesoderm in a rostral to caudal fashion, such that the oldest somites are the most anterior and the most recently formed are the most posterior. Each somite initially gives rise to two populations of committed cells. The cells in the ventral portion of the somite undergo mitosis, subsequently losing their epithelial characteristics, and migrate away from the somite, forming the sclerotome (Christ et al., 1978). The sclerotome contains the precursors of the axial skeleton. The remaining dorsal epithelial cells of the somite constitute the dermomyotome from which both muscle and dermis arise.

Skeletal muscle development begins in the rostral somites of the mouse embryo at E8.0 (Hauschka, 1994). Adjacent to the neural tube, the growing craniomedial corner of the dermomyotome is the first to turn ventrally, forming a dorsomedial lip (DML) (Christ and Ordahl, 1995; Kaehn et al., 1988). The subjacent translocation of muscle precursors, or myoblasts, from the edges of the dermomyotome forms a new layer of cells, called the myotome (Christ et al., 1978; Denetclaw et al., 2001). The myotome is composed of post-mitotic myoblasts that are committed to the skeletal muscle lineage, but that are not yet differentiated. The cells of the myotome elongate cranially and caudally, forming fibers that eventually span the somite (Christ and Ordahl, 1995; Denetclaw et al., 1997; Kaehn et al., 1988; Ordahl et al., 2001). The myotome gives rise to the epaxial muscle of the deep back (Ordahl and Le Douarin, 1992). The DML also drives the growth and morphogenesis of the dermomyotome (Ordahl et al., 2001), which also contains muscle precursors. The myoblasts of the ventrolateral dermomyotome invade the somatic mesoderm to form the hypaxial musculature of the trunk. At the level of the limb, the ventrolateral dermomyotomal cells

invade the limb, eventually giving rise to limb muscle (Christ et al., 1983; Ordahl and Le Douarin, 1992).

ROLE OF THE MYOGENIC BHLH TRANSCRIPTION FACTORS IN MYOGENESIS

Skeletal muscle formation is controlled by the MyoD family of skeletal muscle-specific myogenic basic-Helix-Loop-Helix (bHLH) transcription factors. Evidence for the existence of multiple cell-autonomous muscle determination factors comes from a variety of studies in which non-muscle cells are converted to the muscle phenotype (Konieczny and Emerson, 1984; Pinney et al., 1988). This conversion to muscle coincides with the activity of myogenic bHLH family members, which are expressed in undifferentiated myoblasts and differentiated myocytes *in vitro* (Braun et al., 1989a; Braun et al., 1990a; Braun et al., 1989b; Davis et al., 1987; Edmondson and Olson, 1989; Miner and Wold, 1990; Schafer et al., 1990; Tapscott et al., 1988; Wright et al., 1989). The vertebrate myogenic bHLH family of transcription factors is made up of four members - MyoD, myogenin, Myf5 and MRF4. Members of this myogenic gene family have also been identified in *Drosophila*, *C. elegans* and sea urchin (Chen et al., 1992; Michelson et al., 1990; Paterson et al., 1991; Venuti et al., 1991), indicating that the myogenic program has been highly conserved throughout evolution.

The forced expression of any of the myogenic bHLH factors in non-muscle cells *in vitro* induces their conversion to stable myoblasts, as demonstrated by the expression of muscle-specific genes, and their fusion into multinucleate syncytia (Braun et al., 1989b; Weintraub et al., 1989). The inhibition or absence of myogenic bHLH expression leads to defects in myogenesis (Brennan et al., 1990; Tapscott et al., 1989). In addition to activating the expression of muscle differentiation markers, members of this family are also able to activate one another's expression as well as their own (Braun et al., 1990a; Miner and Wold, 1990; Thayer et al., 1989). This cross- and auto-regulatory loop provides a mechanism by which myogenic differentiation can be maintained and amplified *in vivo*.

Myogenic bHLH transcription factors are exclusively expressed in differentiated skeletal muscle and skeletal muscle precursors (Braun et al., 1990a; Edmondson and Olson, 1989).

During development, *Myf5*, *MyoD*, *myogenin* and *MRF4* have overlapping, but distinct, spatiotemporal expression patterns (Fig. 1). *Myf5* is the first of the myogenic bHLH genes to be expressed during mouse embryogenesis, with transcripts appearing in the rostral dermomyotomes at embryonic day E8.0 (Ott et al., 1991). *Myf5* is first detected in the forelimb and hindlimb buds at E10.5 and E11.0, respectively. Expression of *Myf5* in developing myotomes is maintained at high levels until E11.5. From this point its expression declines until E14.5, at which time *Myf5* becomes undetectable. *Myogenin* and *MyoD* are expressed in the myotome beginning at E8.5 and E10.0, respectively (Sassoon et al., 1989; Wright et al., 1989). At E11.5, when *Myf5* levels begin to decline, *myogenin* and *MyoD* are still expressed strongly in developing myotomes. *Myogenin* is detectable in the forelimb bud at E11.0. By E11.5, *myogenin*, *MyoD* and *MRF4* are detectable in both the forelimb and hindlimb buds (Hannon et al., 1992; Sassoon et al., 1989). *MRF4* is expressed transiently in the myotome between E9.0 and E11.5 and is subsequently down-regulated until later in development when it becomes expressed in differentiated muscle fibers (Bober et al., 1994; Hinterberger et al., 1991). In the forelimb bud, *MRF4* is first detectable at E10.5 (Hannon et al., 1992). *MRF4* is the highest expressed MyoD family member in adult muscle (Miner and Wold, 1990).

The differences in the gene expression patterns of the four myogenic bHLH factors suggest that they may have different functions *in vivo* in spite of their apparent functional redundancy in tissue culture. Loss-of-function experiments *in vivo* have allowed for the investigation of such redundancies. Somatic inactivation of *Myf5* in mice did not result in any apparent skeletal muscle phenotype (Braun et al., 1992). However, the formation of the myotome was delayed, as were the expression of *myogenin* and *MRF4* (Braun et al., 1994).

This indicates that Myf5 plays a role in the early development of the myotome and the induction of *myogenin* and *MRF4*. The eventual formation of normal muscle in this mutant suggests that other of the myogenic bHLH factors is able to substitute for Myf5. In fact, myotomal factors, including *myogenin* and *MRF4,* accumulated in the myotome of *Myf5-/-* embryos immediately following the normal activation of *MyoD* at E10.5 suggesting that MyoD is able to substitute for Myf5. The targeted inactivation of *MyoD* also

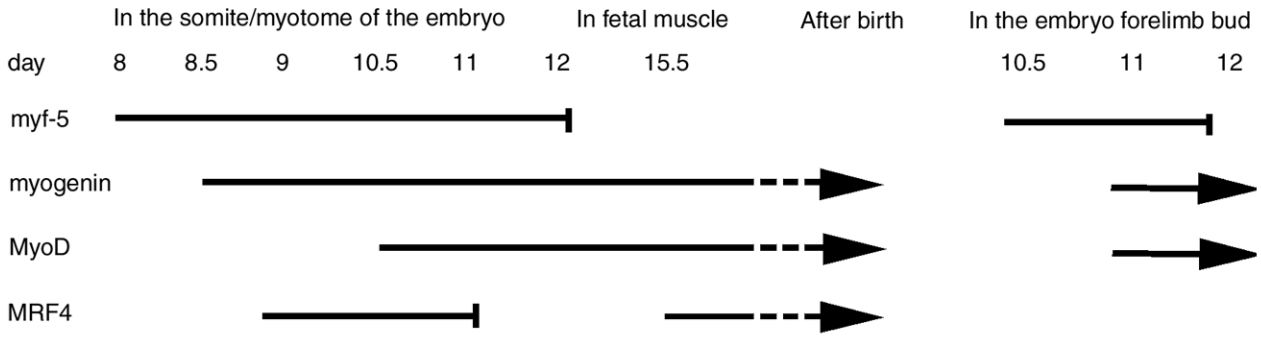

Figure 1. Expression patterns of myogenic bHLH transcription factors during mouse development (see text).

demonstrated no overt skeletal muscle phenotype, although *Myf5* levels do not decrease at E11.5, as they do in wild-type muscle (Rudnicki et al., 1992). The persistence of *Myf5* expression in postnatal muscle suggests that MyoD negatively regulates *Myf5 in vivo*. *MRF4-/-* embryos also show no gross defects in myogenesis, although *myogenin* levels in adult muscle are five-fold higher than normal in mutant animals (Olson et al., 1996; Zhang et al., 1995). Additionally a subset of muscle-specific genes are down-regulated in post-natal skeletal muscle. These data suggest that myogenin may be compensating for the lack of *MRF4*, but that gross muscle development is still not achieved at quite wild-type levels. In simplest terms it appears that Myf5, MyoD and MRF4 are each individually dispensable for myogenesis, due to the potential overlap in function of other of the myogenic bHLH factors. In fact, additional knock-out experiments have shown that MyoD and Myf5 play redundant roles in establishing myoblast identity. Animals lacking both *Myf5* and *MyoD* die at birth due to a complete lack of skeletal muscle (Fig. 2A) (Rudnicki et al., 1993). There is also no evidence of skeletal muscle precursors or the expression of skeletal muscle specific genes. Thus, Myf5 and MyoD appear to have overlapping roles in the commitment of mesodermal precursors to the myogenic lineage. In contrast, myogenin acts later to control myoblast differentiation (Hasty et al., 1993; Nabeshima et al., 1993). In mice lacking *myogenin*, myoblasts are specified and primary muscle fibers are formed, but secondary myogenesis fails to occur (Fig. 2B) (Venuti et al., 1995). The remainder of the presumptive muscle forming regions of *myogenin-/-* mice are populated by undifferentiated myoblasts. As expected there are significant decreases in the expression of various muscle-specific genes in the *myogenin-/-*. Interestingly, primary myoblasts derived from *myogenin-/-* mutant muscle differentiate as efficiently as those of wild-type. However *MRF4* levels in these cultured myoblasts are elevated four-fold, suggesting that MRF4 can compensate for the lack of *myogenin in vitro*, and potentially *in vivo*. Myogenin does not overlap in function with either Myf5 or MyoD, since combining the *myogenin*-null allele with either the *Myf5*- or *MyoD*-null alleles does not result in an exacerbated skeletal muscle phenotype over that of the *myogenin-/-*.

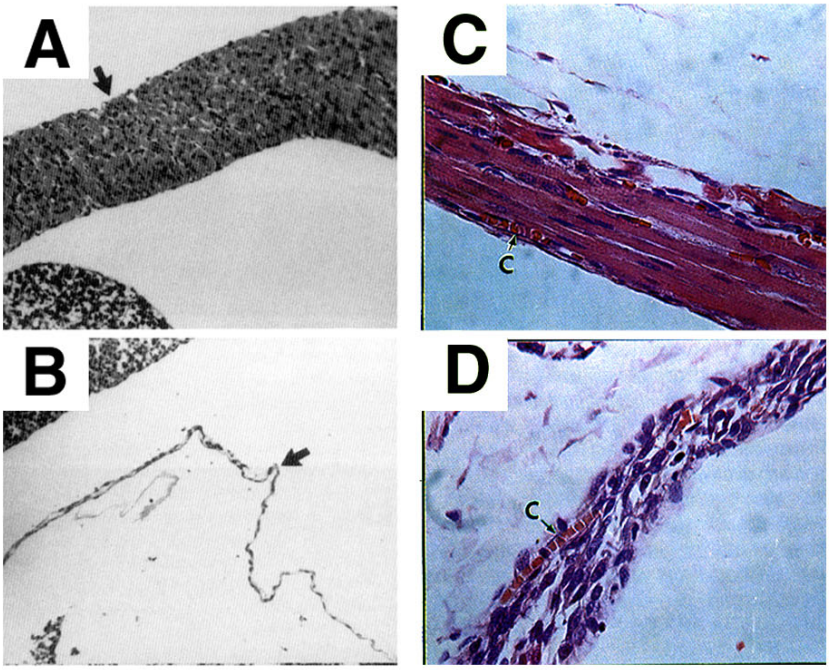

Figure 2. Skeletal muscle defects in two different myogenic bHLH mutants.
H&E sections through the diaphragm of (A) *Myf5+/+MyoD+/-*, (B) *Myf5-/-MyoD-/-*, (C) *myogenin+/+* and (D) *myogenin-/-* neonates. arrow, diaphragm; c, capillary. (A, B) Rudnicki et al., 1993. (C, D) Hasty et al., 1993.

FUNCTIONAL DOMAINS OF THE MYOGENIC BHLH PROTEINS

The myogenic bHLH proteins are about 80% homologous to one another within an approximately 70 amino acid (aa) domain containing a basic region followed by a helix-loop-helix motif. This bHLH region is the defining feature of the *myc* superfamily of proteins, which have been shown to be involved in transcription, differentiation and proliferation. Members of this family dimerize through the association of their amphipathic α-helices, forming a 4-helix bundle in which the helices are parallel to one another. This dimerization juxtaposes the basic regions of the dimer partners, forming a bipartite DNA binding domain (Anthony-Cahill et al., 1992; Murre et al., 1989a). It was shown previously that nuclear extracts from undifferentiated myoblasts and differentiating myocytes contain DNA-binding activity specific to a sequence in the 5' muscle creatine kinase (MCK) enhancer (Buskin and Hauschka, 1989). The core of this sequence conforms to the CANNTG nucleotide consensus sequence, known as an E-box, bound by members of the *myc* superfamily. E-boxes have been found in the regulatory regions of most skeletal muscle specific genes (Li et al., 1993; Li and Paulin, 1991). Through mutagenesis studies, many of these E-boxes have been found to be required for some or all of the activity of these regulatory regions (French et al., 1991; Li and Capetenaki, 1994; Li and Paulin, 1993; van de Klundert et al., 1994; Wentworth et al., 1991). Required E-boxes are also found in the transcriptional regulatory regions of *MyoD*, *myogenin* and *MRF4* (Asakura et al., 1995; Black et al., 1995; Buchberger et al., 1994; Cheng et al., 1992; Cheng et al., 1993; Edmondson et al., 1992; Malik et al., 1995; Naidu et al., 1995; Yee and Rigby, 1993). This supports the finding that myogenic bHLH factors are able regulate the expression of one another and themselves in tissue culture.

Myogenic bHLH transcription factors are able to bind to consensus E-boxes in the regulatory regions of skeletal muscle-specific genes and activate their transcription (Braun et al., 1990a; Braun et al., 1989b; Chakraborty et al., 1991a; Edmondson and Olson, 1989; Li and Capetanaki, 1993). As would be expected, sequence-specific DNA binding of these myogenic factors requires the *myc* homology domain (Lassar et al., 1989). Mutations that disrupt dimerization also disrupt DNA binding (Voronova and Baltimore, 1990). As homodimers myogenic bHLH transcription factors do not bind DNA with high affinity, nor

do they form homodimers efficiently (Chakraborty et al., 1992). Rather, myogenic bHLH proteins heterodimerize with ubiquitously-expressed bHLH-containing E-proteins. Through heterodimerization with E-proteins, these myogenic transcription factors acquire a high affinity for E-boxes (Brennan and Olson, 1990; Chakraborty et al., 1991b; Murre et al., 1989b). The transcriptional activity of the myogenic bHLH factors requires dimerization with E-proteins, and subsequent DNA binding (Lassar et al., 1991). The bHLH region, however, does not have transcriptional activity (Schwarz et al., 1992). The transactivation domains of these myogenic factors lie in the regions outside of the conserved bHLH domain (Braun et al., 1990b; Chakraborty and Olson, 1991; Mak et al., 1992).

While the basic domains of both the E-proteins and the myogenic bHLH factors contribute to sequence-specific DNA binding, it has been shown that conserved residues within the basic domains of myogenic bHLH transcription factors are required for myogenic specificty (Davis et al., 1990). Swapping the basic region of myogenin for that of a ubiquitous E-protein does not prevent DNA binding, but the chimeric protein can not activate myogenesis (Brennan et al., 1991). Vice versa, mutation of residues in the basic domain of a non-myogenic E-protein to those of MyoD confers myogenic specificity to that E-protein. (Davis and Weintraub, 1992; Weintraub et al., 1991). One of the conserved myogenic residues, a threonine, with surrounding residues constitute a Protein Kinase C phosphorylation site. The phosphorylation of this site in myogenin prevents it from binding DNA and is the basis for the negative regulation of myogenin activity by Fibroblast Growth Factor (Li et al., 1992). Analysis of the crystal structure of the MyoD basic region bound to DNA shows that these conserved residues are buried in the protein-DNA interface (Ma et al., 1994). It is therefore likely that the mechanism of inhibition of DNA binding by phosphorylation of this site is due to a change in the conformation of the DNA binding domain.

THE CONTRIBUTION OF THE MEF2 FAMILY OF MADS-BOX CONTAINING TRANSCRIPTION FACTORS TO MYOGENESIS

A second family of proteins, the MEF2 family of transcription factors, has also been shown to be important in the development of skeletal muscle. Muscle Enhancer Factor-2 (MEF2) activity was initially identified as a DNA-binding activity specific to nuclear extracts from differentiated myotubes and myocytes (Gossett et al., 1989). MEF2 proteins bind an AT-rich consensus sequence, YTA(A/T)$_4$TAR (Black and Olson, 1998; Cserjesi and Olson, 1991; Fickett, 1996b), in the control region of many skeletal muscle-specific genes (Cserjesi et al., 1994; Horlick et al., 1990; Kuisk et al., 1996; Li and Capetenaki, 1994; Li and Paulin, 1993; Parmacek et al., 1994; Wentworth et al., 1991). The occupancy of MEF2 binding sites has been associated with the positive differentiation state of skeletal muscle (Mueller and Wold, 1989). MEF2 is also induced in cells that have been induced to differentiate by the expression of myogenic bHLH transcription factors. MEF2 expression is blocked by mitogens which also block myogenesis (Cserjesi and Olson, 1991; Gossett et al., 1989). The MEF2 family members are encoded by novel early muscle genes, in that they are upstream of structural genes, but downstream of the myobHLH genes. Interestingly, MEF2 proteins are able to directly regulate the expression of some myogenic bHLH transcription factors through MEF2 binding sites in their promoters (Buchberger et al., 1994; Cheng et al., 1993; Edmondson et al., 1992; Naidu et al., 1995; Yee and Rigby, 1993). It is interesting, however, that unlike the myogenic bHLH factors, MEF2 proteins are not able to induce the, myogenic program on their own. Taken together, these finding suggest that the role of MEF2 proteins is to amplify and maintain the myogenic phenotype induced by the myogenic bHLH directly, by activating skeletal muscle-specific genes, and indirectly, by the activation of the expression of myogenic bHLH proteins.

Four vertebrate MEF2 factors have been identified, MEF2A, MEF2B, MEF2C and MEF2D (Breitbart et al., 1993; Martin et al., 1993; Pollock and Treisman, 1991; Yu et al., 1992). Additionally, evolutionarily conserved MEF2 family members have also been identified in *Drosophila* and *C. elegans* (Black and Olson, 1998; Bour et al., 1995; Lilly et al., 1994). MEF2 factors belong to the MADS-box containing family of transcription factors (Shore and Sharrocks, 1995). Members of this family contain a consensus DNA-binding and dimerization domain, known as a MADS-box (Pellegrini et al., 1995). Within this domain,

MEF2 proteins share a high degree of homology with the other MADS-box proteins (Pollock and Treisman, 1991). Among each other the MEF2 proteins share approximately 80% homology within the MADS domain. There is a second region of homology, the MEF2 domain, exclusive to the MEF2 proteins, which is located immediately c-terminal to the MADS box. Together the MADS and MEF2 domains are responsible for the dimerization of MEF2 proteins and their subsequent DNA binding activity. The c-terminal portion of MEF2 proteins serves as a transactivation domain and contains a nuclear localization signal (NLS). In contrast to the skeletal muscle specificity of myogenic bHLH factors, MEF2 factors are expressed in skeletal, cardiac and smooth muscle cells, as well as in neurons (Edmondson et al., 1994; Lyons et al., 1995; Ticho et al., 1996), and at lower levels in several other cell types. During embryogenesis, *MEF2C* is expressed at the onset of differentiation of the cardiac and skeletal muscle lineages (Fig. 3) and is followed by the expression of the other *MEF2* genes (Edmondson et al., 1994). Mice lacking *MEF2C* die at about E9.5 from cardiovascular defects, precluding analysis of the role of *MEF2C* in skeletal muscle development *in vivo* (Lin et al., 1997). Mice homozygous for mutations in *MEF2A* or *MEF2B* are viable, whereas mice lacking *MEF2D* die prior to gastrulation (unpublished results). A loss-of-function mutation in the single *MEF2* gene in *Drosophila* results in a block to differentiation of all muscle cell types (Bour et al., 1995; Lilly et al., 1995; Ranganayakulu et al., 1995), indicating that *MEF2* genes are required for muscle differentiation.

The existence of a second family of transcription factors important in myogenesis was expected, due to the fact that many skeletal muscle-specific genes induced by the myogenic bHLH transcription factors during myogenesis do not contain E-boxes, or contain E-boxes that are not required for transcriptional activation (Bouvagnet et al., 1987; Buchberger et al., 1994; Cheng et al., 1992; Edmondson et al., 1992; Mar and Ordahl, 1990; Peterson et al., 1990; Thompson et al., 1991). Many of these genes were found to have AT-rich sequences in their regulatory regions that are critical for their transcriptional activation. These AT-rich sequences have been found to conform to the consensus DNA-binding site for MEF2 factors. Through mutation and deletion analysis, these MEF2 sites have been shown to be required

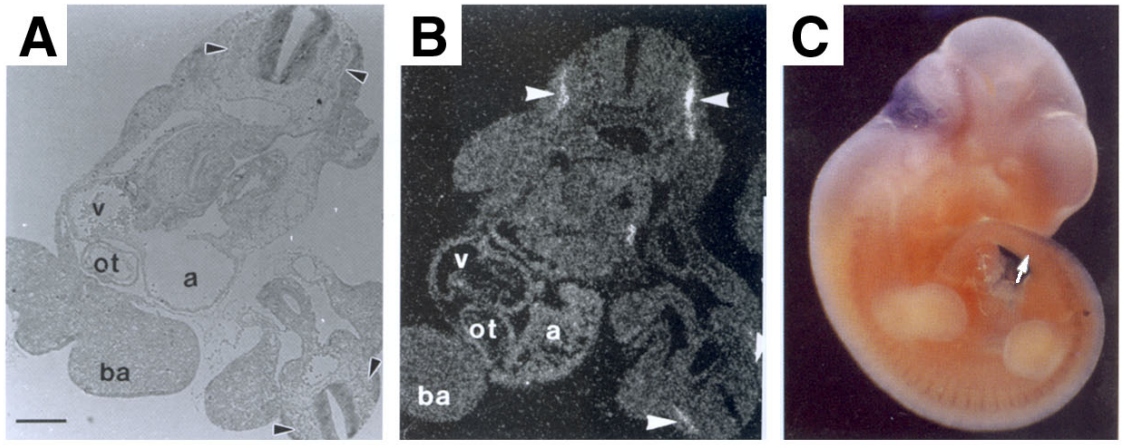

Figure 3. Expression of *MEF2C* in muscle-forming regions. (A) Phase-contrast micrograph of a transverse section through the rostral somites of an E10.5 mouse embryo. This section is adjacent to that shown in (B). (B) Detection of *MEF2C* transcripts in the myotome of an E10.5 mouse embryo. (C) Detection of *MEF2C* transcripts in the myotome of an E11.5 mouse embryo. a, atrium; ba, branchial arch; ot, outflow tract of developing heart; v, ventrical; arrowhead, myotome; arrow, the last somite that shows expression of *MEF2C* transcripts. Bar, in (A), 50 µm. (Edmondson *et al*., 1994.)

for the transcriptional activity of these promoters and enhancers. Additionally, MEF2 sites have been found in close proximity to E-boxes in the regulatory regions of various skeletal-muscle specific genes, implying that MEF2 proteins and myobHLH factors can act in concert during myogenesis (Fickett, 1996a; Wright et al., 1991). MEF2C and MyoD have been shown to synergistically activate myogenesis *in vitro*, as well as synergistically activate transcription through either E-boxes or MEF2 binding sites or a combination of both (Fig. 4) (Black et al., 1998; Molkentin et al., 1995).

OBJECTIVES OF THE DISSERTATION RESEARCH

It is evident that myogenic bHLH and MEF2 transcription factors play an important role in the development of skeletal muscle, however various aspects of muscle development with regard to these factors are still unclear. At the start of this work no specific role had been defined for MRF4. It was not known to what extent a single myogenic bHLH transcription factor could support myoblast specification or differentiation *in vivo*. The lack of characterization of the transcriptional regulatory regions of the *MEF2C* gene had precluded the analysis of its specific role in myogenesis. The objective of this thesis work was to gain a better understanding of the place MRF4 and MEF2C occupy in regulatory networks that control myogenesis by addressing these issues.

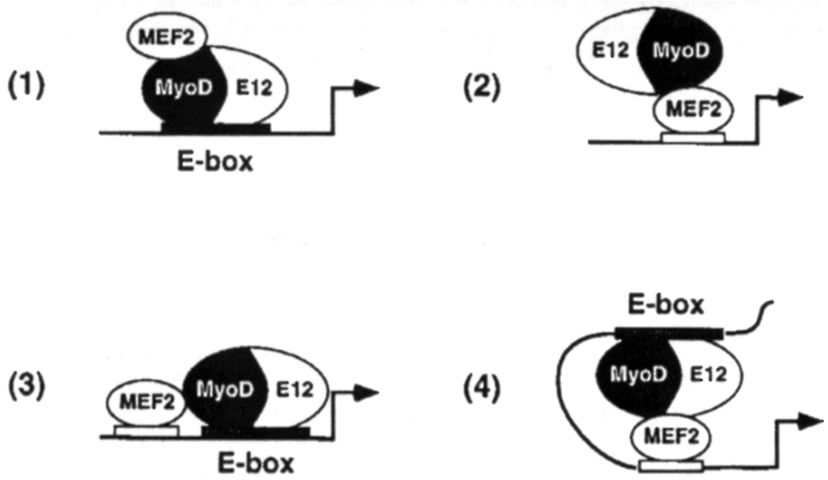

Figure 4. Models of endogenous gene expression directed by various MEF2 and myogenic bHLH factor interactions. (Model 1) some muscle-specific promoters only require E-box elements to direct tissue restricted expression, yet MEF2 may still be part of this regulation via protein-protein interaction. (Model 2) a number of muscle-specific promoters that contain MEF2 binding sites may recruit members of the myogenic bHLH family by protein-protein interaction with MEF2. (Model 3) Many muscle-specific promoters contain both MEF2 sites and E-box sites spaced adjacent to one another in an enhancer or in a basal promoter. A local protein-protein interaction may enhance the affinity of each factor for its site. (Model 4) Some muscle-specific promoters have E-boxes and MEF2 sites spaced far apart from one another. Examples exist in which MEF2 factors recognize the TATA box, and in this situation, it could then interact with myogenic bHLH factors bound to distal enhancer E-box sites, thereby providing a mechanism for promoter-enhancer association. (Molkentin and Olson, 1996.)

CHAPTER II

EXPERIMENTAL METHODS

INTERCROSSES

The *myogenin* mutant mice were described previously (Hasty et al., 1993). Briefly, animals heterozygous for the *myogenin*-null allele (*myogenin+/-*) are viable, whilee mice homozygous for the *myogenin*-null allele (*myogenin-/-*) die within a few hours of birth The *MRF4* mutant mice used for this study have also been described (Zhang et al., 1995). The *MyoD* mutant mice were a gift from Dr. M. Rudnicki (McMaster University) and have also been previously described (Rudnicki et al., 1992). Mice harboring an *MRF4*-null allele or a *MyoD*-null allele are fully viable as heterozygotes and homozygotes. All mutations were maintained in a C57Bl6 background.

To produce the desired *myogenin-/-MRF4-/-* mutants, mice heterozygous for the *myogenin*-null mutation were crossed with animals homozygous for the *MRF4* mutant allele. Resulting *myogenin+/-MRF4+/-* mice were intercrossed to produce the desired double mutant, as well as *myogenin+/-MRF4-/-* mice that could also be bred to obtain double-null animals. During the studies described herein, it was determined that only animals lacking both *myogenin* alleles demonstrated defects in skeletal muscle development and died within hours of birth. We, therefore, interchangeably used animals bearing genotypes 1-4 of Table 4 as wild-type littermate controls (Figs. 5-7 and 11).

To obtain *MyoD-/-MRF4-/-* mutant animals, *MyoD-/-* animals were bred with *MRF4-/-* animals to obtain *MyoD+/-MRF4+/-* mutants, which were then intercrossed. Subsequent *MyoD+/-MRF4+/-* or *MyoD+/-MRF4-/-* progeny were then intercrossed to obtain the desired *MyoD/MRF4* double-null animals. It was observed that, while *MyoD-/-MRF4-/-* animals were not viable, animals of all other genotypes were viable and displayed no obvious skeletal muscle defect. Thus, animals bearing genotypes 1-2 of Table 6 were used interchangeably as wild-type littermate controls (Figs. 8-10).

Bearing in mind the lethality of various genotypic combinations of myogenic bHLH mutants, *myogenin+/-MyoD+/-MRF4-/-* mutant mice were generated and bred in order to obtain the desired *myogenin-/-MyoD-/-MRF4-/-* mutant mice. Viable progeny from these

crosses (see Table 7) were used interchangeably as wild-type littermate controls in Figs. 12-20. Further, *myogenin/MRF4* double mutants are those animals homozygous for mutations in both *myogenin* and *MRF4* (genotypes 7-8 of Table 7). *MyoD/MRF4* double mutants are defined as mice homozygous for mutations in *MyoD* and *MRF4* (genotypes 3 and 6 of Table 7).

GENOTYPING

Mice carrying somatic mutations in *myogenin*, *MyoD* or *MRF4,* or animals containing *lacZ* transgenes, were identified by Southern Blot or PCR (Polymerase Chain Reaction) analysis. Genomic DNA was isolated as described (Zhang et al., 1995). Briefly, tissue from tail biopsies or embryonic yolk sacs was digested in lysis buffer (10 mM Tris (pH 8.0), 25 mM EDTA, 100 mM NaCl, 1% SDS, 0.2 mg/ml Proteinase K (PK)) at 55 ^{o}C overnight (ON), followed by removal of protein by phenol/chloroform extraction and ethanol precipitation.

For Southern Blot analysis, genomic DNA was digested with appropriate restriction endonucleases, fractionated on 0.8% agarose gels and blotted to Zeta-probe GT membranes (BIO-RAD). The presence of the mutant and wild-type alleles were determined by probing the membrane with a gene-specific [α-^{32}P]-labeled DNA fragment, followed by autoradiography. For the *myogenin* mutant allele, genomic DNA was digested with *Sac I* and probed with a 350 bp *Sma I-Kpn I* fragment of the *myogenin* promoter (Edmondson et al., 1992). The probe hybridizes with a 1.3 kb wild-type band and a 2.2 kb mutant band. The *MyoD* mutant and wild-type alleles were detected by digesting genomic DNA with *Xba I* and probing with a 600 bp fragment from the 5' end of the *MyoD* cDNA. This probe recognizes a 4.0 kb wild-type band and a 5.0 kb mutant band. For the *MRF4* mutant allele, genomic DNA was digested with *KpnI* and probed with a 300 bp fragment from the first exon of *Myf5*. This probe hybridizes with an 8.0 kb wild-type band and a 7.0 kb mutant band.

Following are the typical reaction conditions for PCR genotyping: genomic DNA template, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.16 mM of each dNTP, 1 μM of each primer and 2.5U (Units) *Taq* polymerase (Promega) in a 25 μl total volume. A typical temperature profile included 32 cycles of DNA strand melting at 95

^{O}C for 30 seconds (sec), primer annealing at 55 ^{O}C for 30 sec and polymerization at 72 ^{O}C for 30 sec. Genotype analysis performed by PCR on genomic DNA used the primers listed in Table 1.

HISTOLOGY

The preparation, sectioning and staining of embryonic tissue was performed using standard procedures. Prior to fixation, neonates were skinned and eviscerated. Embryos and neonatal mice were then fixed in 4% paraformaldehyde (PF) in phosphate buffered saline (PBS) ON at 4 ^{O}C. Next, these were embedded in paraffin after a stepwise dehydration in progressively higher concentrations of ethanol and two changes of xylene. These were sectioned in increments of 5-7 μm. Sections were stained with hematoxylin and counterstained with eosin (H& E). Embryos stained with lacZ were counterstained with nuclear fast red. Sections used for RNA *in situ* hybridization were counterstained with hematoxylin. Sections were then cover-slipped with cytoseal or permount. Sections to be used for immunohistochemistry (IHC) were left unstained and were not cover-slipped.

IMMUNOHISTOCHEMISTRY

Tissue sections were prepared for immunostaining by deparaffinization in two changes of 100% xylene, 5 minutes (min) each, and hydration through graded ethanols to PBS. Sections were permeabilized in two changes of 0.3% TritonX-100 (Sigma) in PBS, for 5 min each. Quenching of endogenous peroxidases was achieved through the incubation of sections in 0.3% H_2O_2 in methanol for 30 min. Binding to non-specific antigenic sites was blocked by incubation in 1.5% normal horse serum (HS) in PBS for 30 min. The primary antibodies applied to the paraffin-embedded sections were the mouse myosin heavy chain (MHC) antibody MY-32 (Sigma) at a 1:400 dilution in PBS or the mouse desmin antibody (Biogenex) at a 1:300 dilution in PBS ON. According to the Vectastain ABC system (Vector Laboratories), diluted biotinylated secondary horse anti-mouse antibody was applied and then visualized using peroxidase streptavidin and the diaminobenzidine tetrahydrochloride (DAB) chromogen. Sections were then counterstained with hematoxylin.

Table 1. Primers used for PCR genotyping.

Gene	Allele	Forward Primer (5' to 3' direction)	Reverse Primer (5' to 3' direction)	Product Size (bp)
myogenin	WT	GAACAAGCCTTTTCCGACCTGATG	GGTCCACCGACACAGACTTCCTCTT	300
	mutant	GAACAAGCCTTTTCCGACCTGATG	GGTCCACCGACACAGACTTCCTCTT	1450
MyoD	WT	AAGCTCCGCCCTACTACACTCCTATTG	AGGTTCTGTGGGTTGGAATGCACA	231
	mutant	AAGCTCCGCCCTACTACACTCCTATTG	TTGGCTACCCGTGATATTGCTGAAGAG	465
MRF4	WT	ATCTGGGTGGCTCCTCTGGGTTTT	GGCTTCGTTGATTTTCTTTAGTCTCCTC	406
	mutant	ATCTGGGTGGCTCCTCTGGGTTTT	TTGGCTACCCGTGATATTGCTGAAGAG	678
lacZ	WT	CAAACTGGCAGATGCACGGTTAC	CAGTACAGCGCGGCTGAAATC	450

The above primer sets were used for the PCR amplification of allele specific products from mouse genomic DNA derived from tail biopsies or yolk sacs, as described in Experimental Methods (Chapter 2).

Differentiated primary myoblasts to be analyzed by immunostaining were washed in PBS and then fixed in 3.7% PF in PBS for 30-60 min. Cells were washed 3 times with ice-cold 0.1 M glycine in PBS and then permeabilized for 10 min at room temperature (RT) in 0.1% Triton X-100 (Sigma) in PBS. Immunohistochemistry for mouse MHC was performed on fixed cells using MY-32 (Sigma) on fixed cells according to the Vectastain ABC system (Vector Laboratories) as detailed above for paraffin tissue sections. Alternatively, an avidin-conjugated FITC-labeled secondary antibody was used and MHC-expressing cells were detected by immunofluorescence.

IN SITU HYBRIDIZATION

The vector pBSmyf5S containing approximately 90 bp (base pairs) of 5'-UTR (untranslated region) and the entire *Myf5* coding sequence was kindly provided by J.K. Yoon (California Institute of Technology). A *Sal I* linearized fragment of pBSmyf5S was transcribed with T7 RNA polymerase to create an antisense RNA probe. For the control sense RNA probe, an *EcoRI* linearized fragment of pBSmyf5S was transcribed using T3 RNA polymerase. RNA probes were radiolabeled with ^{35}S-UTP (>1, 000 Ci/mmol) (Amersham) using the MAXIscript T3/T7 system (Ambion). *In situ* hybridization on paraffin sections was performed as previously described (Wilkinson et al., 1987) with minor modifications (Frohman et al., 1990). Mouse embryos at E11.0 were fixed and embedded in paraffin as described above for neonates. Following prehybridization procedures, sections were hybridized ON at 50 ^{o}C with sense or antisense probes, 7.5X10^{5} cpm (counts per minute) per slide. Unhybridized probe was then removed through stringent washes and RNAse A treatment. Slides were subsequently coated with K.5 nuclear emulsion (Ilford, UK) and exposed for 7-14 days (dd) at 4 ^{o}C. The slides were developed, counterstained with hematoxylin and visualized by bright- and dark-field optics.

PRIMARY MYOBLAST DIFFERENTIATION

Primary myoblasts were prepared from the forelimbs and hindlimbs of neonates as described (Freshney, 1994), with modifications. Limbs were severed from the neonate,

skinned and placed in 100 U/μl collagenase and incubated at 37 ^{o}C, 5% CO_2 for 30 min. Muscle tissue was then dissected away from the bone and was further incubated in the collagenase at 37 ^{o}C, 5% CO_2 for an additional 30-120 min. Throughout this incubation time tissue was titurated every 10 min, to aid in the disassociation of the myoblasts. 10% fetal bovine serum (FBS) in Dulbecco's Modified Eagle's Medium (DMEM) was added to stop collagenase activity. Cells were pelleted by centrifugation, resuspended in 15% FBS/DMEM and preplated on untreated plates for 60 min at 37 ^{o}C, 5% CO_2 to remove contaminating fibroblasts. Myoblasts were then plated on 0.1% gelatinized plates in growth media (15% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 5 nM basic Fibroblast Growth Factor (bFGF) in DMEM) and allowed to proliferate at 37 ^{o}C, 5% CO_2. Cells were fed every 2-3 days with growth medium until confluent, approximately 7-10 days. To induce differentiation, confluent cultures were transferred from growth medium to differentiation medium (2% HS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in DMEM) and were incubated at 37 ^{o}C, 5% CO_2 for approximately 5 dd.

RNA ISOLATION

Total cellular RNA was isolated from cultured myoblasts and from the carcasses of neonatal mice by homogenizing in TRIzol (Gibco BRL) for 1.5 min in 30 second (sec) intervals. Prior to homogenization, head, skin and internal organs were removed from neonatal carcasses. Phase separation was performed through the addition of chloroform and centrifugation at 12,000 X g for 15 min at 4 ^{o}C. 100% isopropanol was used to precipitate the aqueous phase. RNA was precipitated by centrifugation at 12,000 X g for 15 min at 4 ^{o}C. RNA pellets were washed in 70% ethanol, air-dried and resuspended in RNAse-free dH_2O.

NORTHERN ANALYSIS

Northern blot analysis was performed using standard techniques . *Acetylcholine receptor-δ* (*AchR-δ*) transcripts were detected using a full-length cDNA (provided by J. Sanes, Washington University School of Medicine). *MCK, MHC, α-skeletal actin* (*α-SkAct*) and *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* mRNA, whose expression is

independent of the state of muscle development, were detected using partial expressed sequence tag (EST) cDNA clones (Genome Systems).

RT-PCR ANALYSIS

Muscle-specific transcripts were detected by RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction) as previously described (Munsterberg and Lassar, 1995), with modifications (Rawls et al., 1995). Total RNA was used as a template for reverse transcription using Mo-MuLV reverse transcriptase (Gibco BRL) and a random hexamer primer as described previously (Rawls et al., 1998). The number of cycles required to generate a PCR product during linear amplification was determined for each primer pair. PCR reactions were done in the presence of [α-^{32}P]dCTP. PCR products were separated on a 5% non-denaturing polyacrylamide gel, dried, and visualized by autoradiography. The detection of *L7* transcripts was used a control, as the level of *L7* transcripts is independent of the state of muscle differentiation. Previously described primers, as well as those newly designed for these experiments, for detecting muscle-specific transcripts and sizes of PCR products are listed in Table 2.

BONE AND CARTILAGE STAINING

Embryos and neonates were stained for bone and cartilage as described (Hogan et al., 1994). Animals were skinned, eviscerated and fixed in 95% ethanol ON. These were stained for cartilage with alcian blue stain for approximately five hours. These were rinsed with 95% ethanol and again fixed ON, twice. Samples were cleared in 1% KOH for approximately 1 hour (hr). Bone is counterstained in alizerin red for 1-3 hrs. Samples were cleared using progressively decreasing strengths of KOH in glycerol to 100% glycerol. In order to determine the average length of the ossified portions of the ribs for any animal, ribs 4, 5 and 6 from both the right and left side of the skeleton were removed, laid flat and images were captured. Color images were enlarged such that a length of the ossified portion of each rib could be easily determined. Lenths of mutant ribs are given as % difference from average wild-type rib length.

Table 2. Primers used for RT-PCR

Gene	Forward Primer (5' to 3' direction)	Reverse Primer (5' to 3' direction)	Product Size (bp)
myogenin[1]	TGGAGCTGTATGAGACATCCC	TGGACAATGCTCAGGGGTCCC	184
MyoD[2]	GCAGGCTCTGCTGCGCGACC	TGCAGTCGATCTCTCAAAGCACC	370
MRF4[3]	CTACATTGAGCGTCTACAGGACC	CTGAAGACTGCTGGAGGCTG	234
Myf5[4]	TGTATCCCCTCACCAGAGGAT	GGCTGTAATAGTTCTCCACCTGTT	379
MEF2C[5]	CATGCCGCCATCTGCCCTCAG	CCCTTTCGTCCGGCGAAGGTC	305 or 211*
α-SkAct+	CAGAGCAAGCGAGGTATCC	GTCCCCAGAATCCAACAC	436
eMHC+	CACCTGGAGAGGATGAAGAAGAA	AAGACTTGACTTTCACTTGGAGTTTATC	300
desmin+	GTGGAGCGTGACAACCTGAT	GATGGTCTCATACTGAGCCCG	321
MCK+	CAGACCTCAGCAAGCACAACAATCAC	GCGGAGGCAGAGTGTAACCCTTG	368
AchR-δ+	AGCCATCATTGTTGTTCTC	AAGTCTCACCCTCTCCAA	196
L7[6]	GGAGCTCATCTATGAGAAGGC	AAGACGAAGGAGCTGCAGAAC	202

The above primer sets were used for the PCR amplification of the indicated genes from reverse-transcribed RNA derived from neonatal muscle or differentiated myoblasts, as described in Experimental Methods (Chapter 2). +Primers were designed such that they span an intron. To ensure that equivalent amounts of total RNA were used in different samples, template primers specific to the ribosomal protein L7, which is not affected by myogenesis, were used as a control. Previously decribed RT-PCR primer sets: [1](Edmondson and Olson, 1989), [2](Hannon et al., 1992), [3](Patapoutian et al., 1993), [4](Hannon et al., 1992), [5](Martin et al., 1993) and [6](Hollenberg et al., 1993). *Indicates a different size PCR product due to alternative splicing.

5'-RACE

5'-RACE (Rapid Amplification of cDNA Ends) cloning was performed as described previously (Wang et al., 1999). Briefly, total RNA was isolated from adult mouse skeletal muscle using TRIzol reagent (LifeTech). 5 μg of RNA was used for first-strand cDNA synthesis with random hexamers. A PCR-based RACE procedure was carried out using the Marathon cDNA amplification kit (Clontech) following the manufacturer's protocol. *MEF2C* gene-specific primers were as follows:

2C-RACE1, 5'-GTGTTTCTTCTCTCTCTCGTCCCTG-3'; and

2C-RACE2, 5'-GCACAGCTCAGTTCCCAAATCCCTG-3'.

An aliquot of the first PCR products was also used for subsequent nested PCR. Amplified cDNAs were gel-purified and sub-cloned into the pGEM-T-Easy vector (Promega) and sequenced. Multiple overlapping clones were isolated through this approach.

GENOMIC LIBRARY SCREENING, DNA CLONING, MAPPING AND SEQUENCING

A mouse genomic library (Stratagene) was screened using a cDNA fragment obtained from 5'-RACE as a probe. Three positive clones were isolated and sub-cloned into the pBlueScript vector (Stratagene). Restriction mapping and DNA sequencing were performed as described previously (Lin et al., 1998).

CREATION OF TRANSGENIC MICE AND LACZ STAINING

As previously described (Hogan et al., 1994), transgenes were injected into the male pronuclei of fertilized mouse oocytes. Briefly, DNA was gel purified and eluted using Qiaquick mini columns (QIAGEN). Fertilized eggs from B6C3F1 female mice were collected for pronuclear injections. Injected oocytes were then transplanted into ICR pseudopregnant females. Embryos were harvested at the desired embryonic or neonatal time points.

Detection of *lacZ* transgene expression was previously described (Naya et al., 1999). Briefly, embryos were dissected out of sacrificed mothers. Yolk sacs were removed for *lacZ* PCR genotyping. The amnion was also removed and embryos were fixed at 4 ^{o}C in 2% PF

and 0.2% gluteraldehyde in PBS for variable amounts of time, depending on the size of the embryo. Fixed embryos were washed in PBS at 4 ^{o}C for 30 minutes. Embryos were stained ON at RT in 1 mg/ml X-gal, 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$ and 5 mM $K_4Fe(CN)_6$ in PBS. If necessary, embryos were post-fixed in 4% PF and stored at 4 ^{o}C.

GENERATION OF TRANSCRIPTION FACTOR BINDING SITE MUTANTS

Mutagenesis of the MEF2-like, MEF2 and E-box transcription factor binding sites was achieved using the overlap extension method as previously described (Horton and Pease, 1991). The DNA template used for mutagenesis and cloned into pBlueScript (Stratagene) included the nucleotide region from -512 bp to +41 bp, where +1 represents the transcriptional start site of *MEF2C*. Oligonucleotides used for the mutagenesis are listed in Table 3. Mutant PCR products were then directly sub-cloned into the pGEM-T Easy vector (Promega). Mutated fragments were then excised using endogenous flanking restriction enzyme sites and cloned into the appropriate transgenic expression vector. All mutations were confirmed by DNA sequencing.

IN VITRO TRANSCRIPTION TRANSLATION AND DNA-BINDING ASSAYS

Proteins for electrophoretic mobility shift assays (EMSA) were produced using the TNT T3/T7 Coupled Reticulocyte Lysate System (Promega). The DNA templates used for *in vitro* transcription and translation of mouse MyoD, E12 and myc-tagged MEF2C were: EMSV-MyoD, pCITE-E12 and pcDNA3.1-MEF2C-myc, respectively. pEMSV-MyoD consists of a full length *MyoD* cDNA cloned into the expression vector pEMSVscribe (Harland and Weintraub, 1985). pCITE-E12 is the *E12* cDNA inserted into the pCITE expression vector (Novagen). pcDNA3.1-MEF2C-myc contains the full length *MEF2C* cDNA with a carboxy-terminal *myc* epitope tag (McKinsey et al., 2000) cloned into the pcDNA3.1 (Invitrogen) expression vector. To ensure that proteins were being appropriately translated, parallel transcription-translation reactions were performed in the presence of ^{35}S-methionine, separated by 10% SDS-PAGE and visualized via autoradiography.

Table 3. Primers used in mutagenesis of the MEF2-like site, MEF2 site and E-box in the *MEF2C* skeletal muscle regulatory region.

TF DNA Binding Site	Extension Overlap Primer Designation	Primer sequence (5' to 3' direction)
MEF2-like	primer a	TGACACCGAGTCTCTTAGAGTTACAAGC
	primer b	CACACAGGCTTTATCGATACATTTCCAAGAATA
	primer c	GGAAATGTATCGATAAAGCCTGTGTGAAATGAG
	primer d	TCGCCTCCTCTCCTTTTCTATGAACA
MEF2	primer a	TGACACCGAGTCTCTTAGAGTTACAAGC
	primer b	ACTCTGGAGTATCGATAGCTGTAAAGGTATC
	primer c	CTTTACAGCTATCGATACTCCAGAGTGACAT
	primer d	TCGCCTCCTCTCCTTTTCTATGAACA
E-box	primer a	CACATCAAGGGTCTCCACAGACGTAAGTGTCCC
	primer b	GGCCAGGGTGATCGATTTGATGTCACTCTGGAG
	primer c	GACATGAAATCGATCACCCTGGCCTGCCA
	primer d	CTGCGCTCGCCTCCTCTCCTTTTCTATGAACAC

The above primer sets were used in the PCR mutagenesis of the MEF2-like, MEF2 and E-box transcription factor (TF) binding sites in the -512 bp/+41 bp construct, as previously described (Horton and Pease, 1991) and Experimental Methods (Chapter 2).

The sequences of the sense strands of the oligonucleotides, from the *MEF2C cis*-regulatory region, used as probes in the gel mobility shift assays were as follows:

MEF2 site, 5'-ACC TTT ACA GCT AAA TTT ACT CCA GAG TG-3' and

E-box , 5'-GAG TGA CAT GAA CAG GTG CAC CCT GGC CT-3'.

Control gel mobility shift assays were performed with oligonucleotides corresponding to the high affinity right E-box (Chakraborty et al., 1991a) and MEF2 (Cserjesi et al., 1994) sites from the *MCK* enhancer. The oligonucleotides were all generated with four extra nucleotides, GAGG, on their 5' ends. Oligonucleotides were annealed at a concentration of 100 ng/μl and [α-^{32}P]dCTP end-labeled using the Klenow fragment of DNA polymerase I.

All binding reactions were performed using 2 μg poly(dI•dC), 100,000 cpm of the probe in a total volume of 20 μl of binding buffer (400 mM KCl, 150 mM HEPES (pH 7.9), 10 mM EDTA, 5 mM DTT, 50% glycerol). Where indicated, unlabelled annealed oligonucleotide was used as competitor. Reactions were incubated at RT for 15-20 min. Where indicated, mouse monoclonal IGg_1 c-myc (Santa Cruz) and MyoD (Pharmingen) antibodies were then added to the reactions at a concentration of 45 and 33 ng/μl, respectively, and incubated for an additional 10-15 min. Binding reactions were analyzed by electrophoresis on 4% (MyoD/E12 binding) and 5% (MEF2C-myc binding) non-denaturing polyacrylamide gels in 0.5X TBE. Gels were dried and binding was visualized by autoradiography.

CELL CULTURE AND LUCIFERASE REPORTER ASSAYS

Genomic DNA (from –1058 to +77) was released from construct 7 (see Fig. 22) and then sub-cloned into the pGL3-basic luciferase reporter (Promega). 10T1/2 cells were grown up and transfected as described (Lu et al., 1999). Cells were cultured in growth media. Transfections were performed with FuGENE6 (Roche) according to the manufacturer's instructions. In all transfection experiments, unless otherwise indicated, 200 ng of reporter and 300 ng of each activator plasmid per well were mixed with 3 μl of FuGENE6 and added to cells in six-well plates. After 24 hours, cells were shifted to differentiation media. After an additional 36 hours, cells were then harvested for luciferase assays according to

manufacturer's instructions (Promega). The total amount of DNA per well was kept constant by adding the corresponding amount of empty expression vector without a cDNA insert. CMV-lacZ, under control of the cytomegalovirus promoter, was used as an internal control to normalize for variations in transfection efficiency. C2C12 myoblasts were grown up as described above for the 10T1/2 cells. Construct 7 was transiently transfected into C2C12 cells by calcium phosphate transfection, according to standard methods . 24 hours later, cells were shifted to differentiation media. Cells were then fixed and stained for β-galactosidase at different time points (days 1, 3, and 7).

CHAPTER III

A SECOND, MYOGENIN-INDEPENDENT PATHWAY FOR SKELETAL MUSCLE DIFFERENTIATION

As previously discussed, myogenesis occurs by a two-step mechanism. First, precursor cells become committed to the myogenic lineage. Second, committed myoblasts differentiate to form mature skeletal muscle fibers. As demonstrated by loss-of-function mutations in three of the myogenic bHLH transcription factors, Myf5, MyoD and myogenin all play crucial roles in this two-step mechanism. Briefly, these studies demonstrate that MyoD and Myf5 play redundant roles in both the commitment of multipotential muscle precursors to the myogenic lineage as well as the initiation of *myogenin* and *MRF4* expression *in vivo* (Braun and Arnold, 1995; Braun et al., 1992; Rudnicki et al., 1992; Rudnicki et al., 1993). In contrast, myogenin plays a significant role in the terminal differentiation of myoblasts into myofibers (Hasty et al., 1993; Nabeshima et al., 1993; Rawls et al., 1995). However, the knock-out experiments of the *MRF4* gene yielded no definitive role for this factor (Braun and Arnold, 1995; Patapoutian et al., 1995b; Zhang et al., 1995).

Like the individual loss-of-function mutations of *MyoD* and *Myf5*, the *MRF4* knock-out has no obvious skeletal muscle defect. The only discernable phenotype is that the expression levels of *myogenin* in the *MRF4-/-* mutant are elevated. Interestingly, it has also been demonstrated that the residual muscle fibers in *myogenin*-null mice express *MRF4* at high levels. This led us to the hypothesis that MRF4 was responsible for the terminal differentiation of the residual skeletal muscle fibers in the *myogenin-/-* mice. We further hypothesized that the potential role of MRF4 in the differentiation of skeletal muscle fibers overlaps with that of one of the other myogenic bHLH transcription factors. In order to investigate these possibilities, mice with loss-of-function mutations in both *MRF4* and *myogenin* were generated, as well as double mutant mice for *MRF4* and *MyoD*.

myogenin-/-MRF4-/- MICE DO NOT DISPLAY AN EXACERBATED SKELETAL MUSCLE PHENOTYPE AS COMPARED TO *myogenin-/-* ANIMALS

While *MRF4-/-* animals are viable and able to reproduce, *myogenin-/-* animals are not (Hasty et al., 1993; Zhang et al., 1995). Therefore *myogenin-/-MRF4-/-* mice had to be generated from *myogenin+/-MRF4+/-* intercrosses or from *myogenin+/-MRF4-/-* intercrosses. The first goal was to ascertain whether or not there was any additional lethality associated with any of the newly generated *myogenin/MRF4* genotypic combinations. Because the lethality associated with the *myogenin-/-* phenotype is peri-natal, we obtained tail biopsies for genotype analysis within a few hours of birth or just prior to birth. From the *myogenin+/-MRF4+/-* intercrosses, nine genotypes were expected according to Mendelian genetics. All of the nine expected genotypes were obtained at the expected Mendelian frequency (Table 4). The four expected genotypes from the *myogenin+/-MRF4-/-* intercrosses were also obtained at approximately Mendelian frequencies (Table 5). This indicated that there was no additional embryonic lethality associated with any of the new genotypic combinations. This is consistent with previous studies which demonstrate that mice with even the most severe skeletal muscle deficiency, a complete absence of myoblasts and myofibers, are able to survive until birth (Rudnicki et al., 1993).

Next the progeny from the *myogenin/MRF4* crosses were observed for viability and gross phenotype. It was determined that only those pups lacking both alleles of *myogenin*, including *myogenin-/-MRF4-/-* pups, died peri-natally, regardless of their *MRF4* genotypic status. These non-viable neonates were kyphotic and cyanotic (data not shown), all grossly resembling the *myogenin-/-* animals previously described (Hasty et al., 1993; Nabeshima et al., 1993). Animals of all other genotypes were viable and fertile. The survival of *myogenin+/-MRF4+/-* and *myogenin+/-MRF4-/-* mice indicates that the absence of either one or two alleles of *MRF4* does not sensitize the animal to the level of *myogenin*.
To ascertain whether there was any exacerbation of the *myogenin-/-* phenotype at the level of muscle development in the *myogenin-/-MRF4-/-* animals, muscle from neonates bearing homozygous-null mutations in *myogenin* and *MRF4* was histologically analyzed and compared to that of wild-type and *myogenin-/-* muscle. As reported previously (Hasty et al., 1993; Nabeshima et al., 1993), the muscle-forming regions of *myogenin-/-* mice are severely deficient in differentiated skeletal muscle fibers as compared to wild-type (Fig. 5A, B).

Table 4. Genotypes of offspring from *myogenin+/-MRF4+/-* intercrosses.

	Genotype								
	1	2	3	4	5	6	7*	8*	9*
MRF4	+/+	+/+	+/-	+/-	-/-	-/-	-/-	+/-	+/+
myogenin	+/+	+/-	+/+	+/-	+/+	+/-	-/-	-/-	-/-
Observed no.	12	23	24	50	12	23	12	23	13
Predicted %	6.25	12.5	12.5	25.0	6.25	12.5	6.25	12.5	6.25
Observed %	6.28	12.0	12.6	26.2	6.28	12.0	6.28	12.0	7.0

*Denotes genotypes that were lethal at birth.

Table 5. Genotypes of offspring from *myogenin+/-MRF4-/-* intercrosses.

	Genotype		
	1	2	3*
MRF4	-/-	-/-	-/-
myogenin	+/+	+/-	-/-
Observed no.	25	50	22
Predicted %	25.0	50.0	25.0
Observed %	25.8	51.5	22.7

*Denotes genotype that was lethal at birth.

Residual muscle fibers appear to be somewhat differentiated, while mononuclear, unfused myoblasts populate the remainder of the *myogenin-/-* muscle-forming regions. Mice mutant for both *myogenin* and *MRF4* contain similar numbers of residual fibers and mononuclear myoblasts as *myogenin*-null mice (Fig. 5C). This demonstrates that *MRF4* is not solely responsible for the differentiation of the residual fibers in *myogenin*-null muscle. Further, it suggests that MyoD, Myf5 or both are able to activate myogenesis in the absence of myogenin and MRF4. This is consistent with studies demonstrating that myogenin does not share overlapping functions with MyoD or Myf5 (Rawls et al., 1995). The results of the RNA studies described below, however, deem it unlikely, that Myf5 is responsible for activating myogenesis to a significant degree in the absence of *myogenin* and *MRF4*.

PRIMARY MYOBLASTS DERIVED FROM *myogenin-/-MRF4-/-* DIFFERENTIATE AS EFFICIENTLY AS THOSE OF WILD-TYPE

Previously described work has shown that myogenin-/- (null) myoblasts, although incompetent to fully differentiate *in vivo*, are able to form myofibers as well as wild-type myoblasts *in vitro* (Hasty et al., 1993; Nabeshima et al., 1993). Because *MRF4* is up-regulated as *myogenin*-null myoblasts differentiate (Rawls et al., 1995), it was of interest to determine whether MRF4 was responsible for the differentiation of these myoblasts in tissue culture. Myoblasts from wild-type and mutant animals were cultured under conditions that promote differentiation (Fig. 6; Experiment performed by Wei Zhang). Cultures were observed grossly for the formation of myofibers, fixed and assayed for the presence of MHC, a marker of differentiated muscle, by immunostaining with anti-MHC antibody. Myoblasts derived from *myogenin/MRF4* double mutants differentiated to the same degree as myoblasts from *myogenin-/-* and wild-type animals. These results show that myogenesis *in vitro* can occur in the absence of myogenin and MRF4. This further supports the above *in vivo* results that suggest that MRF4 is not responsible for the differentiation of *myogenin*-null myoblasts.

MYOGENIN AND MRF4 SHARE A ROLE IN REGULATING THE EXPRESSION OF *ACHR-δ* AND *MYF5*

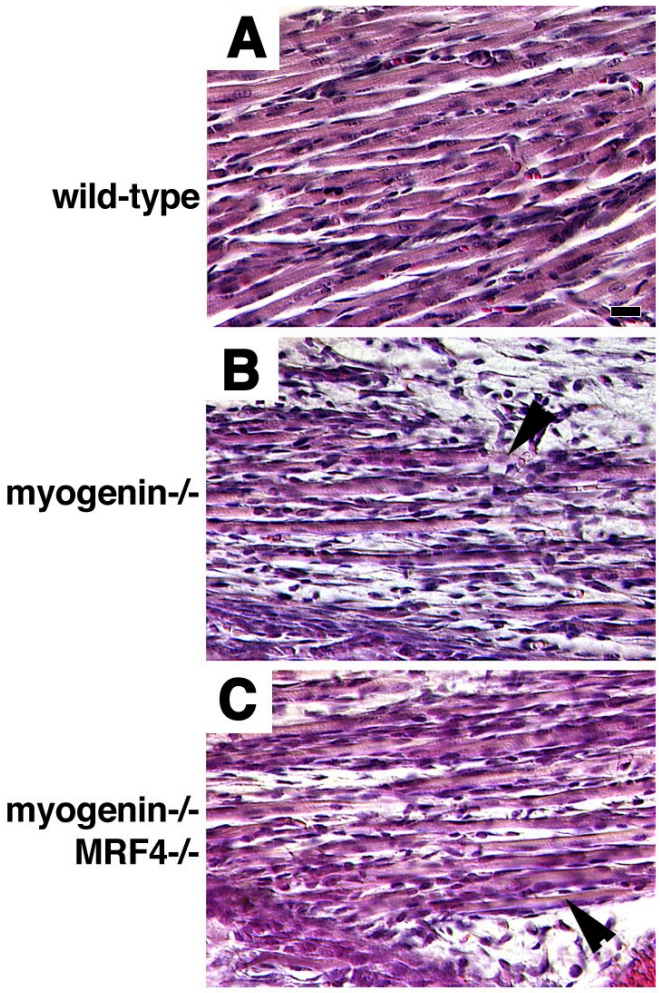

Figure 5. ***myogenin-/-*** **and** ***myogenin-/-MRF4-/-*** **neonates contain comparable numbers of residual muscle fibers.** Thin paraffin sections were cut through the hind-limbs of (A) wild-type, (B) *myogenin-/-* and (C) *myogenin-/-MRF4-/-* neonates. These sections were stained with hematoxylin and counterstained with eosin. Arrowheads, residual muscle fibers. Bar in A, 38 μm.

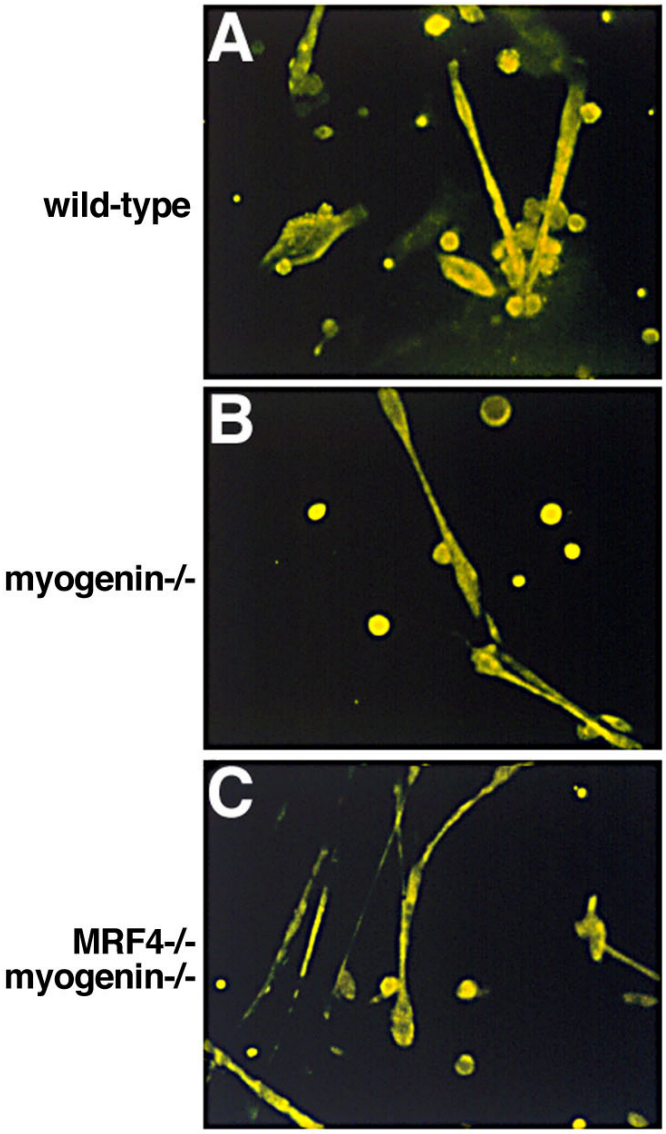

Figure 6. *myogenin/MRF4* double mutant myoblasts differentiate as efficiently as *myogenin-/-* and wild-type myoblasts *in vitro.* Primary myoblasts were isolated from the hindlimbs of (A) wild-type, (B) *myogenin-/-* and (C) *myogenin-/-MRF4-/-* neonates. Primary myoblasts were cultured under conditions that stimulate differentiation, as described in Experimental Methods (Chapter 2). After culturing, cells were immunostained with anti-MHC. (Experiment performed by Wei Zhang.)

Further characterization of the *myogenin/MRF4* double-null mutants was accomplished by assessing the mRNA transcript levels of each of the MyoD family members, as well as those of four other skeletal muscle-specific proteins. These levels were compared to those of *myogenin-/-* or *MRF4-/-* single mutants to determine the potential transcriptional consequences of the *myogenin-/-MRF4-/-* double mutant phenotype. The *myogenin-/-* neonate (Fig. 7A, lane 2) demonstrated a moderate down-regulation in *MRF4* expression and a moderate increase in *Myf5* expression, as compared to wild-type (lane 1). As demonstrated previously (Zhang et al., 1995), *myogenin* levels were up-regulated in the *MRF4* knock-out (lane 3). These results are consistent with the ability of the myogenic bHLHs to cross regulate the expression of one another. Interestingly, *Myf5* expression was almost completely absent in the *myogenin/MRF4* double knock-out (lane 5). This strongly suggests that MRF4 and myogenin have overlapping roles in the induction of *Myf5*. All other myogenic bHLH transcript levels similar to wild-type. The expression levels of *MHC*, *MCK* and *α-SkAct* were significantly reduced in the *myogenin* mutant, but unaffected in the *MRF4-/-* neonate. The levels of these transcripts in the *myogenin/MRF4* double mutant were also significantly decreased, but no lower than those of the *myogenin-/-*. This was not the case with *AChR-δ* expression levels. Both the *myogenin* and *MRF4* single mutant animals expressed *AchR-δ* at wild-type levels. The compound mutant, however, expressed this gene at almost undetectable levels. This suggests that MRF4 and myogenin also share a role in the maintenance of *AChR-δ* expression (Northern Blot analyses performed by Alan Rawls).

MYOD-/-MRF4-/- MUTANTS SHOW A DECREASED LEVEL OF SKELETAL MUSCLE DIFFERENTIATION, COMPARABLE TO THAT OF *myogenin-/-* MICE

Like the *MRF4-/-* animals, *MyoD* knock-out mice are viable and fertile. However, *MyoD/MRF4* double mutants were not viable. Because MyoD had been shown to play a significant role in myogenesis (Rudnicki et al., 1993), whereas MRF4 had not, the decision was made to generate *MyoD* heterozygotes in the background of the *MRF4*-null allele. These were subsequently intercrossed to obtain double mutant mice. The hypothesis that MRF4 and MyoD may have overlapping roles in myogenesis could mean that a combination of the

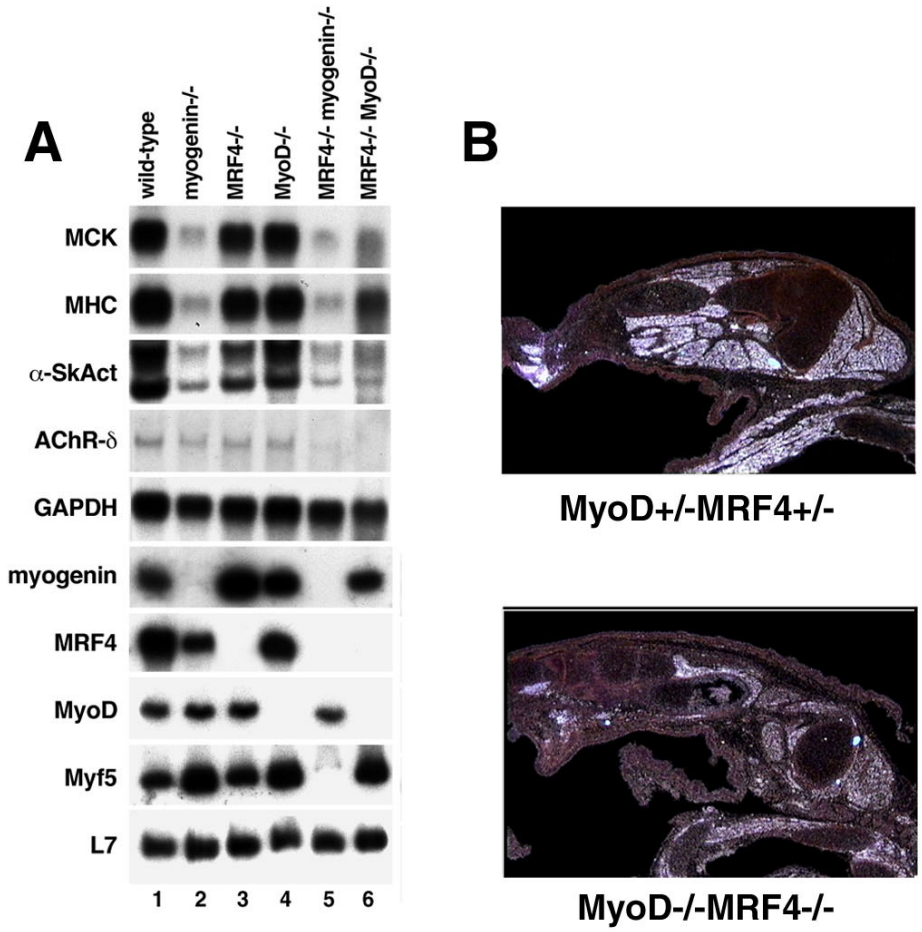

Figure 7. Analysis of muscle transcripts in wild-type and myogenic bHLH single and double mutant neonates. (A) Total RNA was isolated from neonates as described in Experimental Methods (Chapter 2). Expression levels of *myogenin*, *MRF4*, *MyoD*, *Myf5* and control, *L7*, were determined by RT-PCR analysis. Expression levels of *MCK*, *MHC*, *α-SkAct*, *AChR-δ* and control, *GAPDH*, were determined by northern blot analysis. (Northern Blot analysis performed by Alan Rawls.) (B) *myogenin* transcripts were detected by *in situ* hybridization to paraffin sections from the forelimbs of *MyoD+/-MRF4+/-* and *MyoD-/-MRF4-/-*. (Experiment performed by Alan Rawls.)

MyoD- and *MRF4*-null alleles would lead to a deficiency in skeletal muscle development. If severe enough, this muscle deficiency could compromise the viability of neonates. We therefore genotyped progeny within a few hours of, or just prior to, parturition. Each of the three expected genotypes, including *MyoD/MRF4* double mutant mice, were obtained at approximately Mendelian frequencies (Table 6).

The *MyoD/MRF4* double mutant mice were then observed for viability and gross morphology. Animals lacking both *MyoD* and *MRF4* were not viable, unlike the *MyoD-/-* or the *MRF4-/-* mutant mice. Grossly *MyoD-/-MRF4-/-* neonates were cyanotic, showed curvature of the spine and died within hours of birth (Fig. 8). These double mutants additionally appeared to accumulate additional fat at the nape of the neck. None of the other predicted genotypes proved lethal, including *MyoD+/-MRF4-/-* and *MyoD-/-MRF4+/-*. This indicated that a single *MyoD* allele in the background of an *MRF4-/-*, or a single *MRF4* allele in the background of a *MyoD-/-* is sufficient for viability and normal gross development. Interestingly, the lethal phenotype of these double mutant animals was almost identical to that described for animals lacking both alleles of *myogenin* (Hasty et al., 1993; Nabeshima et al., 1993; Rawls et al., 1995). Thus, these data suggested that the *MyoD-/-MRF4-/-* animals were likely deficient in skeletal muscle development, like the *myogenin-/-* mice.

To test this hypothesis, paraffin sections of wild-type and mutant muscle from E16.5 embryos were histologically analyzed. *MyoD*/*MRF4* double mutants demonstrated a significant reduction in skeletal muscle fibers in all regions analyzed (Fig. 9D-F), strikingly similar to that of the *myogenin-/-* phenotype. The accumulation of brown fat adjacent to, and impinging upon, what are normally muscle-forming regions is also clearly evident (Fig. 9D). The few fibers in the muscle-forming regions of the *MyoD-/-MRF4-/-* embryos were poorly differentiated, containing centrally located nuclei or doughnut-shaped cells (arrow in Fig. 9E), both indicative of only moderately differentiated muscle (Fig. 9E, F). The remaining tissue was occupied by mononuclear cells that are likely undifferentiated myoblasts. In contrast, normal muscle was well differentiated, displaying fat myofibers containing peripherally located nuclei (Fig. 9A-C).

Table 6. Genotypes of offspring from *MyoD+/-MRF4-/-* intercrosses.

	Genotype		
	1	2	3*
MRF4	-/-	-/-	-/-
MyoD	+/+	+/-	-/-
Observed no.	8	14	6
Predicted %	25.0	50.0	25.0
Observed %	29.0	50.0	21.0

*Denotes genotype that was lethal at birth.

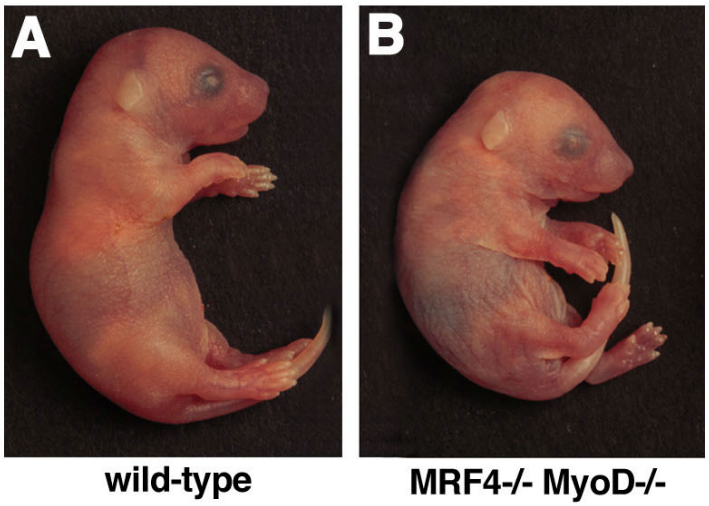

Figure 8. Gross appearance of *MyoD/MRF4* double mutant neonates. Note that the *MyoD/MRF4* double mutant (B) is smaller, demonstrates severe kyphosis and a thickening at the back of the neck as compared to a wild-type littermate (A).

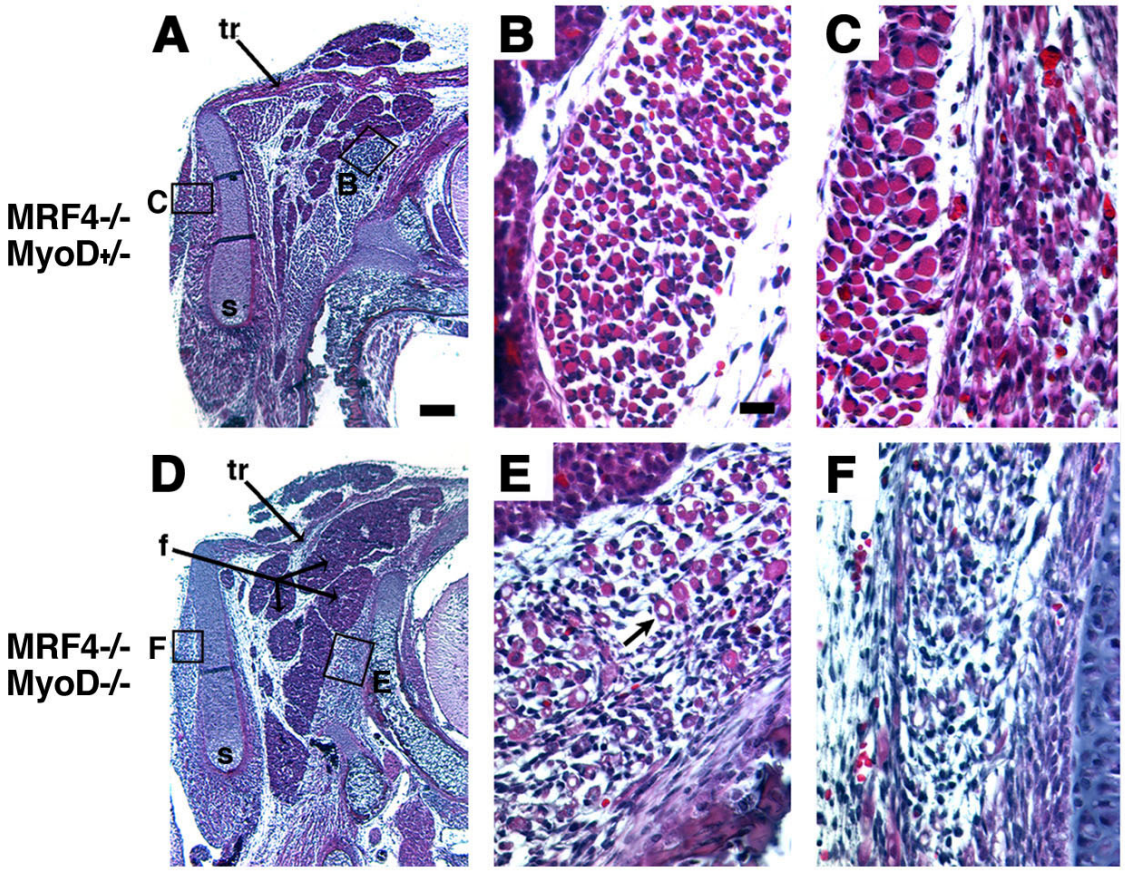

Figure 9. Skeletal muscle defects in *MyoD-/-MRF4-/-* double mutant embryos. H&E-stained transverse sections through the upper thoracic region of E16.5 (A) wild-type and (B) *MyoD-/-MRF4-/-* embryos. (B, C) and (E, F) show enlargements of the regions indicated in (A) and (D), respectively. s, scapula; tr, trapezius; f, brown fat deposits. Bars, 96 µm in (A) and 9 µm in (B).

In order to obtain a more qualitative measurement of differentiation, paraffin sections of wild-type and mutant tongue muscle from E16.5 embryos were immunostained with anti-MHC antibody. Sections of tongue from normal muscle were almost completely comprised of muscle fibers (Fig. 10A). These fibers were very organized and exhibited strong anti-MHC staining, indicating that these fibers were amply differentiated. In contrast, tongue muscle from *MyoD/MRF4* double-null embryos displayed MHC-staining in much less than 50% of the cells. Additionally these sections showed few muscle fibers and little organization (Fig. 10B). This analysis confirmed that the muscle-forming regions of *MyoD-/-MRF4-/-* mutants are filled with undifferentiated mononuclear cells and poorly differentiated residual fibers, as compared to wild-type.

SKELETAL MUSCLE-SPECIFIC TRANSCRIPT EXPRESSION: *myogenin* LEVELS ARE NOT DECREASED IN *MYOD-/-MRF4-/-* DOUBLE MUTANTS

In order to further examine the skeletal muscle defects of these double mutants, northern blot and semi-quantitative RT-PCR analysis was performed on total RNA isolated from wild-type and mutant neonates (Fig. 7A). The expression levels of *MCK*, *MHC* and *α-SkAct* in *MyoD/MRF4* double mutants (lane 6) were moderately decreased compared to wild-type (lane 1), while the levels of these three genes and that of *AChR-δ* in *MyoD* and *MRF4* single mutants (lanes 4 and 3, respectively) were equivalent to those of wild-type. These results are consistent with a deficiency in muscle differentiation in the *MyoD-/-MRF4-/-* mutants. Interestingly, *AChR-δ* levels in the double mutant were undetectable by northern blot analysis, which suggests that MyoD and MRF4 have a shared role in the positive regulation of *AChR-δ* expression. Although *Myf5* levels in the *MyoD-/-* are moderately increased, they are unchanged in both the *MRF4-/-* and the *MyoD-/-MRF4-/-* mutant. This suggests that MyoD, and not MRF4, regulates the expression of *Myf5*, consistent with previously published data (Rudnicki et al., 1992).

Given that the phenotype of *MyoD-/-MRF4-/-* animals so strongly resembles that of the *myogenin* knock-out, despite the fact that these double mutant animals are not missing either *myogenin* allele, we decided to see if there was a decrease in *myogenin* expression that

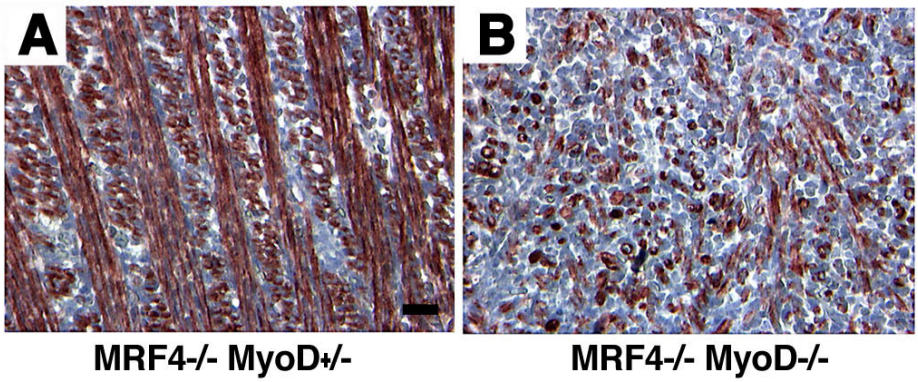

Figure 10. Paucity of muscle differentiation and organization in *MyoD/MRF4* double mutant embryo muscle. The tongue muscle of E16.5 (A) wild-type and (B) double mutant embryos were immunostained with anti-MHC antibody and counterstained with hematoxylin. Bar, 9 μm in (A).

could explain the severe decrease in skeletal muscle development of the double knock-out. Transcript levels for each of the four myogenic bHLH transcription factors were assayed by RT-PCR (Fig. 7A). *Myogenin* levels were slightly up-regulated in the *MRF4-/-* and unaffected in the *MyoD-/-*. Interestingly, the expression of *myogenin* in the *MyoD*/*MRF4* double mutant was unaffected, as compared to wild-type. Detection of *myogenin* transcripts at wild-type levels by RNA *in situ* hybridization in muscle-forming regions of E17.5 animals confirmed this result (Fig.7B; Experiment performed by Alan Rawls). These data show that the level of *myogenin* transcripts in the *MyoD/MRF4* double mutant is in no way perturbed and, therefore, unrelated to the observed phenotype. These data further reveal a previously unidentified myogenin-independent pathway for muscle differentiation, for which MyoD and MRF4 are critical. These results are consistent with the presence of residual muscle fibers in the muscle-forming regions of the *myogenin-/-* animal.

RIB DEFECTS IN *myogenin-/-MRF4-/-* AND *MYOD-/-MRF4-/-* MUTANT MICE

It has been shown previously that the individual *Myf5*, *myogenin* and *MRF4* loss-of-function mutants have rib malformations (Braun et al., 1992; Hasty et al., 1993; Zhang et al., 1995). Because the axial skeleton and most skeletal muscle are both derived from the somite, it was of interest to assay the status of rib development in both of the double-null mutants. The skeletons of wild-type and mutant neonates were fixed and stained for cartilage and bone with alizerin red and alcian blue, respectively. In comparison with wild-type (Fig. 11A), the *myogenin-/-* neonate had severely shortened or missing ribs and malformed intersternebral cartilage (Fig. 11B). The skeleton of the *MRF4-/-* neonate demonstrated rib bifurcations, fusions and supernumerary processes (Fig. 11C). The *myogenin*/*MRF4* double mutant animals also had malformations of their rib cage, but they appeared to be much more severe than those of either of the single mutants. These malformations included a failure of the distal portions of the ribs to reach the sternum, malformation of the sternebral bodies and a bifurcation of the upper region of the sternum (Fig. 11D). *MyoD-/-* mutants demonstrate virtually no rib formation (data not shown), whereas the *MyoD-/-MRF4-/-* double knock-out mice show malformations indistinguishable from that of the *MRF4* single mutant (Fig. 11E).

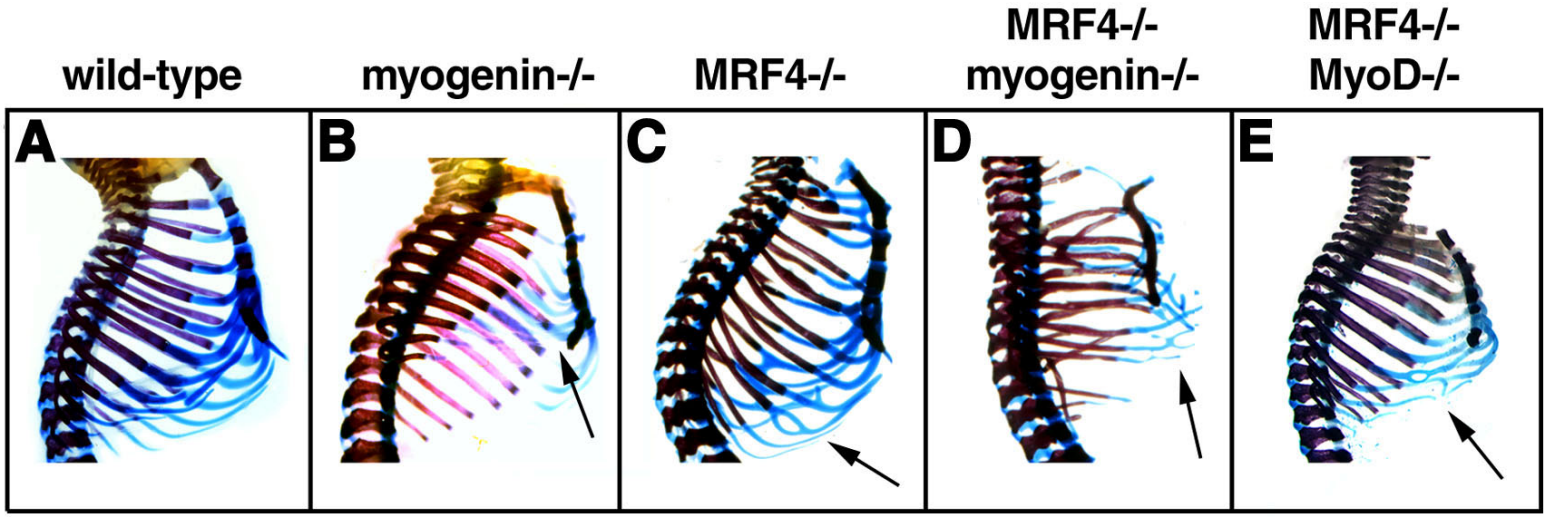

Figure 11. *myogenin-/-MRF4-/-* skeletons show increased rib malformations compared to that of *myogenin* or *MRF4* single mutants. The skeletons of (A) wild-type, (B) *myogenin-/-*, (C) *MRF4-/-*, (D) *myogenin-/-MRF4-/-* and (E) *MyoD-/-MRF4-/-* neonates were fixed and stained for bone and cartilage with alizerin red and alcian blue, respectively. The ribs and vertebrae were then dissected away from the rest of the skeleton and images were captured. Arrows, malformations of the rib cage. (Preps in B, C, D performed by Alan Rawls.)

These data imply that MRF4 is redundant in function with myogenin, but not MyoD, with respect to rib formation. Because neither MRF4 nor myogenin are expressed in the axial skeleton or its precursors, their contribution to rib cage formation must be secondary to their functions in myogenesis.

SUMMARY

There was no significant exacerbation of the *myogenin-/-* phenotype in the *myogenin-/-MRF4-/-* animals. Mice bearing null mutations in *MRF4* and *myogenin* contain a comparable number of residual muscle fibers to *myogenin*-null mice and myoblasts from mice of the single and double mutant genotypes are able to differentiate in culture, indicating that MRF4 does not have an overlapping function with myogenin in gross skeletal muscle development. *Myf5* and *AChR-δ* transcript levels were only barely detectable in the *myogenin/MRF4* double mutants, as compared to wild-type, *myogenin-/-* or *MRF4-/-* mice. Additionally, ribs showed significantly greater malformations in double mutant skeletons, in comparison to that of the single mutants. Thus, MRF4 and myogenin do appear to play compensatory roles in the maintenance of *AChR-δ* and *Myf5* expression levels and in the formation of the ribs.

The *MyoD*/*MRF4* double-null animals showed a phenotype similar to those of the *myogenin-/-*. The presumptive muscle-forming regions of this double mutant contain only a few, poorly differentiated, disorganized myofibers, the remainder of the area being populated with mononuclear cells. This defect is not due to changes in *myogenin* levels, as *myogenin* is expressed at wild-type levels. This shows that MRF4 does overlap in function with MyoD in skeletal muscle differentiation, but not at the level of maintaining *myogenin* expression. In fact, these results indicate that MyoD and MRF4 participate in a myogenin-independent muscle differentiation pathway. It was also found that *AChR-δ* transcripts were undetectable by northern blot analysis in this double mutant, whereas they are normal in the individual mutants. This suggests that MyoD and MRF4 also have redundant roles in the positive regulation of *AChR-δ* expression.

While mice lacking either *myogenin* alone or *MyoD* plus *MRF4* exhibit a lethal deficiency of differentiated skeletal muscle fibers, there are residual muscle fibers present in mutants of these genotypes (Hasty et al., 1993; Nabeshima et al., 1993; Rawls et al., 1998; Venuti et al., 1995). The residual muscle fibers in *myogenin-/-* and *MyoD-/-MRF4-/-* neonates could be explained by the existence of two different myogenic lineages: one dependent on myogenin and the other dependent on MyoD and MRF4. A model for the terminal differentiation of skeletal muscle fibers *in vivo* is given in Fig. 33.

CHAPTER IV

THE TOTAL LEVEL OF MYOGENIC BHLH TRANSCRIPTION FACTORS DICTATES THE DEGREE OF SKELETAL MUSCLE DIFFERENTIATION

Although the majority of myoblasts in mice lacking either *myogenin* or *MyoD* plus *MRF4* are unable to form myofibers *in vivo*, myoblasts from these mutants differentiate normally when cultured *in vitro* (Nabeshima et al., 1993; Rawls et al., 1995; Rawls et al., 1998). These results suggest that the environment is more permissive for myogenesis in tissue culture than in the embryo and that the functional specificity of the myogenic factors revealed from gene knockout experiments does not reflect an absolute requirement for individual factors to activate the differentiation program. In fact, the functions of the myogenic bHLH factors were initially defined by transfection assays, in which each factor was shown to be capable of activating the complete muscle differentiation program. Thus, the specific functions of MyoD and Myf5 in myoblast specification, or the differentiation functions of myogenin, MRF4 and MyoD, as revealed through gene inactivation studies, are not reflected in tissue culture assays. It is important to note, however, that the interpretation of *in vitro* experiments is difficult, owing to the fact that the myogenic bHLH transcription factors are able to auto- and cross-regulate one another's expression.

To determine the extent to which a single myogenic bHLH gene can support any level of myoblast specification or differentiation *in vivo* in the absence of potential contributions from other members of the family, we generated mice lacking *myogenin*, *MyoD* and *MRF4*, but retaining *Myf5*.

myogenin-/-MYOD-/-MRF4-/- TRIPLE MUTANT ANIMALS DEMONSTRATE LITTLE OR NO DIFFERENTIATED MUSCLE FIBERS

Previous studies have shown that mice lacking either *myogenin* alone or *MyoD* plus *MRF4* exhibited a severe skeletal muscle deficiency incompatible with life (Hasty et al., 1993; Nabeshima et al., 1993; Rawls et al., 1998). Taking this into account, we generated *myogenin+/-MyoD+/-MRF4-/-* for use as breeding parents. The fact that these mice were

viable indicates that a single *myogenin* allele and a single *MyoD* allele are sufficient to support normal skeletal muscle development in the absence of *MRF4*. It has been previously shown that even the most severe deficiency of skeletal muscle *in vivo* is not incompatible with the ability of a mouse embryo to reach birth (Rudnicki et al., 1993). Once born, neonates lacking adequate skeletal muscle die within a few hours of birth from suffocation (Hasty et al., 1993; Nabeshima et al., 1993; Rawls et al., 1998). We, therefore, obtained tail biopsies for genotype analysis from mice arising from the *myogenin+/-MyoD+/-MRF4-/-* intercrosses at birth or within a few hours after birth. From these intercrosses, nine genotypes were expected according to Mendelian genetics. As detailed in Table 7, not all of the nine expected genotypes were obtained at the expected Mendelian frequencies. Specifically, *myogenin+/+MyoD+/+MRF4-/-* and *myogenin+/+MyoD+/-MRF4-/-* were observed much less frequently than expected, whereas *myogenin+/+MyoD-/-MRF4-/-* neonates were observed at a much higher frequency than expected. All other genotypes, including the *myogenin/MyoD/MRF4* triple mutant, were present at frequencies consistent with Mendelian predictions. This indicates that there is no additional embryonic lethality associated with the triple mutant genotype. Mice of this genotype were born alive, but died very shortly after birth, presumably from an inability to breathe due to the inadequate development of diaphragmatic muscle.

E18.5 triple mutant embryos were smaller than wild-type littermates and demonstrated a severe curvature of the spine (Fig. 12). Their reduction in body mass and kyphosis appeared to be even more severe than that of a *myogenin/MRF4* double mutant (Fig. 12B). It was expected that these triple mutants would have a myogenic deficiency at least as severe as that of the single and double myogenic bHLH mutants described herein. To test this assertion, muscle from E16.0 *myogenin-/-MyoD-/-MRF4-/-* was histologically analyzed by light microscopy. Tongue, forelimb and deltoid muscle development of these animals was compared to that of *myogenin-/-MyoD+/-MRF4-/-* and wild-type littermates. H&E staining of paraffin sections revealed that in all three regions, wild-type muscle contained organized, fully formed muscle fibers with peripheral nuclei (Fig. 13A-C). Muscle from the *myogenin-/-MyoD+/-MRF4-/-* mutant showed little organization and few residual

Table 7. Genotypes of offspring from *myogenin+/-MyoD+/-MRF4-/-* intercrosses.

	Genotype								
	1	2	3*	4	5	6*	7*	8*	9*
myogenin	+/+	+/+	+/+	+/-	+/-	+/-	-/-	-/-	-/-
MyoD	+/+	+/-	-/-	+/+	+/-	-/-	+/+	+/-	-/-
MRF4	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
Observed no.	3	11	15	15	28	18	9	18	7
Predicted %	6.25	12.5	6.25	12.5	25.0	12.5	6.25	12.5	6.25
Observed %	2.42	8.87	12.1	12.1	22.58	14.52	7.26	14.52	5.65

*Denotes genotypes that were lethal at birth.

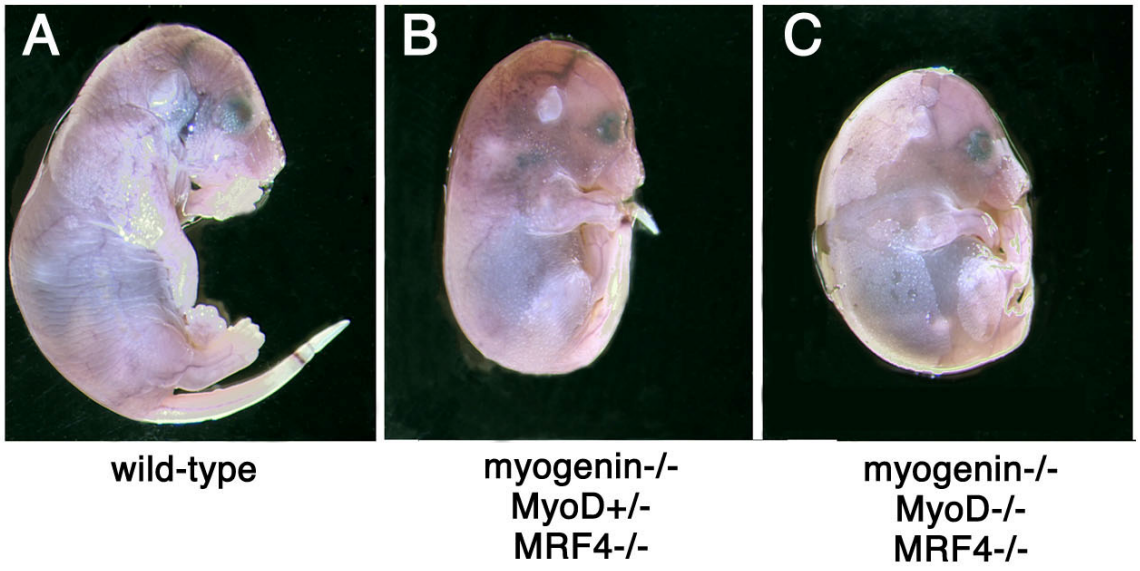

Figure 12. Gross appearance of *myogenin/MyoD/MRF4* triple mutant neonates. Note that the (C) triple mutant shows a reduction in body mass, demonstrates severe curvature of the spine and a thickening at the back of the neck that is slightly more severe than that of a *myogenin/MRF4* double mutant littermate (B). Wild-type littermate (A)is shown for comparison.

muscle fibers (Fig. 13D-F), although the muscle-forming regions were densely populated with what appeared to be mononuclear cells. The existence of differentiated muscle in this mutant demonstrated that a single allele of *MyoD* is sufficient to support the differentiation of residual muscle fibers in animals lacking both *myogenin* and *MRF4*. Of great interest, the extent of myofiber organization and differentiation of the *myogenin/MyoD/MRF4* triple mutant (Fig. 13G-I) appeared to be even less than that of *myogenin-/-MyoD+/-MRF4-/-* littermates. In fact, these triple mutants appeared to have no residual muscle fibers.

To obtain a better quantitative and qualitative analysis of the muscle development of the triple mutant, the extent of differentiation of muscle cells in E16.0 offspring was examined by anti-MHC staining of adjacent histological sections of the tongue, forelimb and deltoid (Fig. 14). In wild-type tongue muscle, both the intrinsic and extrinsic musculature are fully differentiated and well organized (Fig. 14A), while mutants of the *myogenin-/-MyoD+/-MRF4-/-* genotype displayed only residual myofibers that were poorly differentiated and disorganized (Fig 14D). Mononuclear cells that expressed little or no MHC populated the remainder of the muscle-forming region in the tongue of this double mutant. Notably, the muscle-forming region of the *myogenin/MyoD/MRF4* triple mutant tongue was populated only by non-staining mononuclear cells and was essentially unorganized (Fig. 14F). Triple mutant forelimb and deltoid muscle also demonstrated the same lack of differentiation and organization. Thus, different lineages of muscle are equally affected in the triple mutant. That comparable numbers of cells were present in the muscle-forming regions of the triple mutant compared to that of wild-type suggests that myogenic cells were specified, but not differentiated.

MINIMAL MUSCLE GENE EXPRESSION IN *myogenin-/-MYOD-/-MRF4-/-* TRIPLE MUTANTS

As described in Chapter 1, myogenesis is a two-step process consisting of the initial commitment of mesodermal precursors to the myogenic lineage followed by the differentiation of these committed cells. Histological analysis of the muscle-forming regions of triple mutant embryos demonstrated little evidence of muscle differentiation, although

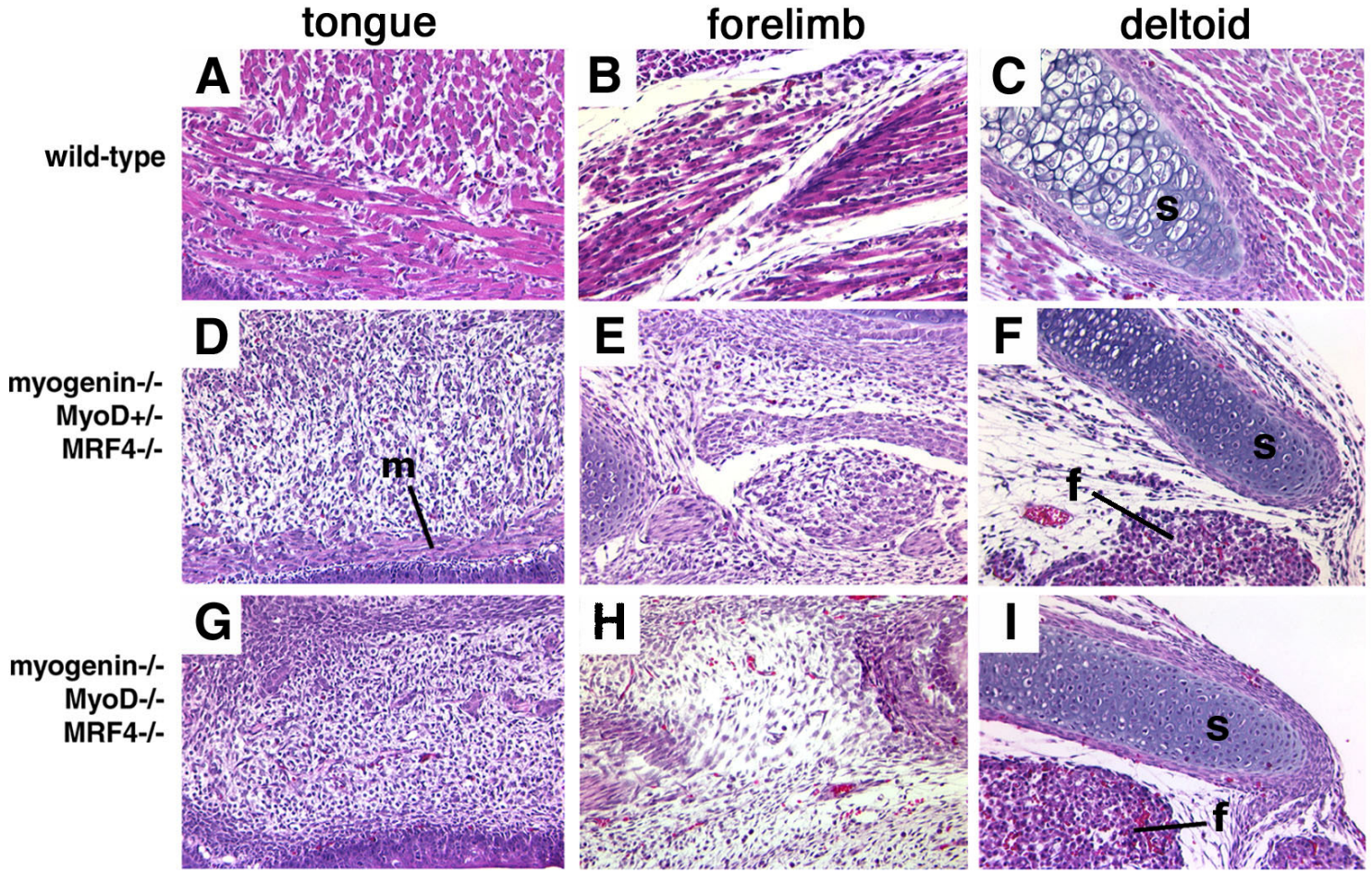

Figure 13. ***myogenin-/-MyoD-/-MRF4-/-*** **neonates demonstrate a deficiency in myogenesis.** Histological analysis of muscle-forming regions of E16.0 (A-C) wild-type, (D-F) *myogenin/MRF4* double and (G-I) *myogenin/MyoD/MRF4* triple mutant neonates. Transverse sections through the tongue (A, D, G), forelimb (B, E, H) and deltoid (C, F, I) were stained with hematoxylin and eosin. m, residual muscle fibers; f, brown fat; s, scapula. Original magnification 20X.

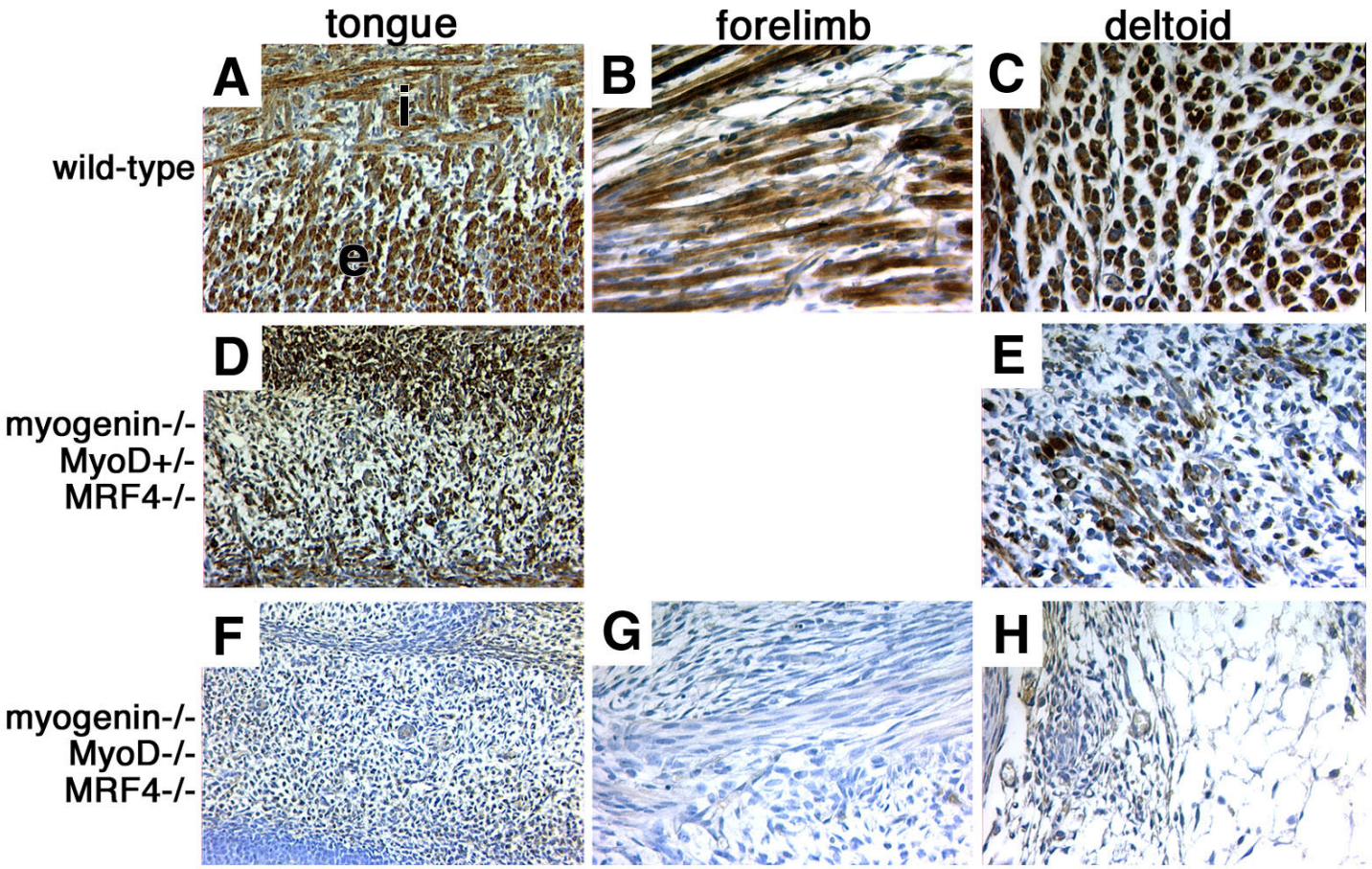

Figure 14. ***myogenin/MRF4/MyoD*** **triple mutant muscle displays almost no differentiated organized muscle.** Anti-MHC immunostaining of muscle from E16.0 (A-C) wild-type, (D, E) *myogenin/MRF4* double and (F-H) *myogenin/MyoD/MRF4* triple mutants. Transverse sections through (A, D, F) tongue, (B, G) forelimb and (C, E, H) deltoid muscle was immunostained with anti-MHC and counterstained with hematoxylin. Original magnification of sections through forelimb and deltoid, 40X. Original magnification of tongue sections, 20X. i, intrinsic muscles; e, extrinsic muscles.

commitment appeared to have occurred, as demonstrated by the number of cells occupying muscle-forming regions (Fig. 14F-H). To further characterize the level of myogenesis in the triple-null mutant versus that of wild-type, *myogenin+/+MyoD+/-MRF4-/-*, and double mutant littermates, we examined the expression of muscle-specific mRNAs in neonates by semi-quantitative RT-PCR. As shown in Fig. 15, transcripts for *myogenin*, *MyoD*, and *MRF4* were expressed as predicted in the various mutant backgrounds. *Myf5* was expressed in the *myogenin/MRF4* double mutant (lane 2) at levels comparable to that of wild-type (lane 1), whereas the *MyoD*/*MRF4* double mutant expressed it at slightly higher levels (lane 3). Interestingly, there was no detectable *Myf5* expression in the triple knock-out (lane 4). This suggests that myogenin, MyoD and MRF4 serve to regulate the maintenance of *Myf5* expression *in vivo*.

Muscle differentiation genes, including *α-SkAct*, *eMHC*, *MCK* and *AChR-δ* are present, but down-regulated in *myogenin*/*MRF4* mutants, as well as in *MyoD*/*MRF4* double mutants. In contrast, neither *AChR-δ* or *eMHC* were detectable in the triple-null neonate and only trace amounts of *α-SkAct*, *desmin* and *MCK* were present, consistent with the apparently negligible level of muscle differentiation demonstrated by the *myogenin/MyoD/MRF4* triple mutant. Interestingly, *MEF2C* transcripts are only minimally decreased in all mutants, indicating that myogenic bHLH factors play a role in *MEF2C* regulation, but that they are not the only component. Together these data support the conclusion that the *myogenin/MyoD/MRF4* triple mutant phenotype is considerably more severe than any of the myogenic bHLH single or double mutants previously described. These results also reflect that myogenesis is almost negligible in the triple mutant.

EARLY COMMITMENT OF TRIPLE MUTANT MYOBLASTS IS COMPARABLE TO THAT OF WILD-TYPE, ALTHOUGH MYOBLASTS DO NOT SIGNIFICANTLY DIFFERENTIATE

To assess the level of myoblast commitment at both early and late time points in the development of the triple mutant, we further characterized two known markers of commitment, Myf5 and desmin. *Myf5* is expressed strongly in the myotome until E11.5 and

marks committed myoblasts (Ott et al., 1991). The results from RT-PCR indicated that *Myf5* expression was dramatically down-regulated in triple mutant neonates. To investigate the potential dependence of early *Myf5* expression on the other myogenic bHLH factors, RNA *in situ* hybridization for *Myf5* was performed on transverse thoracic sections through rostral somites of triple mutant E11.0 embryos, and compared to those of wild-type and *myogenin/MRF4* double mutants (Fig. 16). The level and spatial extent of *Myf5* expression was comparable in all three embryos. The appropriate formation of the myotome in the triple mutant indicates that myoblasts are appropriately specified early in myogenesis.

We next sought to determine what degree of myogenesis these committed myoblasts are able to achieve. Histologically the muscle-forming regions of the triple mutant contain as many nuclei as those of wild-type and *myogenin/MRF4* double mutants (Figs. 13 and 14). This suggests that the muscle-forming regions of the triple mutant are occupied by committed, but undifferentiated, myoblasts. To further characterize the mononucleated cells populating presumptive muscle-forming regions of triple mutants, we stained deltoid and forelimb sections with anti-desmin antibody. Desmin marks both undifferentiated myoblasts and differentiated muscle. Sections from wild-type E16.0 muscle showed myofibers with high levels of desmin expression and peripheral nuclei, indicative of fully differentiated fibers (Fig. 17A, B). In contrast, mononuclear cells of the triple mutants showed desmin staining barely above background (Fig. 17C, D). There were occasional regions in the triple-null mutants where wispy multinucleated structures appeared to form, but they did not stain for desmin any more strongly than adjacent mononuclear cells (Fig. 17D, arrows). The detection of desmin, albeit weak, in the muscle-forming regions of the triple mutant suggests that these regions are occupied, at least in part, by specified myoblasts. This is consistent with the level of desmin expression detected in the triple mutant (Fig. 15, lane 4). The extremely low level at which desmin is detected, however, further indicates that these myoblasts are not differentiated to any meaningful degree.

PRIMARY MYOBLASTS DERIVED FROM *myogenin-/-MYOD-/-MRF4-/-* MUTANT ARE UNABLE TO DIFFERENTIATE *IN VITRO*

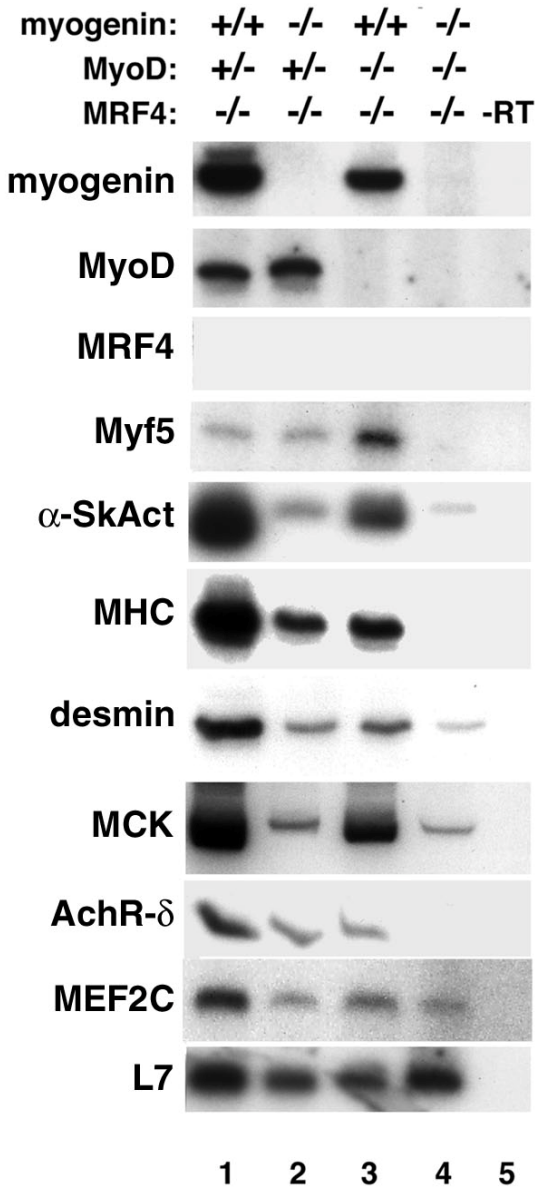

Figure 15. Analysis of muscle transcripts in wild-type and *myogenin/MRF4* double, *MyoD/MRF4* double and *myogenin/MyoD/MRF4* triple mutant neonates. Total RNA was isolated from neonates as described in Experimental Methods (Chapter 2). Expression levels of muscle-specific transcripts were determined by semiquantitative RT-PCR. *L7* transcripts were measured as a loading control. PCR reactions using RNA that was not subjected to reverse transcription are included in lane 5.

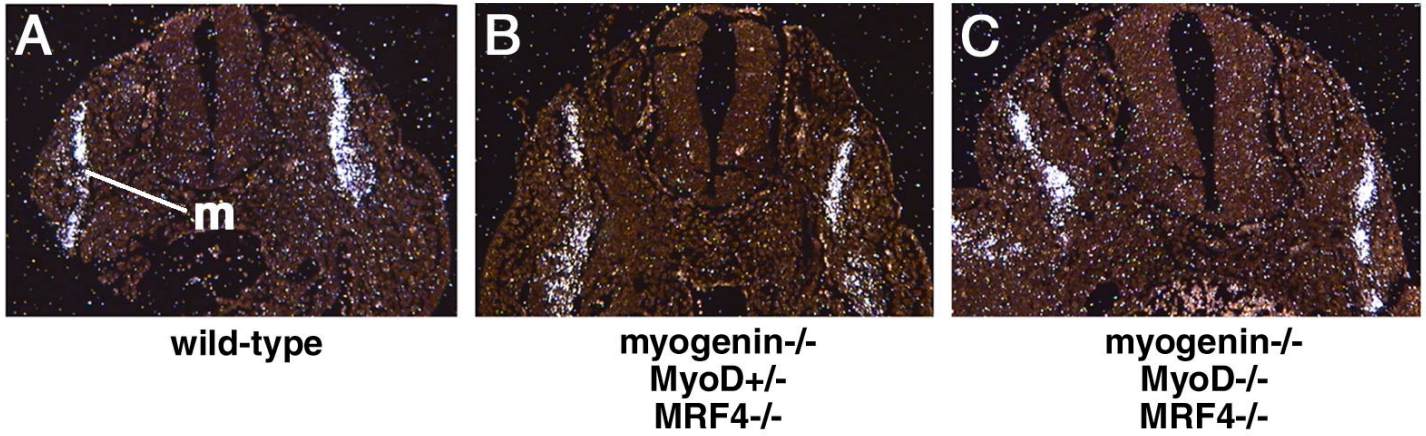

Figure 16. The myotomes of E11.0 *myogenin/MyoD/MRF4* triple mutant embryos display wild-type levels of *Myf5* transcripts. Transcripts for *Myf5* were detected by *in situ* hybridization to transverse thoracic sections through the rostral somites of (A) wild-type, (B) *myogenin/MRF4* double mutant and (C) *myogenin/MyoD/MRF4* triple mutant E11.0 embryos. m, myotome.

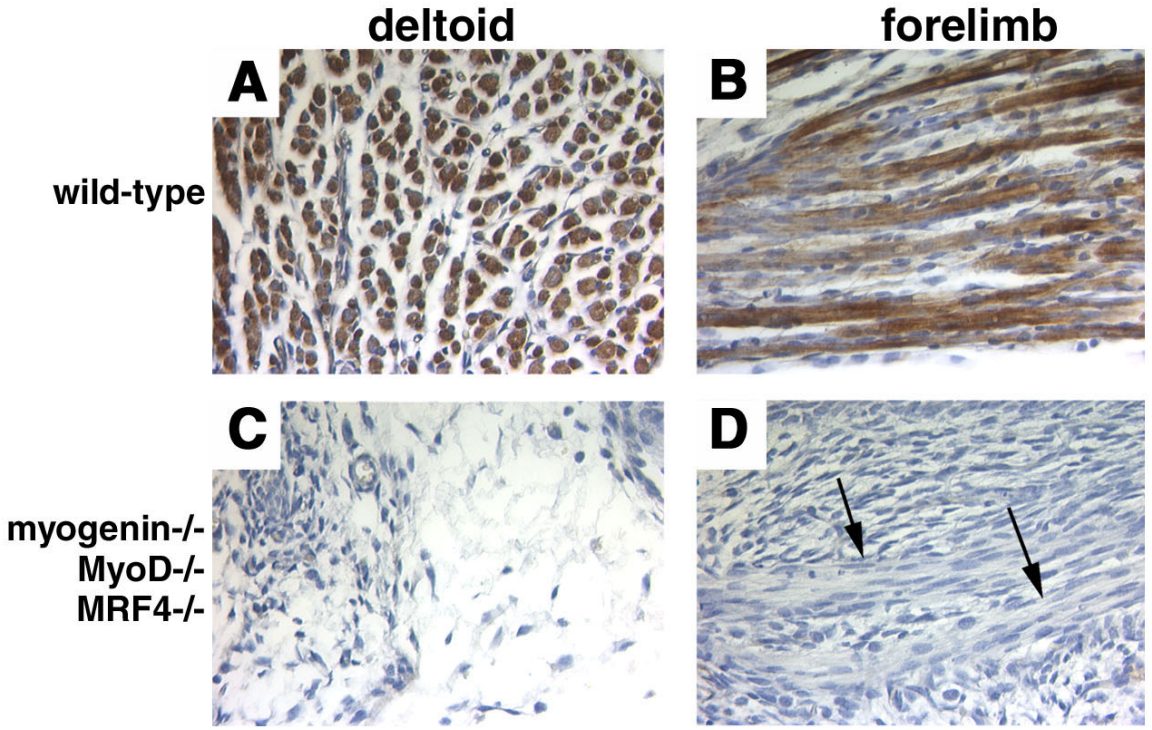

Figure 17. ***myogenin/MRF4/MyoD*** **triple mutant muscle shows little or no committed or differentiated muscle cells.** Anti-desmin staining of (A, B) wild-type and (C, D) *myogenin/MyoD/MRF4* triple mutant muscle. Transverse sections through deltoid (A, C) and forelimb (B, D) muscle from E16.0 embryos were stained with anti-desmin and counterstained with hematoxylin. arrows, multinucleate structures. Original magnification 40X.

It has been previously demonstrated that myoblasts derived from *myogenin-/-* (Hasty et al., 1993; Nabeshima et al., 1993; Rawls et al., 1995) and *myogenin-/-MRF4-/-* (Chapter 3) mice are able to differentiate as well as wild-type myoblasts *in vitro*, yet are unable to fully differentiate *in vivo*. This indicates that the environment is more permissive for myogenesis in tissue culture than in the embryo. It further suggests that the individual myogenic bHLH factors are intrinsically similar in function, but that this similarity is modified by spatiotemporal circumstances *in vivo*. In light of the fact that the triple-null animals have an even more severe muscle defect as compared to the aforementioned mutants, is was of interest to ascertain whether *myogenin-/-MyoD-/-MRF4-/-* myoblasts are able to differentiate in culture.

Primary myoblasts derived from the limbs of wild-type, *myogenin-/-MRF4-/-* and *myogenin-/-MyoD-/-MRF4-/-* E18.5 embryos were cultured under conditions that induce differentiation. These were then assayed for differentiation by immunostaining for MHC. Although the rate of myoblast fusion in *myogenin-/-MRF4-/-* myoblasts is slightly less than that of wild-type myoblasts (data not shown), the ultimate extent of differentiation is no different (Fig. 18B and A, respectively). In contrast, myoblasts derived from the triple mutants showed no evidence of fusion and did not express MHC (Fig. 18C). To further characterize the differentiation defect of triple mutant myoblasts, muscle-specific RNA expression of cultured myoblasts was analyzed by semi-quantitative RT-PCR (Fig. 19). Transcripts for myogenic bHLH factors were expressed in a manner consistent with genotype. As demonstrated for neonatal muscle (Fig. 15, lane 4), *Myf5* expression in the triple-null myoblasts was barely detectable (Fig. 19, lane 3). *MEF2C* was also expressed, but at levels much less than that of wild-type. Consistent with their failure to form myotubes in culture, triple mutant myoblasts did not express muscle differentiation markers, including *α-SkAct, eMHC*, *desmin*, *MCK* and *AchR-δ*. These data indicate that in the absence of *myogenin*, *MyoD* and *MRF4*, *Myf5* is not sufficient to direct myogenesis *in vitro*.

RIBS OF THE TRIPLE MUTANT ARE NOT COMPLETELY FORMED

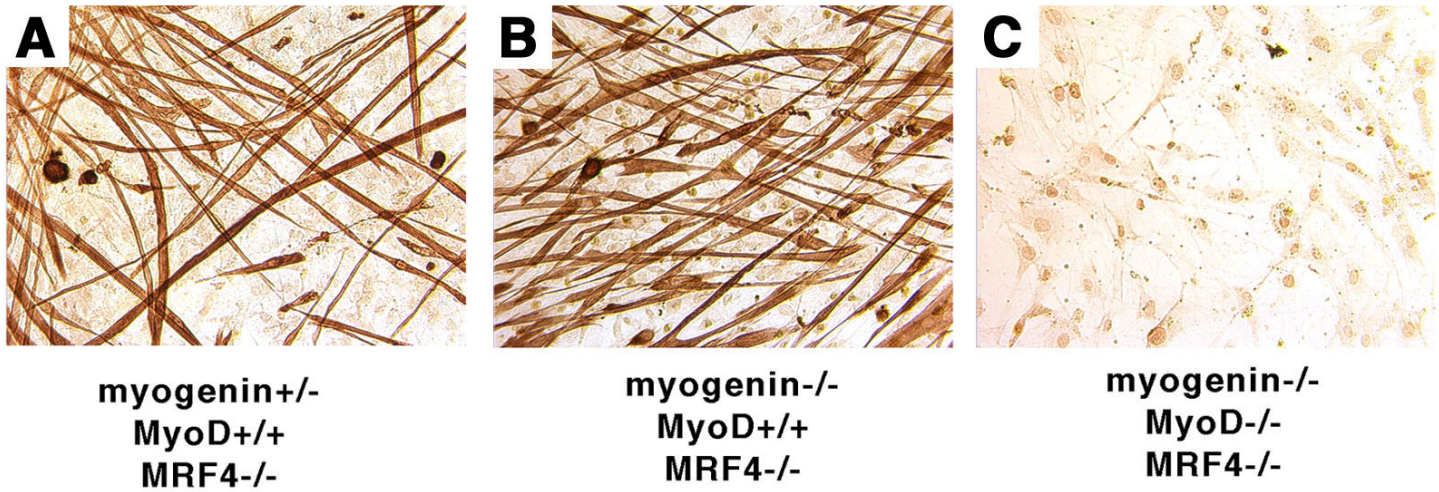

Figure 18. *myogenin-/-MyoD-/-MRF4-/-* primary myoblasts demonstrate little no differentiated muscle fibers *in vitro*. Primary myoblasts were isolated from the limbs of E18.5 (A) wild-type, (B) *myogenin/MRF4* double mutant and (C) *myogenin/MyoD/MRF4* triple mutant embryos. Primary myoblasts were cultured under conditions that induce differentiation, as described in Experimental Methods (Chapter 2). After culturing, cells were immunostained with anti-MHC.

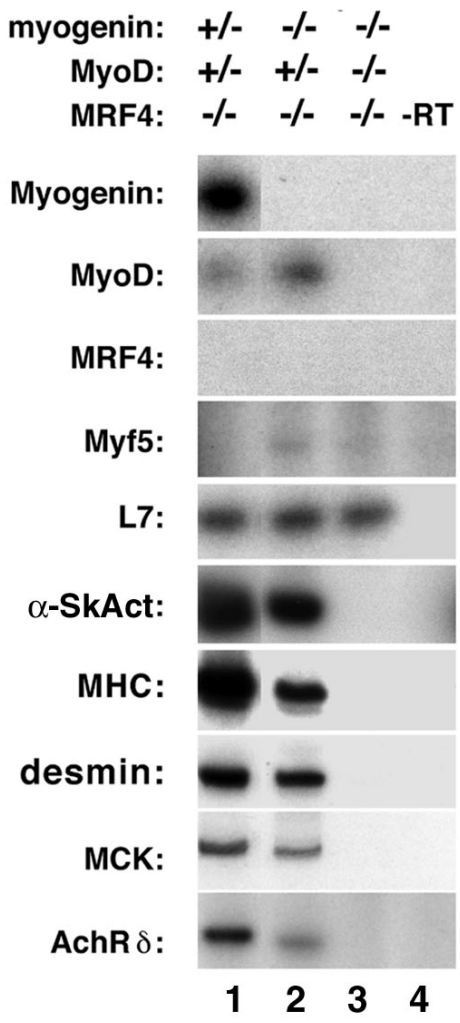

Figure 19. Analysis of muscle transcripts from wild-type and *myogenin/MRF4* double and *myogenin/MyoD/MRF4* triple mutant primary myoblasts cultured under conditions of differentiation. Total RNA was isolated from cultured myoblasts as described in Experimental Methods (Chapter 2). Expression levels of muscle-specific transcripts were determined by semiquantitative RT-PCR. *L7* transcripts were measured as a loading control. PCR reactions using RNA that was not subjected to reverse transcription are included in lane 4.

In mice lacking either *myogenin* or *MRF4*, there are abnormalities in rib morphogenesis (Braun and Arnold, 1995; Hasty et al., 1993; Patapoutian et al., 1995b; Zhang et al., 1995) that are exacerbated in *myogenin*/*MRF4* double mutants (Chapter 3). To determine whether the absence of *MyoD* further enhanced the rib abnormalities of *myogenin*/*MRF4* double mutants, we stained embryos at E15.5 of the *myogenin-/-MyoD-/-MRF4-/-* mutant genotype for bone and cartilage. As described in Chapter 3, the ribs of the *myogenin/MRF4* double mutant embryos demonstrated fusions and bifurcations of various ribs (Fig. 20C, D). The triple-null mutants demonstrated more severe rib defects (Fig. 20E, F). Although some of the ribs reached the sternum, the average lengths of the ossified portions of the ribs of *myogenin-/-MyoD-/-MRF4-/-* mutants were 30% shorter than normal at E15.5. This result was surprising, as *MyoD-/-MRF4-/-* neonatal skeletons did not have an additional rib phenotype over that of *MRF4-/-* neonates (Fig. 11E and C, respectively). All data taken together indicates that MyoD has an overlapping role with myogenin, but not MRF4, in rib development. This seems likely in view of the fact that the rib defect of the triple mutant is different in character than that of the *myogenin/MRF4* double mutant.

SUMMARY

The *myogenin/MyoD/MRF4* homozygous triple knockout mice exhibited a more pronounced defect in muscle differentiation and rib formation than *myogenin-/-*, *myogenin-/-MRF4-/-* or *MyoD-/-MRF4-/-* mice. Histological and immunohistochemical analysis demonstrated that muscle fibers *in vivo* were neither organized nor differentiated to a meaningful degree, although some skeletal muscle-specific transcripts are minimally detectable. Analysis of myotome formation showed that myoblasts are appropriately specified early in development. These data also suggest that the muscle-forming regions of the *myogenin-/-MyoD-/-MRF4-/-* mutant are likely populated with poorly differentiated myoblasts. Thus, the muscle deficiency of the triple knock-out appears to be a defect of differentiation rather than one of commitment. Moreover, myoblasts from these *myogenin/MyoD/MRF4* triple mutant mice were unable to differentiate *in vivo*. These findings suggest that Myf5 is insufficient to carry out the entire myogenic program in the absence of the other myogenic bHLH transcription

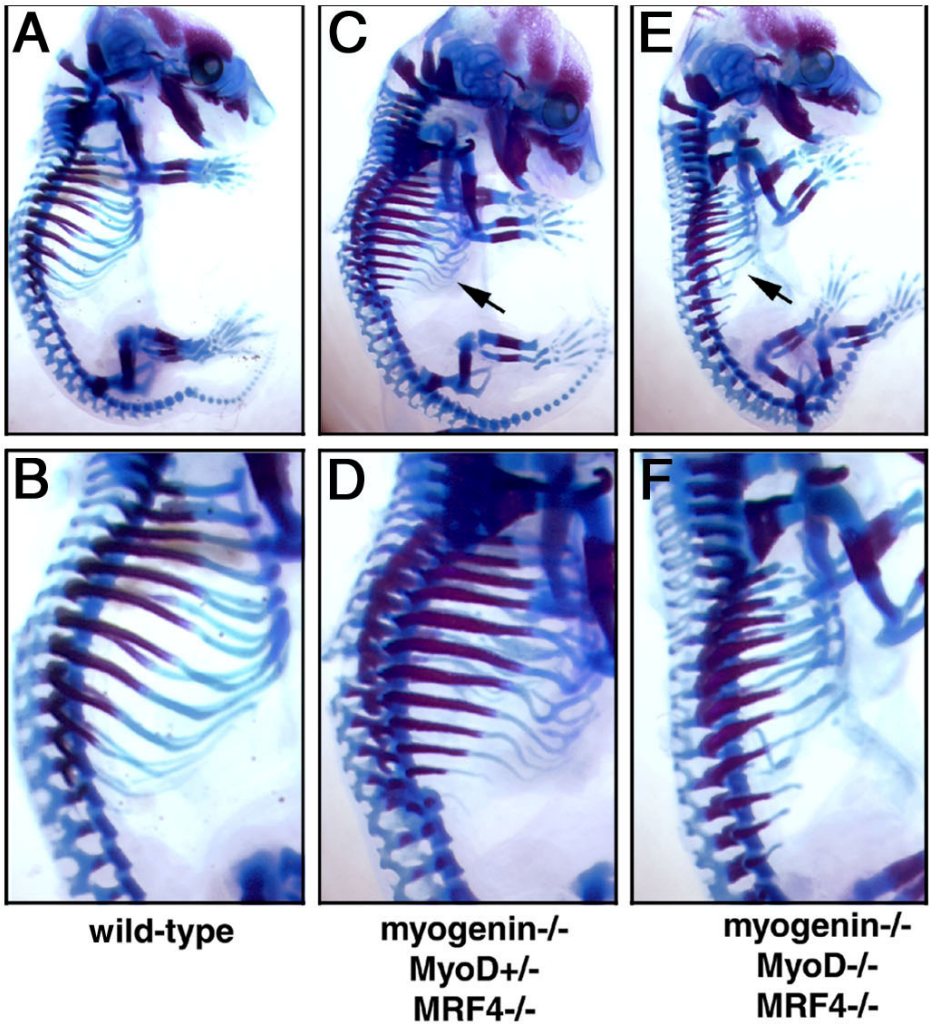

Figure 20. The ribs of *myogenin/MyoD/MRF4* triple mutant neonates are distally truncated. The skeletons of (A, B) wild-type, (C, D) *myogenin/MRF4* double mutant and (E, F) *myogenin/MyoD/MRF4* triple mutant neonates were fixed and stained for bone and cartilage with alizerin red and alcian blue, respectively. (B, D, F) show enlargements of rib cages in (A, C, E), respectively. Arrows, malformations of the rib cage.

factors. This is consistent with the idea that the myogenic bHLH factors have evolved somewhat specialized roles in skeletal muscle development. Alternatively it is conceivable that there exists a threshold level of myogenic bHLH, below which myogenesis in unable to occur (see Fig. 34).

CHAPTER V

MYOGENIC BHLH TRANSCRIPTION FACTORS AND MEF2 PROTEINS DIRECTLY REGULATE THE EXPRESSION OF *MEF2C*

Like the myogenic bHLH transcription factors, MEF2 family members are expressed in myogenic precursors as well as in developing skeletal muscle (Edmondson et al., 1994). *MEF2C* is particularly interesting because it is the first to be expressed in myogenic precursors and it is maintained at high levels in adult skeletal muscle (Martin et al., 1993). MEF2 factors are important in the development of skeletal muscle. Although MEF2 proteins are unable to induce myogenesis *in vitro*, they have been shown to synergistically activate muscle-specific gene expression with members of the myogenic bHLH family through protein-protein interactions (Black et al., 1998; Molkentin et al., 1995). MEF2 binding sites have been found in the regulatory regions of many skeletal muscle-specific genes (Black and Olson, 1998). Through mutational analysis, MEF2 sites have been shown to be required for full transcriptional activation of many skeletal muscle-specific genes, including those of myogenic bHLH transcription factors (Black et al., 1995; Cheng et al., 1993; Edmondson et al., 1992; Naidu et al., 1995; Yee and Rigby, 1993). Conversely, myogenic bHLH transcription factors are able to induce the expression of *MEF2* mRNA and protein (Cserjesi and Olson, 1991; Martin et al., 1993; Olson and Klein, 1994). The MEF2 family of transcription factors are novel early muscle genes, in that they are upstream of muscle structural genes, but downstream of the myogenic bHLH proteins. It is unknown, however whether myogenic bHLH act proteins directly or indirectly on *MEF2* genes to up-regulate their expression, since regulatory elements for vertebrate *MEF2* genes have not been identified. To further understand the mechanisms that regulate MEF2 expression during myogenesis, we sought to identify *cis*-regulatory elements responsible for the transcription of the mouse *MEF2C* gene during skeletal muscle development. Towards this end, a 6.6 kb fragment of *MEF2C* 5'-untranslated flanking DNA was identified which drives the expression of a lacZ reporter in a manner that recapitulates the embryonic expression pattern of endogenous *MEF2C*. Described here is the identification and characterization of this regulatory region.

CLONING OF THE *MEF2C* 5'-UTR AND DETERMINATION OF *MEF2C* GENOMIC STRUCTURE

The 5'-untranslated sequences of the *MEF2C* gene have not been previously defined. Therefore, as a first step toward identifying regulatory regions responsible for *MEF2C* transcription in skeletal muscle, 5' RACE was performed on mouse skeletal muscle mRNA using primers from the cDNA sequence immediately 5' of the translation initiation codon. Several overlapping cDNA clones containing extended 5'-untranslated sequence were obtained. One of the 5'RACE clones was then used to screen a mouse genomic library, resulting in 3 overlapping genomic clones.

The structure of the mouse *MEF2C* gene was characterized by genomic DNA sequencing and restriction mapping, as well as by comparison of human and mouse genomic DNA sequences from several databases. The deduced structure of the mouse *MEF2C* gene is shown in Fig. 21A. The protein coding region of the gene is comprised of 11 exons (exons 4-14) distributed over approximately 200 kb of genomic DNA. As reported previously, several exons are contained in all *MEF2C* transcripts, while others are used alternatively (Martin et al., 1993; McDermott et al., 1993). Based on RT-PCR analysis of RNA from different tissue sources and on the presence of exon sequences in expressed sequence tags, exons 6 and 7 appear to be mutually exclusive, with exon 6 being utilized in transcripts from heart and brain, and exon 7 being specific to skeletal muscle. Exons 9 and 14b are also detected in heart and brain transcripts, but not in skeletal muscle transcripts; and exon 11 is specific to brain. The AUG codon is contained in a 193 bp exon (exon 4) which, in skeletal muscle transcripts, is spliced to an approximately 250 bp exon (exon 1), located about 80 kb (kilobases) upstream. The contributions of exons 4-14 to the translated MEF2C protein are detailed in Fig. 21B.

We also performed 5' RACE with RNA from mouse heart and brain and identified two additional 5' exons that appear to be preferentially utilized in those tissues (Fig. 21A). We did not identify any additional 5' exon sequence in RACE products from these tissues, which leads us to conclude that different promoters are used in skeletal muscle, brain and

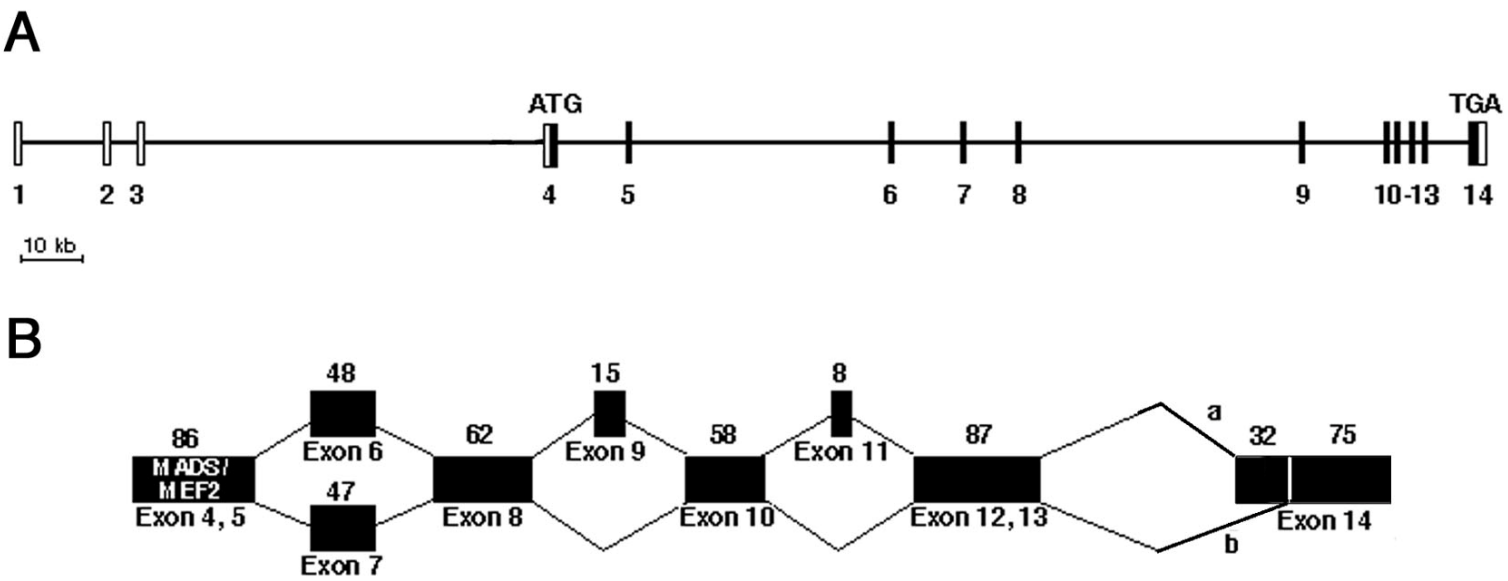

Figure 21. Schematic diagram of the mouse *MEF2C* gene. The genomic structure of the mouse *MEF2C* gene is shown. Open boxes represent UTR. Closed boxes represent translated sequence. Skeletal muscle-specific transcripts contain exon 1 spliced directly to exon 4. Exons 2 and 3 are specific to heart and brain, respectively. The translational start site for *MEF2C* is located in exon 4 (ATG) and the stop codon (TGA) is located in exon 14. The contributions of exons 4-14 to the translated MEF2C protein are detailed in the black boxes below the genomic structure. Numbers above the boxes indicate the number of amino acids in each exon. The MADS/MEF domain is encoded by exons 4 and 5. Exons 6, 7, 9, 11 and 14 are used alternatively (see text). (Determination of genomic structure done by Da-Zhi Wang.)

heart to generate the distinct 5'-untranslated regions of the transcripts from these tissues. (Determination of *MEF2C* genomic structure was done by Da-Zhi Wang).

IDENTIFICATION OF THE *MEF2C* 5' REGULATORY REGION FOR SKELETAL MUSCLE EXPRESSION

To search for the regulatory region responsible for skeletal muscle expression of *MEF2C*, a series of 5' genomic fragments were fused to the *hsp68* basal promoter upstream of a *lacZ* reporter gene and tested for expression in F_0 transgenic mouse embryos (Fig. 22). As shown in Fig. 23, the 6.6 kb region immediately 5' of the skeletal muscle-specific exon 1 was sufficient to direct strong skeletal muscle-specific expression *in vivo* (construct 1; All work with construct 1 done by Da-Zhi Wang). As a positive control for *lacZ* transgene expression, a well-defined neural crest enhancer (NCE) of the mouse *dHAND* gene, which has been shown to be specifically expressed in the branchial arches of developing embryos (J. Charité and E. Olson, unpublished), was fused upstream of this 6.6 kb genomic sequence. The NCE gave rise to an expression pattern in the branchial arches and their derivatives, as predicted. In addition, expression from this construct was localized to the somite myotomes at E9.5-11.5 (Fig. 23A-D). At E14.5, this construct was highly active in differentiated skeletal muscle fibers throughout the body (Fig. 23E). No expression in other cell types, including cardiac and smooth muscle, was detected at any developmental stage examined. Once we had identified the region of genomic DNA with skeletal muscle regulatory activity, we no longer included the NCE in subsequent transgenes.

The next goal was to identify the minimal skeletal muscle enhancer. Using MatInspector V2.2 the identified 6.6 kb genomic sequence was searched for consensus transcription factor binding sites. A total of 5 potential MEF2 sites and 7 potential myogenic bHLH E-boxes were identified. Using these potential binding sites as landmarks, candidate minimal enhancer regions were defined and fused to the *hsp68* basal promoter driving a *lacZ* reporter gene. The resulting constructs were assayed for their ability to direct *lacZ* expression in the muscle-forming regions of transgenic mice in a pattern consistent with that of endogenous MEF2C (Fig. 22, constructs 2-10). Bisection of the 6.6 kb region into

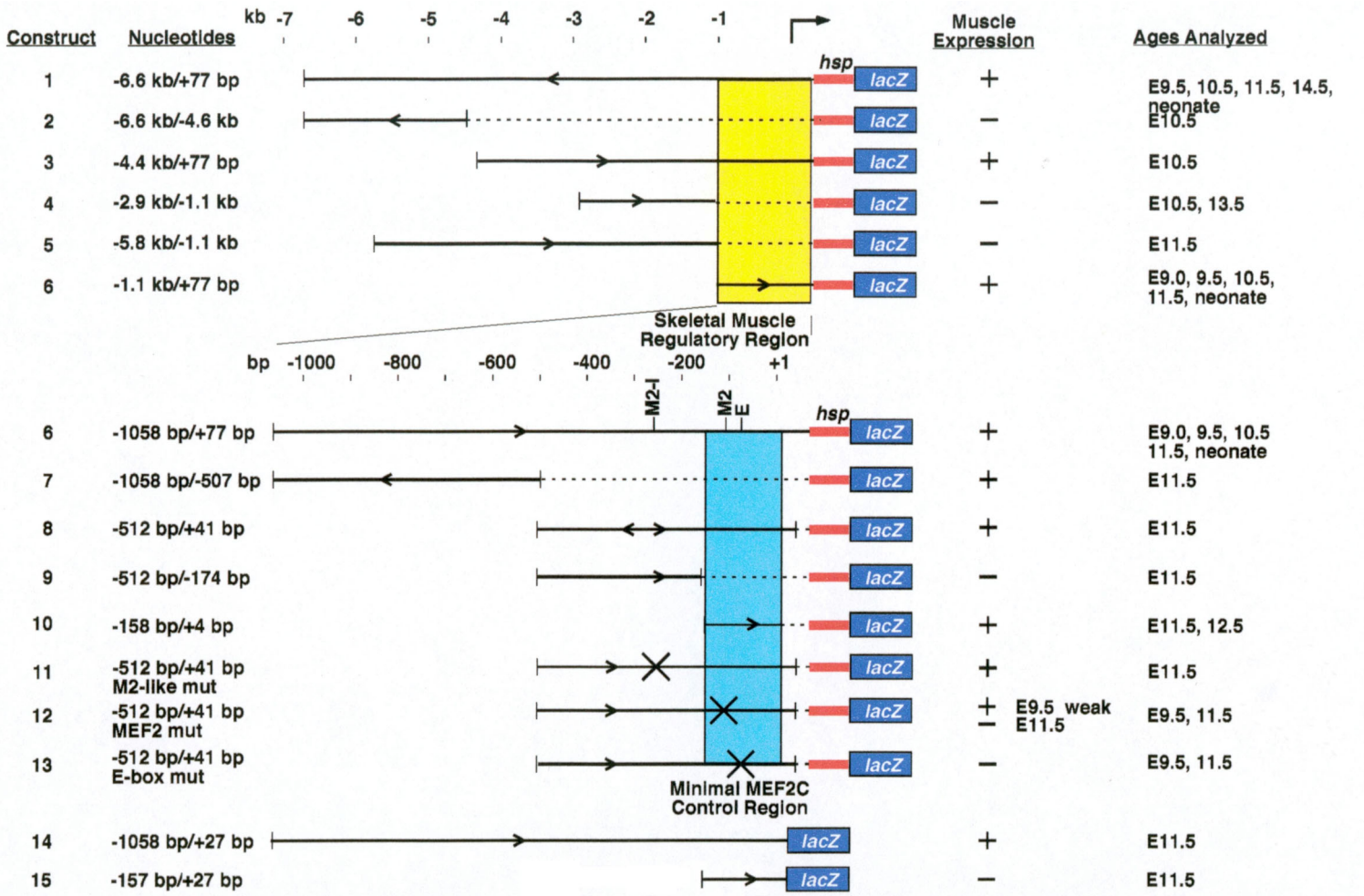

Figure 22. Transgenes used to identify the *MEF2C* skeletal muscle control region. Regions of the *MEF2C* 5' flanking DNA used in *lacZ* transgenes are shown. "+1" denotes the transcriptional start site for skeletal muscle-specific transcripts. The region containing endogenous skeletal-muscle specific promoter activity is indicated in yellow. The minimal skeletal muscle control region is indicated in turquoise. The location of the MEF2-like site at -273, MEF2 binding site at -64 and the E-box at -38 are indicated in the enlarged construct 6. ✖, mutation; >, sense orientation; <, anti-sense orientation; < >, sense or antisense orientation.

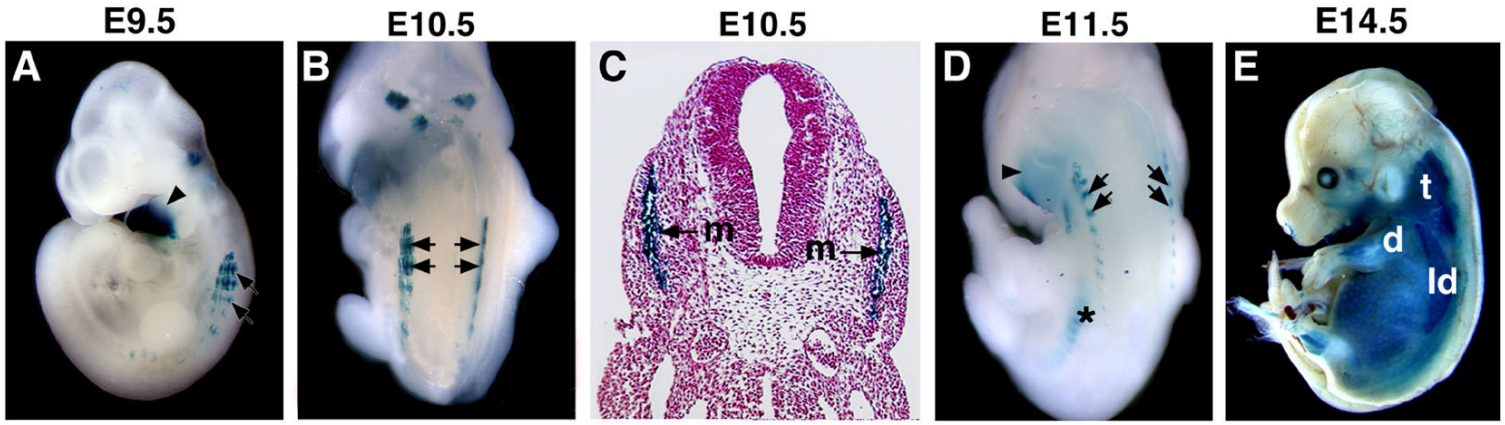

Figure 23. β-galactosidase staining of mouse embryos harboring the *MEF2C* 6.6 kb *lacZ* transgene throughout embryonic development. F_0 embryos harboring construct 1, containing the region from -6.6 kb to +77 bp (see Figure 22), were stained for lacZ expression. A neural crest enhancer (NCE) was also included in this construct as a positive control for lacZ activity. (A) An E9.5 transgenic embryo shows lacZ expression in rostral somites and weaker activity in more caudal somites. (B) Dorsal view of an E10.5 embryo shows expression in rostral and caudal somites. (C) Transverse section of an E10.5 embryo at the level of thoracic somites demonstrates lacZ staining in the myotome. (D) Dorsal view of an E11.5 embryo shows staining in somites and ventral myoblasts. (E) At E14.5, transgene expression is evident throughout much of the embryonic musculature. Also evident is branchial arch expression driven by the NCE (A), (B) and (D). arrows, somites; arrowheads, branchial arch expression; *, ventral myoblasts; m, myotome; t, trapezius; d, deltoid; ld, latissimus dorsi. (Experiment performed by Da-Zhi Wang.)

fragments from -6.6 to -4.6 and from -4.4 to the first exon (constructs 2 and 3, respectively) showed that all skeletal muscle activity was localized to the 3' DNA fragment (construct 3, Fig. 24C). Further dissection of this region localized the skeletal muscle control region to a fragment extending from exon 1 to -1.1 kb (construct 6, Fig. 24F).

There is a precedent in other muscle genes for modularity of regulatory elements in which individual control regions direct only a portion of the muscle expression pattern. In such cases the complete expression pattern requires combinations of independent regulatory regions (Firulli and Olson, 1997). We therefore carefully analyzed the expression pattern of construct 6 at various stages of development from E9.0 through E16.5 (Fig. 25). This construct was active in all embryonic skeletal myocytes that express *MEF2C*. The construct was also expressed at extremely high levels in all post-natal skeletal muscle fibers (Fig. 26). These results suggest that this upstream DNA region contains the *cis*-regulatory elements sufficient to direct the complete skeletal muscle expression pattern of *MEF2C in vivo*.

When the proximal 1.1 kb fragment (construct 6) was cut approximately in half, each portion directed a distinct pattern of *lacZ* expression within the myotome. At E11.5 the distal portion (-1058/-507, construct 7) was expressed only in the dorsomedial lip of the myotome and in ventrolateral myoblasts in the limb (Fig. 27A, B and C). In contrast, the proximal portion (-512/+41, construct 8) was expressed in the entire myotome (Fig. 27D, E). Thus, these two DNA fragments identify distinct, but overlapping myogenic precursor populations. The expression pattern of construct 8 was indistinguishable from the expression pattern of construct 6, from which it was derived. We therefore attempted to further localize the *cis*-regulatory elements within this fragment. Bisection of construct 8 into two fragments (-512/-174, construct 9, and -158/+4, construct 10) demonstrated that all of its transcriptional activity was contained in the 3' fragment (Fig. 27 F, G). Together, the above results demonstrate the existence of two independent *MEF2C* regulatory regions capable of directing transcription in the skeletal muscle lineage. The region from -158/+4 appears to be primarily responsible for the skeletal muscle-specific expression of *MEF2C*. (Promoter activity studies done by Da-Zhi-Wang.)

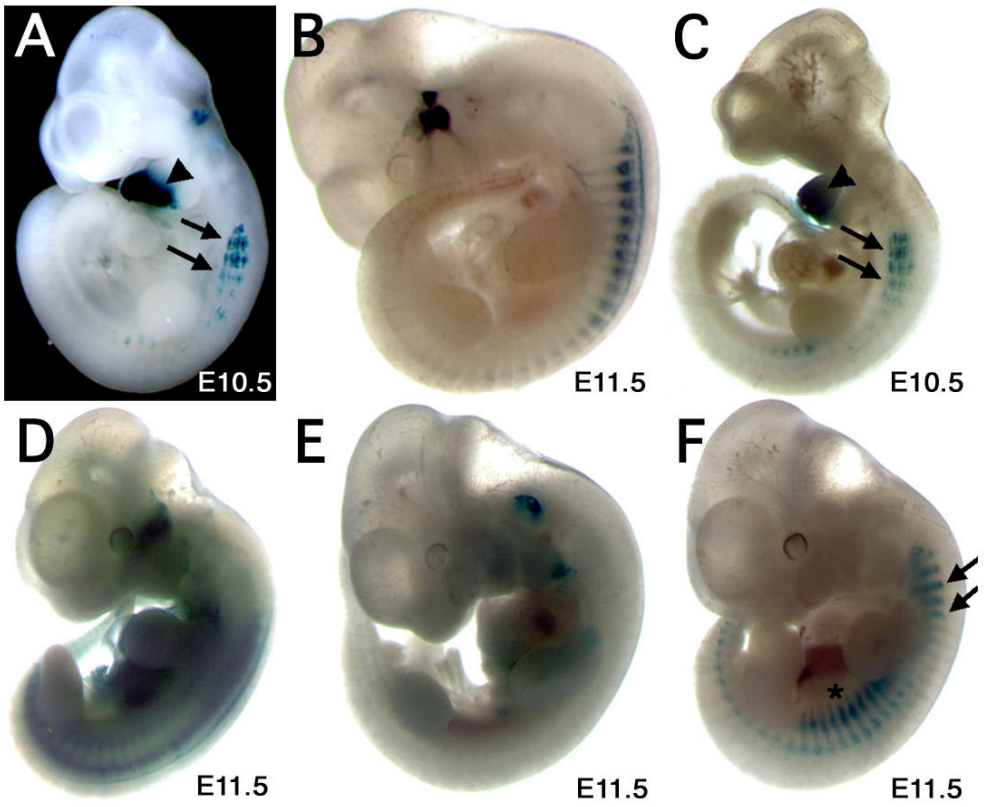

Figure 24. Gross mapping of the skeletal muscle regulatory region of *MEF2C*. As detailed in Figure 22, a series of *MEF2C* 5' genomic fragments were fused to the *hsp68* basal promoter driving a *lacZ* reporter gene and tested for expression in F_0 transgenic mouse embryos. Shown are representative embryos from these experiments. (A) Construct 1 at E10.5. (B) Construct 2 at E11.5. (C) Construct 3 at E10.5. (D) Construct 4 at E11.5. (E) Construct 5 at E11.5. (F) Construct 6 at E11.5. A neural crest enhancer (NCE) was also included in this construct as a positive control for lacZ activity in construct 1 (A) and construct 3 (C). arrows, myotomal staining in somites; arrowheads, NCE-driven expression; *, ventral myoblasts. (Panel A from experiments performed by Da-Zhi Wang.)

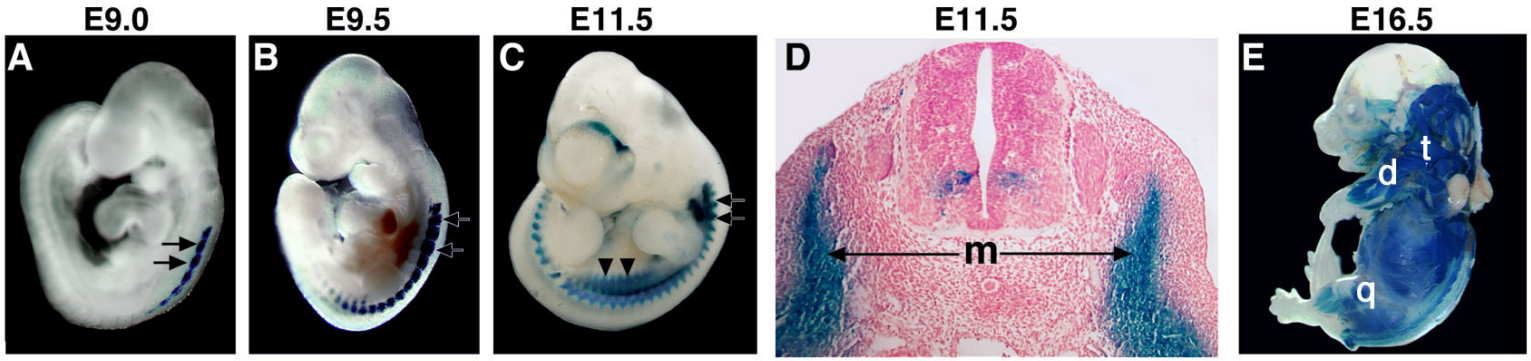

Figure 25. Expression of construct 6 during embryogenesis. The nucleotide region from -1.1 kb to +77 bp was fused to hsp68-lacZ and used to create transgenic mice (see Figure 22). (A) At E9.0, lacZ expression is detected in rostral somites. (B) An E9.5 embryo shows lacZ reporter expression from the most rostral somites to some somites in the region of the hindlimb. (C) At E11.5, transgene expression is evident throughout the somites and in ventral myoblasts. (D) Transverse section through somites at the level of the forelimb shows lacZ expression in the myotome. Neural tube staining is not reproducible. (E) At E16.5 expression of the transgene reporter is seen throughout embryonic musculature, including facial, epaxial, hypaxial and limb muscle. arrows, somites; arrowheads, ventral myoblasts; m, myotome; d, deltoid; t, trapezius; q, quadriceps.

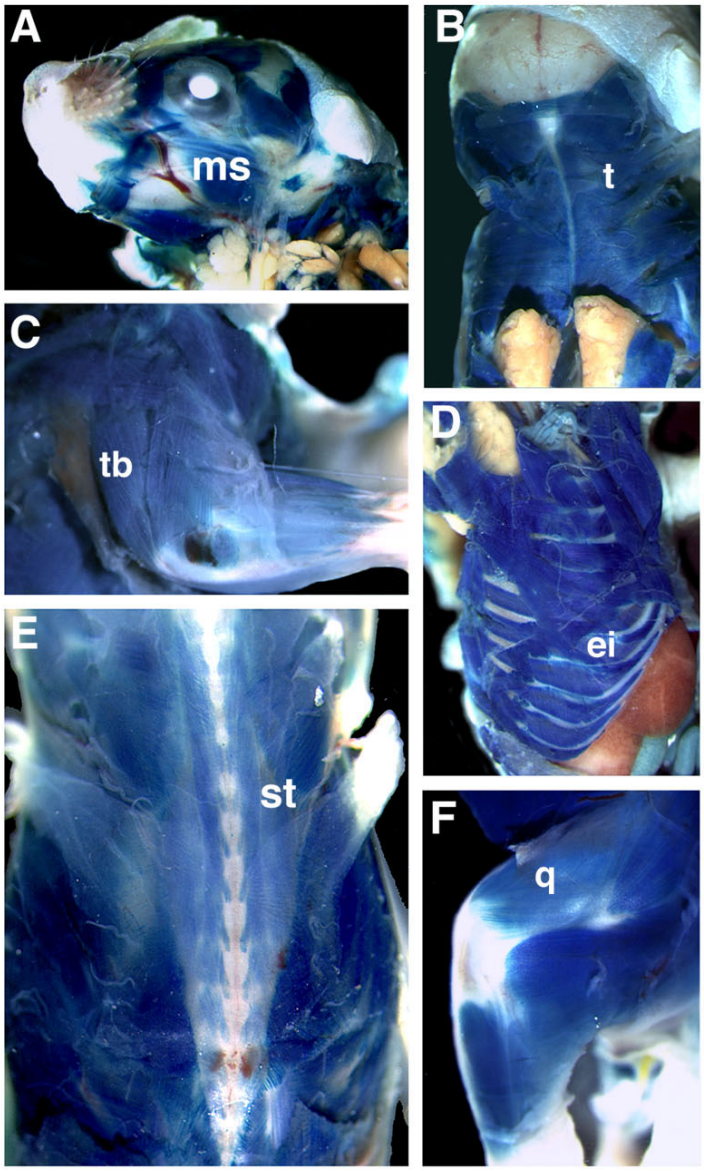

Figure 26. Expression of construct 6 in postnatal skeletal muscle. Expression of construct 6 was analyzed in F_0 neonatal transgenic mice. High expression was observed in muscles of the (A) face, (B) neck, (C) forelimb, (D) rib cage, (E) back and (F) hindlimb. ms, masseter; t, trapezius; tb, triceps brachii; ei, external intercostals; st, spinalis thoracis; q, quadriceps.

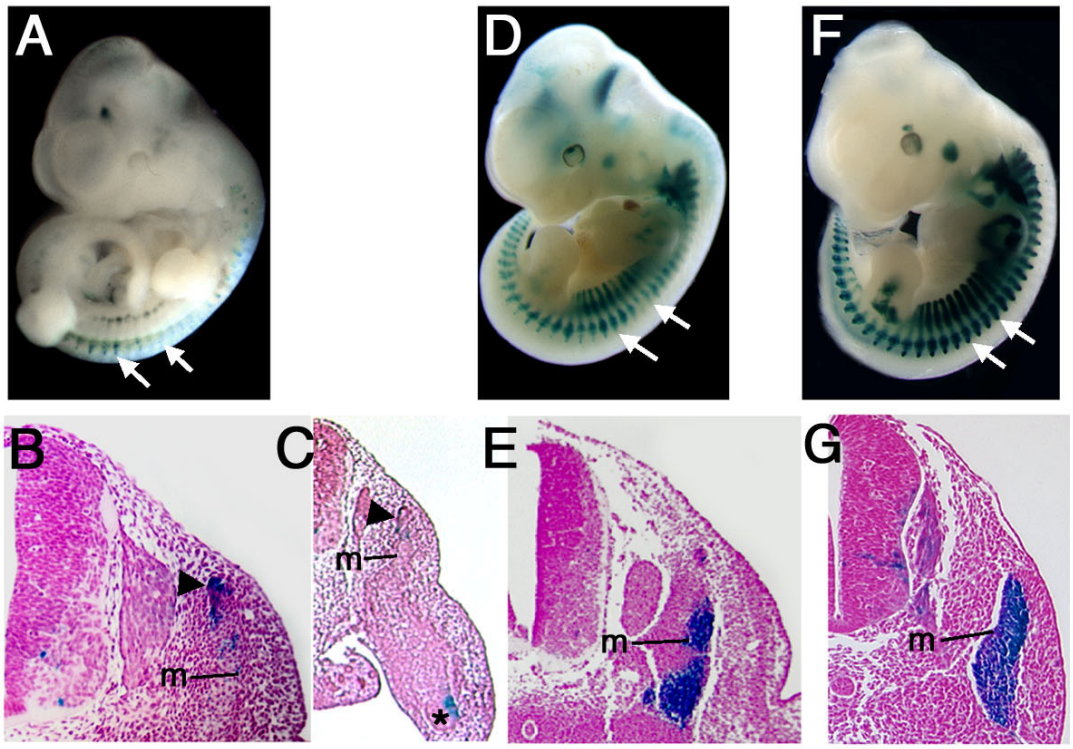

Figure 27. Delineation of the minimal *MEF2C* skeletal muscle regulatory region. Whole-mount E11.5 transgenic embryos expressing constructs 7, 8 and 10 are shown. (A) The nucleotide region from -1058 bp to -507 bp fused to *hsp68-lacZ*. Small, discrete, regions of β-galactosidase staining are seen in a metameric pattern throughout rostral and caudal somites (construct 7). (B, C) Transverse sections at the level of forelimb somites of the embryo in (B) demonstrate that the lacZ staining marks the extreme dorsomedial aspect of the myotome and ventrolateral myoblasts in the limb. (D) The nucleotide region from -512 bp to +41 bp was fused to *hsp68-lacZ* (construct 8). Strong lacZ expression is evident throughout the somites and in ventral myoblasts. (E) Transverse section through the somites of an E11.5 embryo expressing construct 8. Expression is evident throughout the myotome. (F) The nucleotide region from -157 bp to +4 bp fused to hsp68-lacZ (construct 10). Somites and ventral myoblasts show strong lacZ expression. (G) Transverse section through the somites of an E11.5 embryo expressing construct 10. Section shows expression throughout the myotome. arrows, somites; m, myotome; arrowheads, dorsomedial myotome; *, limb myoblasts.

ANALYSIS OF THE *MEF2C* REGULATORY REGION FOR PROMOTER ACTIVITY

The proximal position of the *MEF2C* control region relative to exon 1 suggested that this region might function as a skeletal muscle-specific promoter, although no consensus TATA binding sites are found in this 1.1 kb region. To test this, we fused the region from -1058 bp to +27 bp directly to a promoter-less *lacZ* transgene (Fig. 22, construct 14). This transgene showed an expression pattern at E11.5 (Fig. 28A) that was the same as that of construct 6, although its level of expression was weaker, which we presume reflects the stronger potential activity of the *hsp68* basal promoter, which was included in construct 6. This indicated that the 1.1 kb proximal regulatory region could also function as a skeletal muscle-specific promoter for *MEF2C*.

To determine whether the promoter activity of the 1.1 kb regulatory region was muscle differentiation-dependent, C2C12 myoblasts were transfected with construct 14. Transiently transfected cells were shifted to differentiation media and the activation of this promoter was monitored by β-galactosidase staining at different time points. As shown in Fig. 28C, the *MEF2C* skeletal muscle promoter is not activated in undifferentiated myoblasts. However, as myoblasts begin to differentiate into myotubes, the *MEF2C* skeletal muscle promoter-driven *lacZ* reporter begins to be expressed. This expression is further enhanced as cells approached full differentiation.

To further examine whether the *MEF2C* skeletal muscle promoter could be directly transactivated by myogenic bHLH or MEF2 proteins, we performed a transfection reporter assay using 10T1/2 fibroblast cells transiently transfected with a *luciferase* reporter driven by the 1.1 kb *MEF2C* skeletal muscle promoter. When MyoD or MEF2C expression vectors were co-transfected, a clear transactivation of the *luciferase* reporter was observed (Fig. 28B). Similar *in vivo* and *in vitro* studies were performed with the -158/+4 regulatory region (Figure 22, construct 15). This construct demonstrated no promoter activity in these assays (data not shown). This is likely due to the small size of the nucleotide region, but it is formally possible that *cis*-elements are missing in this construct. Taken together, the above data indicate that the 1.1 kb proximal regulatory region could also function as a skeletal muscle-specific promoter for *MEF2C*. This region can also direct muscle-specific expression

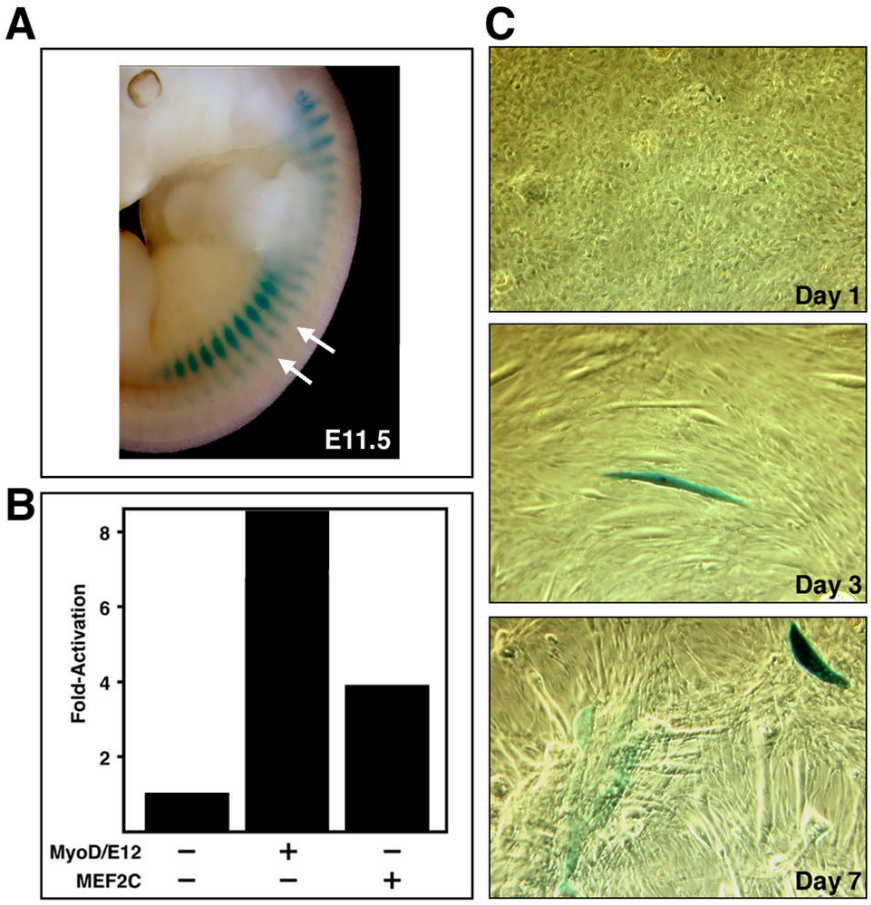

Figure 28. -1.1 kb/+27 bp has skeletal muscle-specific promoter activity. (A) The nucleotide region from -1058 to +27 was fused directly upstream of the promoter-less LacZ cassette (construct 14) and used to generate F_0 transgenic embryos. β-galactosidase staining was analyzed at E11.5. (B) 10T1/2 fibroblasts were transiently transfected with a *luciferase* reporter driven by the -1.1 kb/+27 bp MEF2C skeletal muscle regulatory region. MyoD/E12 or MEF2C were co-transfected with the luciferase reporter construct under conditions that stimulate differentiation. Background luciferase activity was determined by co-transfection of empty vector. Fold activation over background levels are shown. (C) C2C12 myoblasts were transiently transfected with construct 14. Once confluent, these cells were shifted to differentiation media. The activation of this promoter was monitored by β-galactosidase staining 1 day, 3 days and 7 days after transfer to differentiation media. arrows, somites. (Experiments performed by Da-Zhi Wang.)

in the opposite orientation when combined with the *hsp68* promoter, which suggests that it can also act as an enhancer.

REGULATION OF THE *MEF2C* SKELETAL MUSCLE REGULATORY REGION BY MYOGENIC BHLH AND MEF2 PROTEINS

The sequence of the -512/+41 *MEF2C* skeletal muscle regulatory region (construct number 8) is shown in Fig. 29A. Comparison of the sequence from the mouse and human genes showed extensive cross-species homology (data not shown). Within this regulatory region, we identified an E-box, a MEF2 site and a MEF2-like site (Fig. 29A, B; designated as E, M2 and M2-l, respectively in Fig. 22). To determine whether myogenic bHLH or MEF2 proteins could bind these sequences, we performed gel mobility shift assays using oligonucleotide probes containing these sequences and *in vitro*-translated proteins. As shown in Fig. 30A, MEF2C-myc bound to the MEF2 site-containing oligonucleotide and binding was competed by the cognate sequence, but not by a nonspecific sequence (data not shown). The identity of the MEF2C-containing complex was confirmed by its supershift with anti-myc antibody. Similarly, the E-box-containing sequence was bound by MyoD/E12 heterodimers, which were supershifted with anti-MyoD antibody (Fig. 30B). Homodimers of MyoD or E12 showed only weak binding to this site. Oligonucleotides containing the MEF2-like site were not bound by *in vitro* translated MEF2C (data not shown).

The potential roles of the transcription factor binding sites in *MEF2C* transcriptional regulation were determined by mutagenesis of each site individually within the context of the -512/+41 bp region (Fig. 22, constructs 11, 12 and 13). As shown in Fig. 31A, the MEF2-like site mutant was fully active at E11.5, while the MEF2 site and E-box mutants were inactive (Fig. 31B and C, respectively). The E-box mutant was also inactive at E9.5 (Fig. 32C and D). A total of nine F_0 transgenic embryos harboring the E-box mutant construct were analyzed. None showed expression in the skeletal muscle lineage, but several showed ectopic expression in other cell types, reflecting random integration sites of the transgene. These data indicate that the E-box is necessary for initiation, as well as maintenance, of *MEF2C* expression *in vivo*. In contrast to the complete inactivity of the E-box mutant, the

-512 AAGCTTTCTAATTTGGGAGCATGATTAATCCCTCCTATGTGATAAGTTTT
AACCTTCTAATATTTCTTTGGATTGAAAAAAGCAAATGAGCTGCGGCAAA
GAATGGCAAATAACTACAGTGCTTAACACAGTTTAATAACCTGAAATGAA
GCAAGTGTGTGCTATGTTTCCATTAAAAAGTTTCCAGCCACAATTAATTG
AACAAAAACTTGTCTTGTTCCAAGATTATTCTTGGAAATGTAATTTTAAA
GCCTGTGTGAAATGAGGAAACTTAACTTTTTATACCATATGAAAGCAATT
TCATTTTTTAGGAATGATTTTGGATAGACTTCCGATTGGATATTTTCCAT
TGGAACTAACAGTGTAGAGGCTTGGGGTGGGGAGAGAGCAGTTCTGTGTT
CTTTTGCCAGCACTGACAAAGGTCTGGTTGTCAATGATACCTTTACAGCT
AAATTTACTCCAGAGTGACATGAACAGGTGCACCCTGGCCTGCCAGACAC
TTGTGCAGAGGGATCACGCATCTCACCGCTTGACGATCAAGGGGGCAAAG
CTT +41

B

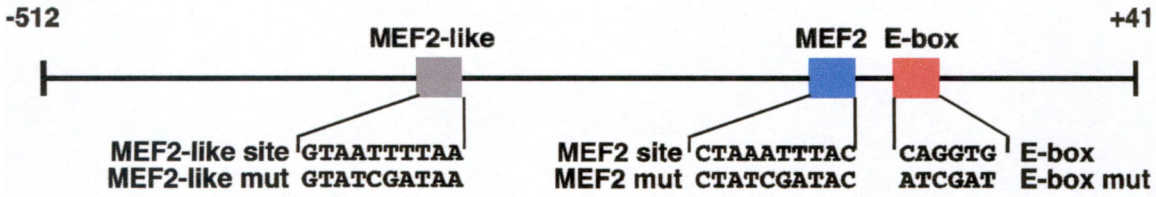

Figure 29. Sequence of the nucleotide region from -512 bp to +41 bp. (A) Sequence of the minimal skeletal muscle regulatory region is underlined. The MEF2-like site is shaded in grey. The MEF2 site is shaded in blue. The E-box is shaded in red. (B) Schematic representation of the -512/+41 *MEF2C* skeletal muscle control region. The MEF2-like site (grey box) includes nucleotides -273 through -264. The MEF2 binding site (blue box) extends from nucleotides -64 to -55. The E-box (red box) includes nucleotides -38 through -33. The specific nucleotides mutated for the experiments shown in Figs. 31 and 32 are indicated beneath the wild-type. All nucleotides are listed in the 5' to 3' direction. mut, mutated sequence.

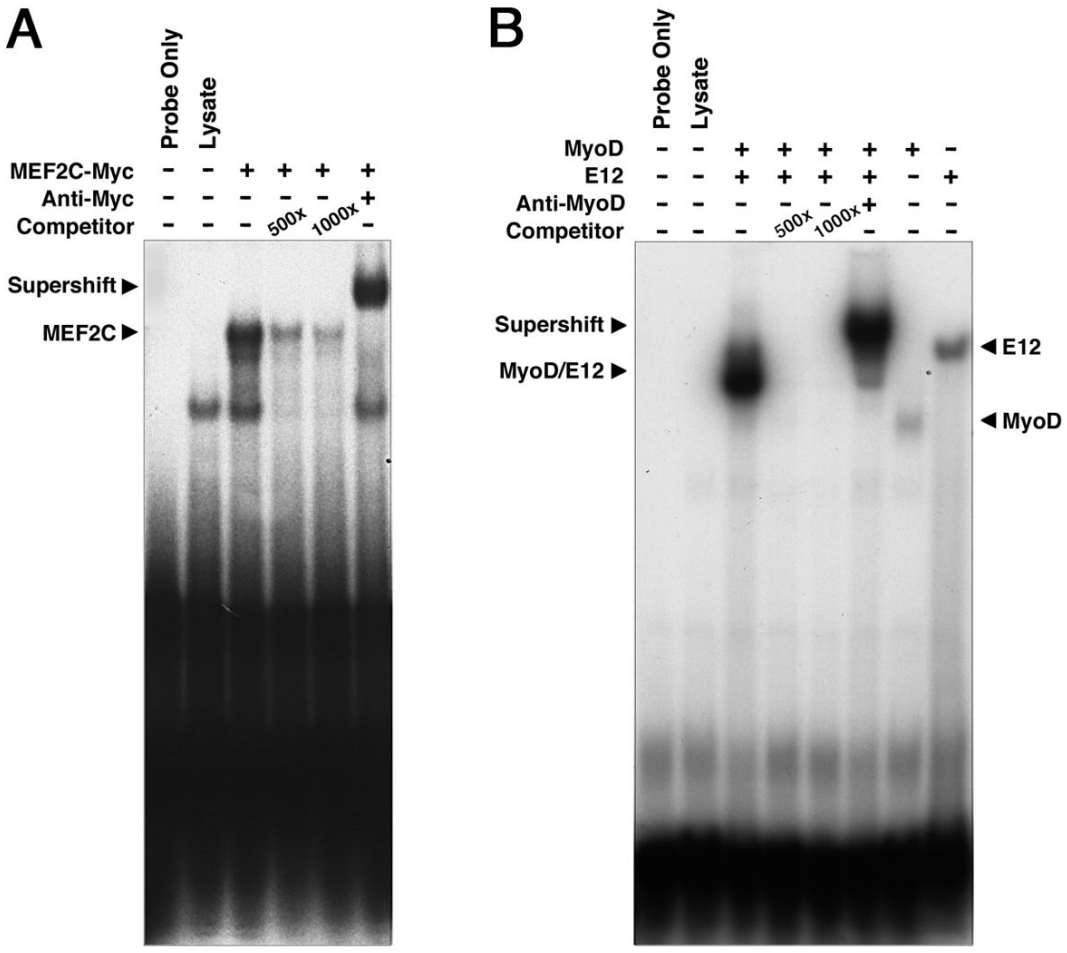

Figure 30. Binding of MyoD/E12 and MEF2C to the *MEF2C* skeletal muscle regulatory region. [α-^{32}P]-labeled oligonucleotides for the (A) MEF2 site and (B) E-box of the *MEF2C* skeletal muscle regulatory region were used as probes in gel mobility shift assays with *in vitro* translated myc-tagged MEF2C (A), MyoD (B) and E12 proteins (B). Gel shift reactions using unprogrammed reticulocyte lysate are included in parallel lanes. A 500-fold or a 1000-fold excess of unlabeled (B) MEF2 site or (B) E-box oligonucleotides were used as competitors. (A) Anti-myc and (B) anti-MyoD antibodies were used for supershifts. Labeled arrows indicate the positions of the various protein-DNA complexes.

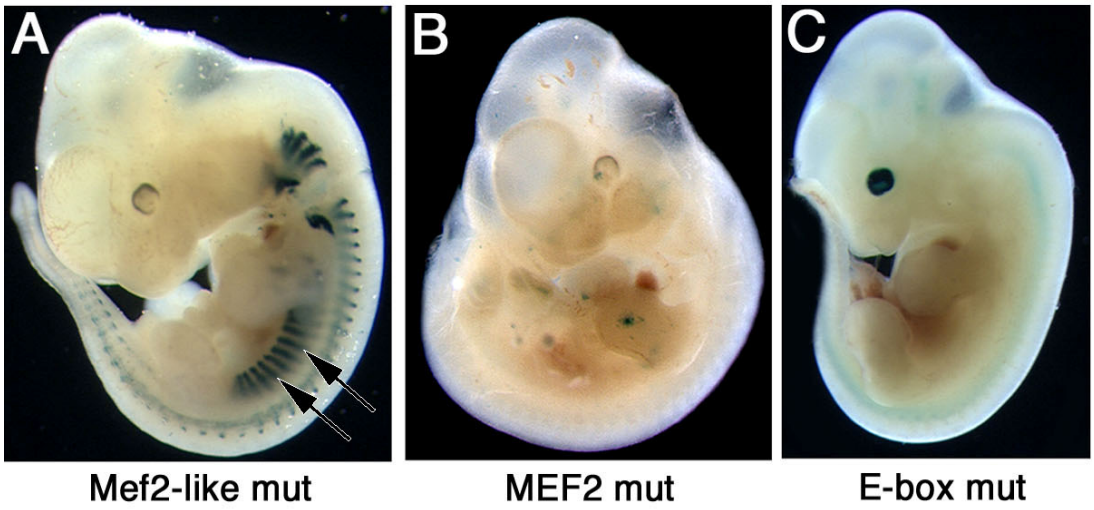

Figure 31. Inactivation of the MEF2C skeletal muscle enhancer at E11.5 by individual mutations in the MEF2 site and E-box. Mutations of the (A) MEF2-like site (MEF2-like mut), (B) MEF2 site (MEF2 mut) and (C) E-box (E-box mut) were made according to the schematic in Figure 29B. These mutations were individually made within the context of the nucleotide region from -512 bp to +41 bp. These nucleotide regions were then individually sub-cloned upstream of the hsp68-lacZ cassette (constructs 11, 12 and 13, respectively). These constructs were then used to create F_0 transgenic embryos and were analyzed for β-galactosidase expression at E11.5. (A) Only the MEF2-like mut construct is active in somites at E11.5. Constructs were inactive at all other time points. arrows, somites.

MEF2 site mutant was weakly active in the myotome at E9.5 (Fig. 32A and B). The weak activity of the MEF2 site mutant at E9.5 and its inactivity at E11.5 suggest that the MEF2 site is not required for the initiation of *MEF2C* transcription, but is essential for the maintenance and amplification of *MEF2C* expression *in vivo*.

SUMMARY

The skeletal muscle regulatory region of *MEF2C* was identified as a 1.1 kb genomic region located immediately 5' of the skeletal muscle-specific exon 1 of *MEF2C*. This region, as well as an approximately 165 bp region derived from its 3' end, was able to direct *lacZ* expression in the muscle-forming regions of transgenic mice in a pattern consistent with that of endogenous MEF2C. The identified skeletal muscle regulatory region was able to direct muscle-specific expression in both sense and anti-sense orientations when combined with the *hsp68* promoter, which suggests that it acts as an enhancer. This 1.1 kb region was also able to direct muscle-specific reporter expression in the absence of the *hsp68* promoter, indicating that it can also act as a muscle specific promoter for *MEF2C*. This control region is a direct target for the binding of myogenic bHLH and MEF2 proteins. Our findings further reveal that an E-box in this control region is required for the initiation and amplification of *MEF2C* gene expression, while a MEF2 binding site is necessary for the amplification and maintenance of *MEF2C* expression *in vivo* (see Fig. 35A). A model describing the regulatory circuit between these two classes of transcription factors during early and late myogenesis is shown in Fig. 35B.

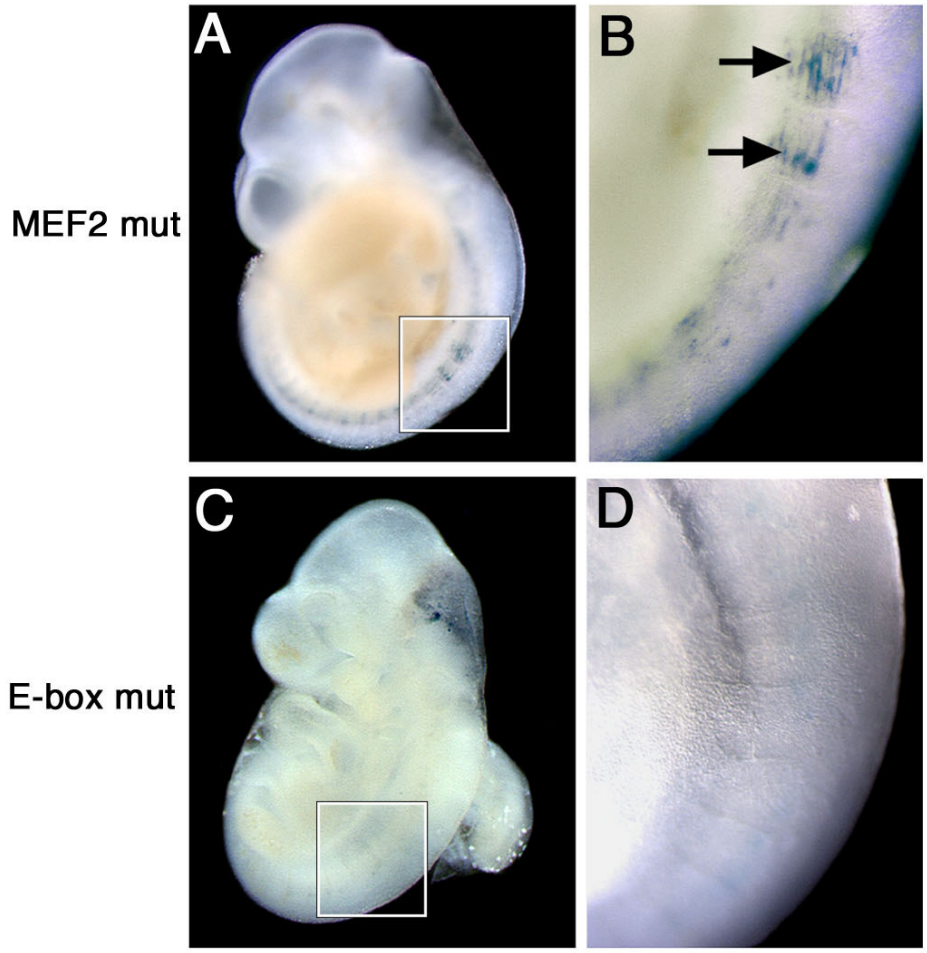

Figure 32. The MEF2 site, but not the E-box, mutant is weakly active at E9.5. Mutations of the (A, B) MEF2 site (MEF2 mut) and (C, D) E-box (E-box mut) were made according to the schematic in Figure 29B. These mutations were individually made within the context of the nucleotide region from -512 bp to +41 bp. These nucleotide regions were then individually sub-cloned upstream of the hsp68-lacZ cassette (constructs 12 and 13, respectively). These constructs were then used to create F_0 transgenic embryos and were analyzed for β-galactosidase expression at E9.5. (B and D) are enlargements of the regions indicated by the boxes in (A) and (B), respectively. (A, B) Only the MEF2 mut-hsp68-lacZ construct was weakly active in somites at E9.5. arrows, somites.

CHAPTER VI

DISCUSSION

The general goal of these studies has been to gain a better understanding of the transcription factors involved in skeletal myogenesis. As discussed in Chapter 1, the myogenic program is primarily dictated by members of the myogenic bHLH transcription factor family. Members of this family are expressed in muscle-forming regions throughout development. They directly activate the transcription of skeletal muscle-specific genes and are ultimately responsible for dictating the development of skeletal muscle. Prior to these studies, individual and combination loss-of-function mutations in each of the MyoD family members led to a model defining roles for three of the four transcription factors. Previous studies indicate that MyoD and Myf5 play overlapping roles in myoblast specification (Rudnicki et al., 1993), whereas myogenin was assigned a role in the terminal differentiation of myofibers from myoblasts (Hasty et al., 1993; Nabeshima et al., 1993). However, analysis of *MRF4-/-* knock-out mice yielded no definitive information about the role of MRF4 in skeletal muscle (Braun and Arnold, 1995; Patapoutian et al., 1995b; Zhang et al., 1995). Thus, our first goal was to further investigate the role of MRF4 in vertebrate skeletal muscle development.

MYOBLAST DIFFERENTIATION IN THE ABSENCE OF *myogenin* AND *MRF4*.

Based on the preferential expression of *MRF4* in mature muscle fibers (Rhodes and Konieczny, 1989), the up-regulation of *myogenin* in *MRF4* mutant mice (Zhang et al., 1995), and the expression of *MRF4* in residual differentiated muscle fibers in myogenin mutant mice (Rawls et al., 1995), it has been suggested that MRF4 plays a role in late stages of muscle development that may overlap with the functions of myogenin. If this were the case, we would expect that a combination of these two alleles would lead to an exacerbation of the skeletal muscle phenotype observed in the *myogenin-/-* animals. The results of the present study demonstrate, however, that this is not the case and that muscle fibers do form in *MRF4/myogenin* double mutants at a level similar to that of the *myogenin-/-*. The presence

of residual differentiated muscle fibers in *myogenin* mutant mice reveals the existence of a myogenin-independent pathway for myoblast differentiation *in vivo*. The finding that residual fibers are also present in *myogenin/MRF4* double mutants indicates that MyoD or Myf5, in addition to their role in myoblast specification, are also capable of activating differentiation in a subset of myoblasts *in vivo*. Because residual fibers are also found in *myogenin/MyoD* and *myogenin/Myf5* double mutants (Rawls et al., 1995), it appears that no single myogenic factor is clearly essential for the differentiation of most myoblasts.

It has been previously demonstrated that myoblasts derived from *myogenin-/-* mice are able to differentiate as well as wild-type myoblasts *in vitro*, in spite of the fact that they are unable to fully differentiate *in vivo* (Hasty et al., 1993; Nabeshima et al., 1993). It was shown here that *myogenin-/-MRF4-/-* myoblasts are also able to differentiate as efficiently *in vitro* as *myogenin-/-* and wild-type myoblasts. These results further support our conclusions that MRF4 is not responsible for the differentiation of *myogenin*-null myoblasts under any circumstances. Further it appears that the individual myogenic bHLH factors are intrinsically similar in function, but that this similarity is modified by spatiotemporal circumstances *in vivo*, such that myoblasts competent to differentiate *in vitro* are unable to do so fully *in vivo*. This indicates further that the environment is more permissive for myogenesis in tissue culture than in the embryo.

The only qualitative difference detected between *myogenin-/-* and *myogenin-/-MRF4-/-* fibers is that *AChR-δ* and *Myf5* transcript levels appear to be lower in the *myogenin/MRF4* double mutant. These findings suggest that MRF4 and myogenin play redundant roles in the maintenance of *Myf5* and *AChR-δ* expression during the period of muscle fiber maturation. Early expression of *Myf5* in the myotome, however, must be independent of MRF4 and myogenin because *Myf5* is expressed before MRF4 and myogenin (Bober et al., 1991; Hinterberger et al., 1991; Ott et al., 1991; Sassoon et al., 1989). Enhancers that control *Myf5* expression have been identified within the region between the *Myf5* and *MRF4* genes (Patapoutian et al., 1995b), within the body of the *MRF4* gene (Yoon et al., 1997), as well as within a region more than 45 kb upstream and within 500 kb downstream of the *Myf5* gene (Zweigert et al., 1997). However, none of these enhancers appears to direct the late

expression of the *Myf5* gene in differentiated muscle fibers, a time when *Myf5* expression is predicted from our results to fall under myogenin and MRF4 control. Recently, regulatory regions for *Myf5* expression have been identified that direct *Myf5* expression in back muscle as late as E13.5, but not in other muscle groups (Summerbell et al., 2000). When all of the regulatory regions responsible for the late expression of *Myf5* are identified, it will be interesting to see if myogenin and MRF4 are able to directly regulate them.

MUSCLE DEFECTS IN *MRF4/MYOD* DOUBLE MUTANTS.

A surprising result from this study was that while neither MyoD nor MRF4 alone is required for muscle development (Rudnicki et al., 1992; Zhang et al., 1995), *MRF4/MyoD* double mutants exhibited severe skeletal muscle defects, similar to those seen in *myogenin* mutants. Since *myogenin* and *Myf5* were expressed in these double mutants, these findings suggested that the overall concentration of myogenic bHLH factors may need to reach a critical threshold to induce myoblast differentiation. In the absence of *MRF4* and *MyoD*, myogenic bHLH protein levels are insufficient to trigger the differentiation program. For this hypothesis to be true, myogenin would have to be a stronger activator of the myogenic program, since loss of its two alleles causes the same level of muscle defects as the loss of both alleles of *MyoD* and *MRF4*. If myogenin is a stronger activator of the muscle differentiation program than either MyoD or MRF4, but all three factors contribute to achieving a threshold level of myogenic bHLH protein expression required for initiating myogenesis, reducing the level of myogenin, as in a *myogenin+/-* background, might sensitize the animal to the level of MyoD and MRF4 and result in muscle deficits when animals were homozygous for one of these mutant alleles and heterozygous for the other. However, we found that *myogenin+/-MyoD+/-MRF4-/-* mice were normal. Thus, the level of bHLH protein expression from a single *myogenin* and *MyoD* allele is sufficient to support normal muscle development, whereas the level of expression from two *myogenin* alleles is not.

Alternatively, the severe muscle deficiency in *MyoD/MRF4* double mutants could indicate that MRF4 shares a specific myogenic function with MyoD that can not be

compensated for by myogenin or Myf5. If the latter explanation is correct, it might be expected that the residual fibers present in the *myogenin* mutants would be distinct from those in the *MyoD/MRF4* mutants. In fact, there are some slight differences in skeletal muscle-specific gene expression between these two mutants. *MCK*, *MHC* and *α-SkAct* levels are higher than those in the *myogenin*-null, but lower than wild-type levels. This could indicate that the residual myofibers of the *MyoD/MRF4* double mutant are more completely differentiated than those of the *myogenin-/-*. It could also mean that there are higher numbers of residual fibers in the *MyoD-/-MRF4-/-*. However, *AChR-δ* levels in the *MyoD/MRF4* mutant are lower than both wild-type and *myogenin-/-* levels. This suggests that the residual fibers that persist in each mutant are molecularly distinct from one another. We therefore favor the model that MyoD and MRF4 share overlapping roles in the differentiation of skeletal muscle independent of the role of myogenin in differentiation (Fig. 33).

A potential complication in interpreting phenotypes of MRF4 mutants is that Myf5 levels are affected in *cis* by the *MRF4* mutation (Olson et al., 1996). Thus it is conceivable that the *MyoD/MRF4* double mutant could reflect a reduction in *Myf5* expression, as well. While this possibility can not be formally ruled out, it is unlikely for several reasons. First, in the *MRF4* mutant mice used in these experiments, there is only a transient reduction in *Myf5* expression at E10.5, but by a day later, *Myf5* expression is normal (Yoon et al., 1997; Zhang et al., 1995) and muscle development is unaffected. The muscle phenotype in the *MRF4/MyoD* double mutants is not observed until the late fetal and neonatal period, which is several days later than the transient reduction in *Myf5* expression. Second, since MyoD and Myf5 have overlapping roles in the specification of myoblasts, if the severe muscle defects in *MyoD/MRF4* double mutants arose from a reduction in *Myf5* expression, we would expect to see a reduction in myoblasts in the double mutant. Instead, the muscle defects seen in the *MyoD/MRF4* double mutants are very similar to those observed in the *myogenin-/-* phenotype, with unfused myoblasts populating the presumptive muscle-forming regions of the mice.

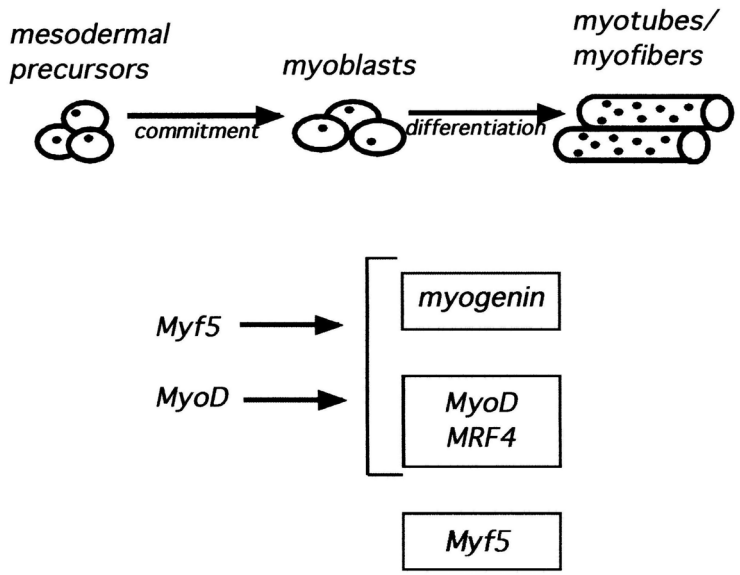

Figure 33. A model for the terminal differentiation of skeletal myofibers *in vivo*. Myogenesis occurs through a two-step mechanism. First, mesodermal precursor cells become committed to the myogenic lineage. Second, committed myoblasts differentiate to form mature skeletal muscle fibers. MyoD and Myf5 have been previously shown to play redundant roles in both the commitment of multipotential muscle precursors to the myogenic lineage as well as the initiation of myogenin and MRF4 expression *in vivo* (Braun et al., 1992; Rudnicki et al., 1992; Rudnicki et al., 1993). In contrast, myogenin has been shown to play a significant role in the terminal differentiation of myofibers from myoblasts (Hasty et al., 1993; Nabeshima et al., 1993). The current studies demonstrate that mutants lacking *MyoD* and *MRF4* have a deficiency in myogenesis similar to that of the *myogenin-/-*, although *myogenin* transcripts are at wild-type levels. Thus, MyoD and MRF4 share overlapping roles in a myogenin-independent pathway in the differentiation of skeletal muscle *in vivo*. Animals lacking *myogenin*, *MyoD* and *MRF4* were also able to initiate differentiation, indicating that Myf5 may also functions in the initiation of differentiation.

UNIQUE EXPRESSION VERSUS UNIQUE FUNCTIONS OF THE MYOGENIC BHLH FACTORS.

A central question in skeletal myogenesis is whether the different myogenic bHLH factors in vertebrates have evolved specialized functions or whether the different myogenic phenotypes that result from inactivation of these genes reflect their distinct expression patterns. Collectively, the results of *in vivo* and *in vitro* studies suggest that both the levels of expression and unique functional activities of the individual factors are important for precisely orchestrating muscle determination and differentiation during embryogenesis.

In transfection assays in which the individual factors are over-expressed, the different factors show similar myogenic activities, although subtle differences in their abilities to transactivate certain muscle promoters have been reported (Brennan et al., 1990; Chakraborty and Olson, 1991; Yutzey et al., 1990). Recent gene replacement studies suggest that certain of the myogenic bHLH genes can only partially compensate for one another's functions *in vivo*. For example, expression of a transgene in which *MRF4* expression is controlled by the *myogenin* promoter in a *myogenin*-null background results in only some rescue of muscle differentiation (Zhu and Miller, 1997), suggesting that MRF4 has the ability to partially, but not completely, substitute for the functions of myogenin if it is expressed in the same temporospatial pattern as myogenin. In other experiments, mice have been created in which the *Myf5* gene was replaced with the *myogenin* coding region (Wang et al., 1996). When this *myogenin* knock-in allele was bred into a *MyoD*-null background, it was able to support the early functions of Myf5 in myoblast specification (Wang and Jaenisch, 1997). These conclusions are also consistent with the finding that myoblasts from the various mutants can differentiate in culture with no apparent requirement for any specific myogenic bHLH factor.

MUSCLE DEVELOPMENT IN MYOGENIC BHLH MUTANTS MISSING THREE MYOGENIC BHLH GENES.

While it seems clear that the different myogenic factors can compensate, at least partially, for each other's functions and that their unique expression patterns contribute to their roles in muscle development, it remains unclear whether a single myogenic bHLH

transcription factor is able to direct the entire myogenic program *in vivo* or *in vitro*. *Myf5* is the first myogenic bHLH gene to be expressed during mouse embryogenesis, with transcripts appearing in the dermomyotome at E8.0 (Ott et al., 1991). Since the onset of *Myf5* expression precedes that of all other myogenic bHLH genes, initial activation of the gene must be independent of myogenic bHLH factors. For this reason we chose to investigate the myogenic functions of Myf5 *in vivo* and *in vitro*, without complications from functional redundancy and cross-regulatory interactions with other myogenic bHLH genes. In order to do this, we generated mice lacking *myogenin*, *MyoD*, and *MRF4*. This is the first description of a mouse with only a single myogenic bHLH gene.

The skeletal muscle phenotype of these mice is much more severe than those of the other single or double myogenic bHLH gene mutants previously described. *myogenin-/-MyoD-/-MRF4-/-* demonstrate almost negligible skeletal muscle development *in vivo*. Presumptive muscle-forming regions are populated with undifferentiated mononuclear cells and only traces of anything resembling residual muscle fibers. Analysis of muscle specific-transcripts confirmed that little or no differentiation had occurred in these regions. Transcripts for *α-SkAct*, *desmin* and *MCK* are only barely detectable and *eMHC* and *AChr-δ* are undetectable. Additionally, primary myoblasts derived from the limbs of the triple mutant are unable to differentiate *in vitro*, in comparison to primary myoblasts derived from all other myogenic bHLH mutants described, which are able to differentiate as efficiently as wild-type. Of the skeletal muscle-specific transcripts analyzed for these cultured triple mutant myoblasts, only *Myf5* and *MEF2C* are even remotely detectable. Thus, although Myf5 has the potential to activate myogenesis in transfection assays, it cannot act autonomously to maintain the myogenic phenotype in the absence of other myogenic factors *in vivo* or *in vitro*.

DIFFERENT REQUIREMENTS FOR ACTIVATION AND MAINTENANCE OF MYF5 EXPRESSION.

The low levels of *desmin* expression in triple mutant neonatal muscle and the undetectable levels of *desmin* in cultured triple mutant myoblasts suggested that the primary

defect of the triple mutant could be at the stage of commitment to the myogenic lineage. it has been shown that Myf5 is important in the commitment of mesodermal precursors to the myogenic lineage (Rudnicki et al., 1993). In *Myf5* mutants, somite myogenesis is delayed until the onset of *MyoD* expression, also reflecting the requisite and at least partially redundant roles of Myf5 and MyoD in specification of the myogenic lineage (Braun et al., 1994; Kablar et al., 1997). However, the level and extent of *Myf5* expression in the *Myf5*-expressing regions of the somite of the triple mutant were the same as those in wild-type embryos. This is consistent with the finding that neonates of the triple mutant genotype contained a normal number of cells in presumptive muscle-forming regions, suggesting that myoblast specification occurred normally. However, by the neonatal stage, we detected little, if any, *Myf5* expression in presumptive myoblasts of triple mutant neonates. The reduction in *Myf5* expression by the neonatal stage suggests that other myogenic bHLH genes are required for maintenance of *Myf5* expression. This is consistent with myogenin and MRF4 sharing a role in the maintenance of *Myf5* expression, as described above. The down-regulation of *Myf5* in the triple mutants also suggests that Myf5 does not efficiently auto-regulate its own expression. Thus, different signals are responsible for the initiation versus the maintenance of *Myf5* expression *in vivo*.

THRESHOLDS OF MYOGENIC FACTORS VERSUS DIFFERENT MYOGENIC LINEAGES.

The phenotype of *myogenin-/-MyoD-/-MRF4-/-* triple mutants, in which myoblasts are specified but cannot differentiate, differs from that of *MyoD/Myf5* double mutants, in which myoblasts are not specified, or *myogenin* and *MyoD/MRF4* mutants, in which a subset of specified myoblasts can differentiate *in vivo*. In addition, cultured myoblasts from *myogenin* and *MyoD/MRF4* mutants differentiate normally *in vitro*, whereas myoblasts from the triple mutants do not. The virtual absence of myoblast differentiation in *myogenin-/-MyoD-/-MRF4-/-* mutants could reflect the existence of an appreciable functional redundancy among these three factors such that the absence of either *myogenin* or the combination of *MyoD* and *MRF4* result in a partial block to myogenesis, whereas the

combined absence of all three factors prevents differentiation altogether. However, there is some evidence that an extremely minimal level of myofiber differentiation does occur *in vivo*, as demonstrated by the presence of trace amounts of *α-SkAct* and *MCK* transcripts in neonatal muscle from triple mutants. Therefore it appears that Myf5 can initiate differentiation, but apparently it alone is not competent to carry the process of differentiation to completion. It is important to note, however, that *Myf5* levels are undetectable in neonatal muscle of the triple mutant, presumably because the other myogenic bHLH transcription factors are not present to maintain its expression. Therefore it seems that all four of the myogenic bHLH transcription factors are each functionally able to initiate differentiation (Fig. 33), but it is the absolute level of total myogenic bHLH transcription factors that dictates the degree of differentiation (Fig. 34). Indeed it has been shown, using a hypomorphic *myogenin* allele, that the degree of differentiation of skeletal muscle is exquisitely sensitive to the absolute levels of myogenin *in vivo* (Vivian et al., 1999). It is still formally possible, however, that Myf5 is not functionally able carry out normal differentiation, and that this is the reason why muscle development is so severely handicapped in *myogenin/MyoD/MRF4* triple mutants.

MEF2C EXPRESSION AND SKELETAL MUSCLE DEVELOPMENT: IDENTIFICATION OF A *CIS*-REGULATORY ELEMENT THAT FUNCTIONS AS A SKELETAL MUSCLE SPECIFIC ENHANCER AND PROMOTER FOR *MEF2C*

The necessity of transcriptional co-factors for the myogenic bHLH proteins is demonstrated by the fact that E-boxes are not present in the regulatory regions of skeletal muscle-specific genes shown to be regulated by the myogenic bHLH transcription factors during myogenesis or are not required for the activity of these promoters (Bouvagnet et al., 1987; Buchberger et al., 1994; Cheng et al., 1992; Edmondson et al., 1992; Mar and Ordahl, 1990; Peterson et al., 1990; Thompson et al., 1991). It has since been shown that MEF2 transcription factors play a central role in the control of skeletal muscle development by enhancing the muscle-inducing activity of myogenic bHLH proteins (Black et al., 1998; Molkentin et al., 1995). Like the myogenic bHLH transcription factors, MEF2 family

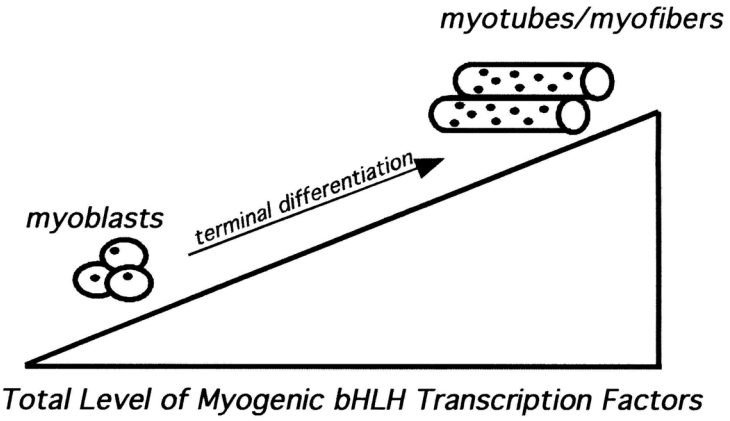

Figure 34. Myogenic bHLH "levels" model for the terminal differentiation of skeletal muscle. While mice lacking either *myogenin* alone or *MyoD* plus *MRF4* exhibit a lethal deficiency of differentiated skeletal muscle fibers, there are residual muscle fibers present in mutants of these genotypes (Hasty et al., 1993; Nabeshima et al., 1993; Rawls et al., 1998). In contrast, mutants lacking *myogenin*, *MyoD* and *MRF4* have almost undetectable levels of differentiated muscle, although myoblasts are efficiently committed early in development. The fact that an increasing loss of myogenic bHLH proteins *in vivo* demonstrates a decreasing level of skeletal muscle development suggests that it is the total level myogenic bHLH transcription factors that dictates the general level of skeletal muscle differentiation.

members are expressed early in myogenic precursors and in developing skeletal muscle. *MEF2C* is the first member of the MEF2 family to be expressed in skeletal muscle; its expression is initially detected at E9.0 in the rostral myotomes at the onset of myocyte differentiation (Edmondson et al., 1994) and expression is maintained throughout development and into adulthood (Martin et al., 1993).

While much has been learned about the mechanisms whereby MEF2 proteins activate muscle-specific transcription, little is known of the mechanisms that regulate expression of *MEF2* genes and no *cis*-regulatory elements that control transcription of vertebrate *MEF2* genes have been previously identified. This has been a difficult problem because the 5'-UTR of *MEF2* genes are each encoded by multiple alternative exons distributed over approximately 80 kb of genomic DNA. To locate the control region responsible for the expression of *MEF2C* in skeletal muscle, we began by performing tissue-specific 5'-RACE to identify 5'-exons encoding skeletal muscle transcripts. We also took advantage of the completed human genome sequence by searching the human genome database using sequences from the tissue-specific 5'-untranslated sequences that we identified. Our results demonstrate that alternative splicing of exons 1, 2 and 3 gives rise to *MEF2C* transcripts that are specific to skeletal muscle, heart and brain, respectively.

In the current study, we were able to detect *MEF2C-lacZ* transgene expression in the myotome as early as E9.0, throughout skeletal muscle development, and into adult muscle, fully recapitulating that of the endogenous *MEF2C* gene. This region was able to do so in both sense and anti-sense orientations indicating that it functions as an enhancer. The proximity of the regulatory region to the transcriptional start site of *MEF2C* led us to investigate its potential promoter activity. In fact the -1.1/+77 regulatory region of MEF2C was able to drive the muscle specific expression of a *lacZ* reporter both *in vivo* and *in vitro*. Thus, this proximal regulatory region can also function as a promoter.

REGULATION OF *MEF2C* GENE EXPRESSION IN SKELETAL MUSCLE: INITIATION VERSUS AMPLIFICATION/MAINTENANCE

Forced expression of myogenic bHLH proteins in non-muscle cells is sufficient to up-regulate *MEF2* expression (Cserjesi and Olson, 1991; Lassar et al., 1991). Our results clearly show that this reflects the direct binding of myogenic bHLH proteins to an E-box in the proximal promoter of the *MEF2C* gene. Further, our data demonstrate that this E-box is essential for the initial activation of *MEF2C* transcription in the skeletal muscle lineage. An E-box mutation in the *MEF2C* promoter completely abolished *lacZ* reporter transcription at E9.5. Since the E-box mutation also abolished transcription at later stages of development, binding of myogenic bHLH proteins to this site also appears to be required for the maintenance of *MEF2C* expression. As Myf5 and myogenin are expressed prior to *MEF2C*, these factors are potential initiators of *MEF2C* transcription. In mature skeletal muscle fibers, it is likely that it is MRF4 that maintains *MEF2C* expression, as this factor is the most highly expressed in post-natal muscle.

In contrast to the requirement of the E-box for initiation of *MEF2C* transcription, a mutation of the MEF2 site did not affect the timing for initial activation of the reporter gene such that weak *lacZ* expression was detected in the somites at E9.5. However, at later stages this MEF2 site mutant was unable to maintain *lacZ* expression and was completely inactive at E11.5. Together, these results suggest that MEF2C acts on its own promoter to amplify and maintain its expression in differentiating myoblasts and differentiated muscle. Other members of the MEF2 family also become expressed at high levels in the somite myotome after E9.5 (Edmondson et al., 1994), which could further reinforce the expression of *MEF2C*. Our results demonstrate that myogenic bHLH transcription factors are required for the initial activation of *MEF2C* expression *in vivo*. Although MEF2 proteins are not sufficient to activate *MEF2C* transcription, they cooperate with the myogenic bHLH proteins, which provide the muscle-specificity necessary to direct the expression of *MEF2C* in cells of the myogenic lineage throughout development and into adulthood. A model of how myogenic bHLH and MEF2 proteins cooperatively regulate the expression of *MEF2C* during skeletal myogenesis is shown in Fig. 35A. This integration of myogenic activity and auto-regulation explains why this promoter region is inactive in other cell types, such as cardiac myocytes

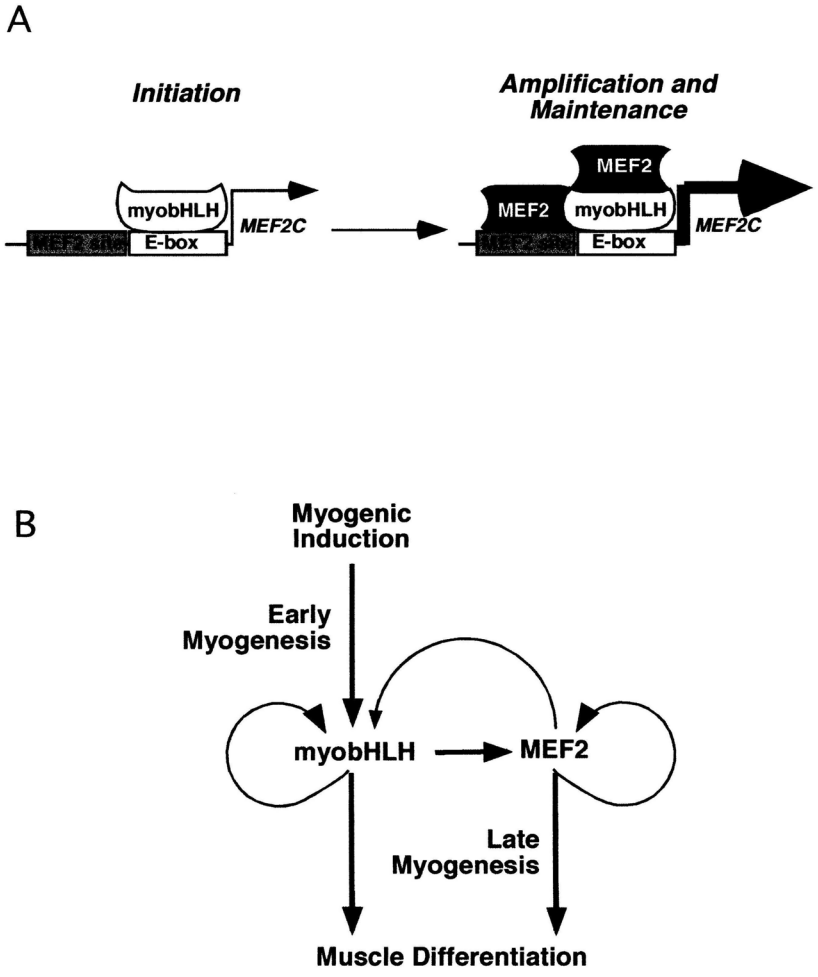

Figure 35. A model for the regulation of *MEF2C* transcription during skeletal muscle development (A) Regulation of *MEF2C* expression during myogenesis. The skeletal muscle regulatory region of *MEF2C* contains binding sites for myogenic bHLH and MEF2 proteins. The E-box is required for initiation and maintenance of *MEF2C* expression. In contrast, the MEF2 site is required for amplification and maintenance of *MEF2C* expression. (B) Auto- and cross-regulation of myobHLH and MEF2 proteins during early and late myogenesis.

and neurons, in which MEF2 is also expressed at high levels, because myogenic bHLH proteins are exclusively expressed in cells of the skeletal muscle lineage.

CROSS-REGULATION OF *MYOGENIC BHLH* AND *MEF2* GENES

The structure of the *MEF2C* skeletal muscle promoter/enhancer is remarkably similar to that of the *myogenin* and *MRF4* promoters, both of which contain MyoD and MEF2 binding sites (Black et al., 1995; Cheng et al., 1993; Edmondson et al., 1992; Naidu et al., 1995; Yee and Rigby, 1993). Like *MEF2C*, *myogenin* and *MRF4* are up-regulated during myocyte differentiation presumably through direct transactivation by bHLH and MEF2 factors. This type of cross-talk provides a powerful mechanism for amplification of both types of regulatory genes, thereby reinforcing and stabilizing the transcriptional program for myogenesis.

In transfected cells, MyoD and MEF2 can cooperatively activate transcription through a binding site for either factor alone (Molkentin et al., 1995). In contrast, mutation of either the MEF2 or MyoD site in the *MEF2C* promoter severely diminished transcriptional activity *in vivo*. These findings suggest that the levels of expression of the endogenous factors are insufficient to activate transcription solely through protein-protein interactions and without binding sites for both factors. This further demonstrates the existent cross-regulation between the myogenic bHLH transcription factors and the MEF2 proteins in skeletal muscle development. A model to account for the cross-regulation of myogenic bHLH and *MEF2* proteins during early and late myogenesis is shown in Fig. 35B.

The *Drosophila MEF2* gene, *D-mef2*, is also expressed at high levels in differentiated skeletal muscle fibers (Bour et al., 1995; Lilly et al., 1995). Previously, we and others showed that transcription of *D-mef2* is controlled by an array of independent enhancers that are activated in specific subsets of myocytes at distinct developmental stages (Cripps et al., 1999; Nguyen and Xu, 1998). Recently, we identified an enhancer that directs *D-mef2* transcription specifically in differentiated myofibers of the *Drosophila* embryo (R. Cripps and E. Olson unpublished). This enhancer contains both a high-affinity MEF2 binding site that is essential for transcriptional activity and an E-box that is necessary for full activity.

Thus, the type of positive auto- and cross-regulatory loops identified in the present study seems likely to reflect an evolutionarily conserved mechanism for the control of *MEF2* gene expression in the skeletal muscle lineage.

MODULAR REGULATION OF *MEF2C* TRANSCRIPTION

A common theme that has emerged through the analysis of muscle gene transcription is the modularity of *cis*-regulatory elements, in which multiple independent regulatory regions are required to generate the complete spatiotemporal expression pattern of a gene throughout development (Firulli and Olson, 1997). Our results demonstrate the existence of two independent regulatory regions that direct *MEF2C* transcription in discrete, but overlapping, sets of embryonic skeletal muscle cells.

A distal upstream region (-1058/-507) activates transcription specifically in cells from the dorsomedial myotome and ventrolateral dermomyotome, which give rise to extreme epaxial, as well as hypaxial and limb muscles, respectively. To our knowledge, these two populations of muscle cells have not been previously recognized as being molecularly distinct. Thus, this *MEF2C* transgene has revealed unique compartments of the myogenic lineage. The specific transcription factors that activate this distal regulatory region remain to be identified.

The proximal regulatory region of *MEF2C* acts independently of this distal enhancer and is sufficient to direct expression of *lacZ* in a pattern that appears to fully recapitulate the expression pattern of the endogenous *MEF2C* gene during pre- and post-natal skeletal muscle development. While our studies do not allow us to conclude that these two regions are solely responsible for *MEF2C* transcription in the skeletal muscle lineage, we have found no other skeletal muscle regulatory elements within 24 kb of genomic DNA analyzed.

In addition to its expression in the skeletal muscle lineage, *MEF2C* is expressed in the developing heart, in specific sets of neurons in the brain, and in the spleen (Edmondson et al., 1994; Martin et al., 1993). Unlike many other muscle-specific enhancers, the *MEF2C* enhancer described here is absolutely specific for the skeletal muscle lineage with no expression in the heart or other organs, suggesting the presence of completely separate

regulatory mechanisms for expression in tissues other than skeletal muscle. We have not yet identified the regulatory elements that control *MEF2C* transcription in the latter cell types. However, our studies suggest that cardiac and neural transcripts for *MEF2C* contain unique 5' exons that likely reflect alternate promoters in these tissues. The mechanisms that regulate *MEF2* expression in these cell types and whether MEF2 positively auto-regulates its expression in cardiac myocytes and neurons, as in skeletal myocytes, is currently under investigation.

SUMMARY

The purpose of these studies has been to further investigate the roles of myogenin bHLH and MEF2 transcription in the development of vertebrate skeletal muscle. Through the current studies, previously undefined roles for the myogenic bHLH transcription factors, MyoD and MRF4, were determined. Prior to these studies, roles had been defined for Myf5, MyoD in the commitment of precursor cells to the myogenic lineage and for myogenin in the differentiation of those committed cells. No such specific role had been assigned to MRF4. We demonstrate here that MyoD and MRF4 share redundant roles in the differentiation of committed myoblasts. Further, MyoD and MRF4 perform these functions independently of myogenin. We also describe here previously unidentified roles for myogenin, MRF4 and MyoD in the maintenance of *Myf5* expression during late myogenesis.

The discovery that a single myogenic bHLH transcription factor, MyoD, could function during both commitment and differentiation led us to investigate whether a single myogenic bHLH factor, Myf5, could direct the entire myogenic program in the absence of the three other factors. We confirmed previous work that demonstrated that Myf5 is able to cause the commitment of precursor cells. We also found that Myf5 is able to initiate the differentiation of skeletal muscle, which was not known previously. However, in this *myogenin/MyoD/MRF4* triple mutant Myf5 was not able to carry the differentiation program to completion, providing evidence that the induction of differentiation can be separated from the fulfillment of the differentiation program, and further that it is the total level of myogenic bHLH transcription factors that dictate the degree of differentiation *in vivo*.

As for some of the myogenic bHLH transcription factors prior to this work, specific roles for MEF2 proteins had not been identified, although they have been shown to be important in skeletal muscle development by enhancing the myogenic activity of the myogenic bHLH proteins. We therefore decided to search for the regulatory elements responsible for the expression of *MEF2C* in skeletal muscle, with the purpose of more closely examining the place occupied by MEF2C in the genetic hierarchy of the skeletal muscle development pathway. Our studies have resulted in the identification and characterization of a regulatory region immediately 5' to the skeletal muscle-specific transcriptional start site of *MEF2C*. This region is able to direct the expression of a *lacZ* reporter gene in a pattern consistent with the endogenous expression of *MEF2C* throughout development and into adulthood. We show that this region contains both enhancer activity and promoter activity. Prior to this work, no *cis*-regulatory elements that control the transcription of vertebrate *MEF2* genes had been identified. We also show that myogenic bHLH transcription factors are required for the induction and amplification of *MEF2C* gene expression, while MEF2 proteins are necessary for the amplification and maintenance of expression. This is the first time that myogenic bHLH factors have been shown to be responsible for directly activating MEF2 gene transcription in any organism.

FUTURE DIRECTIONS

The completion of this work will allow for a more detailed study of the myogenic bHLH factors during skeletal muscle development. For example, we can now more precisely investigate the functional redundancy of the myogenic bHLH transcription factors, by using the *MEF2C* skeletal muscle regulatory region to specifically direct the expression of any of the myogenic bHLH factors in the muscle lineage in any myogenic bHLH mutant background. The current studies will also aid in the interpretation of such data, as we now know more specifically when and where the myogenic bHLH proteins function. Similarly any proteins of interest, or mutants thereof, can be expressed in cells of the myogenic lineage throughout development. The *MEF2C* control region is already being successfully used to

drive *Cre* expression throughout skeletal muscle development (M. Czubryt and E. Olson, unpublished).

The identification and characterization of the *MEF2C* regulatory region will also allow us to better understand the role of MEF2 proteins in skeletal muscle, as well as other lineages. *MEF2C* knock-out mice die at approximately mid-gestation due to a lethal cardiac phenotype, which precludes the analysis of the function of MEF2C in skeletal muscle (Lin et al., 1997). Somatic deletion of the *MEF2C* skeletal muscle regulatory region, however, should allow for the generation of a skeletal muscle-specific *MEF2C* loss-of-function mutant. Further characterization of the genomic regions 5' to the identified of brain and heart-specific 5'-untranslated exons, is now also possible. Finally, the characterization of the genomic structure of mouse *MEF2C* gives us a model with which the genomic structure and regulation of the other MEF2 family members can be characterized.

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VITAE

Melissa Renee Valdez was born in the large Texas town of San Antonio on December 1, 1971. The daughter of Sonia Vite Valdez and Reynaldo Garza Valdez, Jr., Melissa was (and is) well loved and much encouraged in all her endeavors, both academic and personal. She attended Camelot Elementary School in San Antonio, where she was made Captain of Patrols, despite her tendency to talk a little too much, a characteristic she maintains to this day. In the fall of 1983, Melissa entered Ed White Middle School, San Antonio, Texas, where she promptly changed her name to Renee, and refused to answer to her first name. It has been said that she did this to draw attention to herself during roll call on her first day of classes. Two days before Halloween of that same year, Renee was presented with the most significant gift in her life to date - a brother, Reynaldo Alexander Valdez III. Eleven years and eleven months apart in age, these siblings are still inseparable. Renee next attended Theodore Roosevelt High School, San Antonio, Texas, where she was president of the Marching Band, a National Merit Scholar and graduated *summa cum laude* in May of 1990. That fall she matriculated at Rice University in Houston, Texas. After entering as an Engineering Major, Renee was awarded the degree of Bachelor of Arts in Biochemistry in May of 1994. Having an immense appreciation for research and the Biosciences, Renee was torn between entering Graduate School or Medical School. She solved her dilemma by joining the Medical Scientist Training Program at the University of Texas Health Science Center at Dallas in the fall of 1994. Her first two years in the program were spent in medical school, after which she joined the laboratory of Eric N. Olson, Ph.D. in the summer of 1996. Renee was married to David Lane Scott of Sugar Land, Texas, on March 24, 2001. All signs point to a long and blissful marriage for these two. At the end of her fifth and final year of graduate studies, Renee successfully defended her doctoral thesis on June 25, 2001. On July 1 of the same year she will begin the last two years of her medical school training.

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