The Role of the MyoD Family Genes during Mouse Development

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Abstract

Myogenesis is studied as an example of vertebrate cell type determination and differentiation mainly due to the cloning and characterization of genes, both regulators and downstream structural genes, specifically expressed in this lineage. The studies presented in this thesis describe the regulation and function of the MyoD family of myogenic regulatory factors (MRFs) in the developing mouse embryo.

There are four known MRFs (MyoD, Myf-5, myogenin, MRF4/herculin/myf6) in vertebrates; all are exclusively expressed in skeletal muscle and their progenitors, but each with a unique and dynamic pattern. The individual function of one of these, MRF4, was tested by gene disruption via homologous recombination. MRF4 is required for proper muscle formation in a specific domain of the axial lineage during embryogenesis. Later in development, the muscle phenotype is rescued apparently by cellular compensation, suggesting partial redundancy between MRF members. However, an unexpected rib pattern formation defect was observed that caused the death of MRF4 null mice at birth. An inductive signal from muscle precursors to rib progenitors is postulated to be the cause for this malformation.

A differentiated cell is usually considered to be a terminal phenotype. However, the MRFs, when force expressed, have the unique capacity to transform various differentiated cells into a myogenic phenotype. Such a switch in phenotype seems to occur during normal perinatal development of esophagus muscle, as these cells transdifferentiate from a functional smooth type to skeletal muscle by sequentially expressing the MRFs, and then skeletal muscle-specific structural genes. This is one of the few examples of transdifferentiation that occurs during normal development of vertebrates.

The potent capacity of the MRFs to convert cells to a myogenic phenotype requires tight regulation of MRF expression as well as modulation of their function. Transgenic

mice containing certain regulatory sequences from the Myf-5 locus, the first MRF to be expressed in all muscle lineages studied, drives the expression of a marker gene specifically in the early head but not trunk muscle precursors. This implies distinct regulatory pathways of initiating muscle determination in the two lineages. Furthermore, the head lineage is unique since Myf-5 is expressed at least three days before any of the other MRFs or muscle-specific differentiation genes are detectable, and suggests that Myf-5 function is under negative control.

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Chapter 1

Introduction

The Role of the MyoD Family Regulatory Factors in Vertebrate Myogenesis

How a single cell, the fertilized egg, gives rise to the diverse mature cell types in multicellular organisms is a fundamental question in developmental biology. It is generally accepted that multipotential progenitor cells undergo a series of stepwise changes in cellular phenotype, ultimately leading to a specific differentiated cell type. These changes correspond to the changing repertoire of genes expressed at each step of development, and insight into the molecular pathways that control these developmental progressions has recently emerged. For example, in the skeletal muscle lineage, the cloning and characterization of the MyoD family of myogenic regulatory factors (MRFs) have been crucial in the study of myogenic cell fate determination and differentiation (Weintraub, 1993). The MRFs (MyoD, myogenin, Myf-5, MRF4/herculin/myf6) belong to the basic-helix-loop-helix (B-HLH) class of transcription factors, and have been thoroughly studied in cell culture, where they exhibit the extraordinary ability to convert naive fibroblasts and other permissive cell types into a myogenic phenotype. An underlying goal of my thesis research has been to instead focus on understanding the regulation and function of the MRFs in the context of the intact developing embryo. These and other studies in the animal now highlight three important concepts: first, it has become clear that each of the four MRFs have substantially different roles during myogenesis. Second, within specific groups of muscle cell precursors, the expression and regulation of -- and the requirement for -- individual MRFs seem to be surprisingly heterogeneous. Third, MRFs also seem to have a non-autonomous function in inducing proper rib development. With this new understanding of the MRFs in hand, I will compare mammalian skeletal myogenesis with other regulators and developmental systems that involve cell type specification.

MRFs and Myogenesis:

The study of myogenesis in cell culture has given an impression of relative simplicity for the process by which the MRFs, as potent transcription factors, orchestrate myogenic differentiation. However, myogenesis in vivo occurs within the context of

mesodermal specification and patterning, and involves a hierarchy of cell-cell interactions. These facts, together with the multilineage origin of skeletal muscle in vertebrates, make the complex process of myogenesis difficult to mimic in cell culture experiments. Thus, the need to investigate the MRFs in vivo is critical. For the MRFs to play a direct role in the myogenic determination and/or differentiation process in vivo, it would first be expected that these regulators be expressed in skeletal muscle and their precursors before overt differentiation of muscle. Second, the MRFs must be necessary for myogenesis to occur. Indeed, the MRFs are specifically expressed in the skeletal muscle lineage at all timepoints, and each MRF has a unique and dynamic expression pattern within the developing animal (Buckingham, 1992; Smith *et al.*, 1994). Germline gene disruption experiments in the mouse are now providing further evidence that individual MRFs play a crucial but distinct role in myogenic determination and differentiation.

Origin of muscle in vertebrates:

Before characterizing the molecular pathways of myogenesis, it is necessary to have an understanding of the origin of skeletal muscles in vertebrates. While cardiac and smooth muscle arise from lateral mesoderm, skeletal muscle is mostly derived from dorsal paraxial mesoderm (Wachtler and Christ, 1992). In the trunk and tail regions, the paraxial mesoderm is first segmented into somitic blocks in a rostrocaudal progression on both sides of the neural tube. Under the influence of signals from epidermis, neural tube and notochord, these somites subdivide into dermomyotome (muscle and skin precursors) and sclerotome (cartilage and bone precursors) and subsequently the dermomyotome further segregates into myotome and dermatome. The myotome, like other parts of the somite, is a transient embryonic structure. The skeletal myocytes that differentiate there are few in number relative to the adult musculature, and their specific fate is unknown; however, they are thought to contribute to parts of muscles of the back (Christ and Ordahl, 1995). Lateral somitic cells contain at least one separate lineage of muscle precursors with respect to morphological criteria and molecular markers. These cells migrate laterally and ventrally to

form the muscles of the limbs and the body wall (Christ and Ordahl, 1995). Muscles of the head are derived from a third independent lineage which arise from prechordal plate and anterior (non-segmented) paraxial mesoderm. A majority of these muscle precursors migrate ventrally to visceral arches before they reach their final location and differentiate (Noden, 1991; Couly *et al.*, 1992).

MRFs are expressed in a distinct and dynamic pattern in vivo:

The MRFs are expressed in all three lineages described, and each MRF has a unique spatiotemporal expression pattern. The best studied lineage is that of the myotome. In the mouse myotome, Myf-5 is the first muscle-specific gene to be expressed at embryonic day 8 (E8, 4 somites stage), and its expression precedes overt differentiation of myocytes. RT-PCR studies have also shown low-level expression of Myf-5 prior to somite formation, in the presumptive segmental plate (Kopan *et al.*, 1994). Myogenin expression quickly follows that of Myf-5 at E8.5. MRF4, which is highly expressed in adult musculature, is expressed transiently in myotomes between E9 and E11.5. MyoD expression appears last in myotomes starting at E10.5. More recent studies with MRF antibodies have shown a surprising subdivision of the myotome with respect to MRF expression, so that each MRF is expressed in distinct spatial domains of the myotome; for example, Myf-5 and MyoD are present in separate regions of the myotome, with Myf-5 being expressed dorsomedially, while MyoD expression appears in more ventrolateral myotomal cells (Smith *et al.*, 1994). Hence, although MyoD is the last to be expressed in myotomes, it is turned on in cells that have probably not expressed any other MRFs, making MyoD the first MRF to be expressed in a subpopulation of myotomes.

MRF expression in limb bud and visceral arch expression differ from that of myotomes by at least two different ways. First, MRF4 is not expressed in these lineages during embryonic development, and only appear at fetal stages (E16.5), during the second wave of MRF4 expression; second, the other MRFs are not expressed in the muscle precursors prior to their completion of ventral migration, and are therefore temporally

delayed with respect to MRF expression and differentiation of myotomes. One interesting explanation for the lack of early MRF expression in these lineages is perhaps to protect these cells from prematurely entering the differentiation pathway while they migrate long distances in the embryo. Myotomal cells do not migrate substantially, and differentiate early during somitogenesis.

Role of MRFs in myogenesis: determination vs. differentiation

Despite their complex and distinct expression patterns in the developing embryo, all four MRFs have similar potential to recruit cells to a myogenic phenotype in culture. This apparently simple observation is somewhat complicated because the MRFs have the capacity to auto- and cross-regulate one another; this has made it difficult to discern the individual roles of the MRFs in cell culture experiments (Weintraub *et al.*, 1991). This issue has been partly clarified by recent gene disruption experiments. Although animals lacking either Myf-5 or MyoD have mild muscle phenotypes at birth (Braun *et al.*, 1992; Rudnicki *et al.*, 1992), mice lacking both Myf-5 and MyoD do not contain any skeletal muscle at birth, and lack cell mass where skeletal muscle would normally be present (Rudnicki *et al.*, 1993). This suggests that Myf-5 and MyoD are partially redundant for muscle formation, but at least one of them is required for myogenic determination. Further proof for the role of Myf-5 in muscle determination is coming from ongoing unpublished work in Buckingham lab, where Myf-5 gene was replaced with the bacterial *lacZ* gene. Mice homozygous for this transplantation allele show expression of *lacZ* in non-muscle cells such as dermis and cartilage cells. The simplest interpretation is that, in the absence of Myf-5 function, cells normally determined to become muscle cells are recruited to other cell fates. It is interesting that these cells, initially cued to turn on Myf-5, continue to reflect that myogenic 'assignment' by continuously expressing *lacZ* even after their recruitment to new cell fates.

A different phenotype is observed in mice lacking a functional myogenin gene. These animals form proper muscle precursors in number and morphology, however, there

is a gross lack of differentiation and fiber formation in vivo, which suggests a more 'downstream' (compared to Myf-5 and MyoD) and a direct role of myogenin in carrying out the differentiation process (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993; Venuti *et al.*, 1995).

Since the MRFs have a very similar activity in ectopic expression studies, it is not yet obvious if the differences in MRF knockout phenotypes are due to subtle differences in the function of the particular proteins, or that the differences are simply dictated by the regulatory sequences that control their pattern of expression. Homologous recombination experiments where one of the MRFs is replaced with another will differentiate between these possibilities.

The role of MRF4 appears to be more complicated, and initial analysis show that it might have a dual role during development: an embryonic and a postnatal function in myogenesis. During somitogenesis, as I will discuss below in more detail, MRF4 is necessary for the formation of a subset of myotomal precursors (Patapoutian *et al.*, 1995a); MRF4 seems to have an important function in postnatal muscle development as well, judged by the upregulation of myogenin in adult muscle (Zhang *et al.*, 1995). More detailed analysis of these null mice, together with combination double knockout experiments with other MRFs will clarify the importance of MRFs in determination and differentiation of muscle (Braun and Arnold, 1995). The summary of MRF knockouts is presented in Table I.

Different MRFs are required for distinct myotomal domains:

Detailed studies of the MRF knockouts are reinforcing the idea of cellular heterogeneity within the myotome. Based on the MRF protein expression pattern described above (Smith *et al.*, 1994), as well as the analysis of Myf-5 (Braun *et al.*, 1994) and MRF4 (Patapoutian *et al.*, 1995a) null mice during embryogenesis, we proposed the existence of three distinct myogenic regulatory programs in the somite (My1-My3), which correlate temporally and spatially with three waves of cellular recruitment to the expanding myotome

(primary, secondary, tertiary myotomes). This is illustrated in Fig. 1 and discussed in more detail in the second chapter of my thesis. Each myotomal subdomain expresses a distinct set of the MRFs, and its formation is dependent on those regulators. Myf-5 null mice show a lack of the My1 and My2 programs, and this deficit is rescued later during embryogenesis by MyoD expressing My3 program (Braun *et al.*, 1992; Braun *et al.*, 1994). A similar situation seems to occur in MRF4 null mice: The second of these programs (My2) was severely affected in the $MRF4^{bh1/bh1}$ mice judged by the lack of the secondary myotome at the cellular level, but this deficit was later rescued by the third program. These data suggest that the formation of the primary wave of myogenesis is dependent on Myf-5; the secondary wave, on Myf-5 as well as MRF4; while the third wave, on MyoD. Myogenin, although expressed very early during myotomal formation, seems to play only a later role in differentiation during fetal stages (Venuti *et al.*, 1995). The idea of a compartmentalized myotome raises an important point on the mechanism of compensation observed in the muscle deficits in MRF null mice. There are two possible mechanisms of compensation: 1) molecular compensation, where compensation occurs within a single cell, as one MRF can perform the same function as another; 2) cellular compensation, where a separate lineage of cells expand and repopulate the cellular deficit caused by the lack of a single MRF. Recent evidence from MRF knockout mice all point toward cellular compensation as the mechanism involved in myotomal compensation. However, from ectopic expression experiments both in vivo and in vitro, it is obvious that MRFs are capable of crossregulation within a cell. This leaves open the possibility of molecular compensation in vivo in other settings (Weintraub *et al.*, 1991; Miner *et al.*, 1992). For example, upregulation of Myf-5 in MyoD null adult mice (Rudnicki *et al.*, 1992), and upregulation of myogenin in MRF4 null mice (Zhang *et al.*, 1995) can be explained by either compensatory mechanism. However, cellular compensation seems increasingly attractive for these cases as well, since if compensation was at a molecular level, it would be necessary to postulate that MyoD normally represses expression of Myf-

5, and MRF4 normally represses the expression of myogenin. Although possible, there is presently no evidence to date for such negative regulatory loops within the MRF circuitry.

Non-autonomous function of the MRFs:

One of the most interesting and unexpected results from the MRF knockout studies are the observed rib morphogenetic phenotypes observed in these mice. This was surprising since MRFs are not expressed in ribs or their precursors, and as transcription factors, are thought to act in a cell autonomous fashion. All the MRF null homozygotes, except MyoD, show some rib abnormalities. Myf-5 null mice show very severe truncation of ribs, while myogenin and MRF4 show more of a rib patterning defect (Braun *et al.*, 1992; Hasty *et al.*, 1993; Nabeshima *et al.*, 1993; Braun and Arnold, 1995; Zhang *et al.*, 1995; Patapoutian *et al.*, 1995a). The most straightforward interpretation of the rib phenotypes is to postulate an interaction between muscle and rib precursors during early somitogenesis. Indeed, the myotomal cells are in close proximity to sclerotomal cells (rib precursors) during this time in development, and suggests that the basis for the rib anomalies in the MRF null animals are mainly due to signals or scaffolding produced by the myotome. One such possible signal could be FGF-6 which is expressed specifically in skeletal muscle during embryogenesis and belongs to the family of fibroblast growth factors (Han and Martin, 1993; deLapeyriere *et al.*, 1993). I found that its levels are strongly downregulated in myotomes of MRF4 null mice and is described in chapter 3 of my thesis. It is relevant that FGF family receptors such as FGFR-1 and FGFR-2 are expressed in sclerotomal cells, but no known candidate ligands have been identified within the sclerotome itself (Peters *et al.*, 1992; Yamaguchi *et al.*, 1992). Recent unpublished studies in Arnold lab have also shown that, FGF-6 and TGF-b together, can induce cartilage formation in vitro.

Regulation of MRF Expression and somitic patterning:

Given the varied and dynamic expression patterns of the MRFs, and their developmental capabilities discussed above, a central problem is to understand what

regulates their expression. Isolation and characterization of regulatory sequences of MRFs in transgenic mice have proven useful, and also present a method by which events in myogenic determination temporally preceding expression of the MRFs can be studied. Myogenin regulation seems to be the most straightforward (Cheng *et al.*, 1993; Yee and Rigby, 1993). Approximately 150 bases of 5' flank DNA is sufficient for correct recapitulation of myogenin expression, and two essential motifs in these sequences are consensus binding sites for b-HLH family members and RSRF (related to serum response factor) proteins. This reinforces cell culture experiments that the MRFs are under strong cross-regulatory loops. MyoD, Myf-5, and MRF4 regulation is more complicated. An enhancer for MyoD transcription is found in a region of 258bp localized about 20 kb 5' flank of the site of transcriptional initiation; these sequences contain b-HLH consensus binding sites, but activation of transcription is not dependent on these sites (Goldhamer *et al.*, 1992; Goldhamer *et al.*, 1995), reinforcing the idea that MyoD can be activated independent of previous MRF expression as seen in Myf-5 null mice (Braun *et al.*, 1994). Factors that activate MyoD transcription are not known as of yet.

For Myf-5, regulatory elements that appropriately initiate activation in head but not trunk muscle precursors have been identified (Patapoutian *et al.*, 1993), and this is the focus of the fourth chapter of my thesis. Thus, Myf-5 expression rely on different regulatory regions of this gene in different muscle lineages. This argues that muscle determination might be under separate molecular pathways in different lineages, a view that is not surprising given the different cellular environments in which visceral arch and myotomal cells are induced to express Myf-5 and initiate myogenesis. Regulatory sequences that control the late, but not early, expression of MRF4 has also been isolated (Patapoutian *et al.*, 1993).

MRF4 and Myf-5 are linked in mammalian genome; in the mouse, the two transcript start sites are 7kb apart. The immediate upstream sequences of the two genes' are not sufficient for complete recapitulation of the endogenous expression patterns, and

suggests that this locus might be under complex regulation, where the linked state of Myf-5 and MRF4 is perhaps essential for correct expression. Indeed, unpublished results from Rigby lab have shown that some Myf-5 regulatory sequences reside within the MRF4 gene. This complex regulatory locus provides a possible explanation for the diverse phenotypes achieved by knocking out different regions of the MRF4 gene (Braun and Arnold, 1995; Zhang *et al.*, 1995; Patapoutian *et al.*, 1995a). Three different groups have generated targeted disruption alleles of MRF4, and the phenotypes vary considerably (Fig. 2). The Arnold construct unintentionally created a null allele of Myf-5 gene as well; it acts as a double mutant and the dominant phenotype is a Myf-5 phenocopy. The other two alleles are more comparable, and it is not obvious as of yet if the differences are due to partial cis effects on Myf-5 expression, or perhaps genetic background variations (refer to table I, Fig 2 and chapter 2).

The complicated crossregulatory network between the MRFs, as well as the difficulty of narrowing down regulatory elements in transgenic experiments make it difficult to use the regulatory sequences of MRFs to identify the signaling events that lead to MRF expression. One alternative way of asking this question is by in vivo transplantation or in vitro culture of tissue explant techniques, where undifferentiated and unpatterned somites are put in proximity to potential inducing tissues of myogenesis, and MRF expression. Although such studies are at an early stage at this time and several studies report apparent contradictions, several consensus conclusions appear to be emerging. The notochord and the floor plate, perhaps through the hedgehog protein, specify a sclerotomal (ventral) somitic fate, while signals from the surface ectoderm and neural tube specify a dermomyotomal (dorsal) phenotype (Fan and Tessier-Lavigne, 1994). When tested specifically for myogenic differentiation or MRF expression, the neural tube is the strongest candidate inducer. The role of the notochord in myogenesis is controversial, and results claiming both induction and repression have been reported (Stern and Hauschka, 1995; Munsterberg and Lassar, 1995; Buffinger and Stockdale, 1995).

Although no molecules are yet identified that could induce myogenesis in such a system, it has been recently shown that a diffusible factor from the neural tube is capable of inducing myogenic activity in the somites (Buffinger and Stockdale, 1995).

Negative regulation of MRF activity, and myogenic expansion:

Transcriptional activation of the MRFs is only the first level of regulation. In cell culture, the MRFs are expressed in dividing myoblasts, but their activity is negatively regulated by various mechanisms, until the time of differentiation (Weintraub *et al.*, 1991). One set of negative regulator of the MRFs are the Id family members, which are HLH proteins lacking a functional DNA-binding basic region. Id-1 is present in myoblasts in culture and binds directly to the MRFs or their partners and repress their DNA-binding ability (Benezra *et al.*, 1990; Neuhold and Wold, 1993). Downregulation of Id-1 is observed under differentiation conditions, and this is thought to unleash the MRFs to carry out the myogenic differentiation process. However, the in vivo domain of somitic expression of Id-1 is largely exclusive from and precedes any MRF expression (Wang *et al.*, 1992). This raises multiple questions. First, the technique used (radioactive in-situ hybridization) might not be sensitive enough to pick up a small number of coexpressing cells. Second, only myotomal expression has been examined carefully, where differentiation genes are expressed almost immediately after Myf-5 is first detected, and where there might not be a need for an global negative regulation of MRF expressing cells. Other muscle lineages, such as those of the head precursor visceral arches, express Myf-5 three days before any muscle-specific differentiation gene is expressed, and might be coexpressing positive and negative HLH regulators, and this issue is discussed in more detail in chapter five of my thesis (Patapoutian *et al.*, 1995b). Finally, there are at least three other Id molecules, and hence other closely related negative regulators might be expressed in some MRF-expressing cells. In fact, while no detailed expression studies for the other Id molecules are yet available, comparison of the expression of Myf-5 and m-twist, another b-HLH protein that can repress the action of the MRFs, showed that these

RNAs appear to be expressed in a critical domain at the dorsal lip region of the myotome where new MRF expressing cells are thought to originate (Yun *et al.*, 1995). Alternative mechanisms of MRF activity regulation such as phosphorylation of the MRFs have also been suggested (Li *et al.*, 1992) and are discussed in detail by Lassar *et al.* (1994), where posttranslational modifications of the MRFs could render them inactive until the appropriate time of cell cycle withdrawal and differentiation.

The negative regulation of MRFs is a critical issue, because it provides a plausible mechanism for the muscle expansion that occurs throughout embryogenesis. A model in the field postulates that some of the cells expressing MRF differentiate immediately, while others are kept under negative regulation, continue to divide, and serve as the myoblasts that will populate the next myogenic expansion and differentiation. Whether dividing myoblasts that express MRFs are present within the myotome is not yet known. Another possibility that has been largely ignored is that MRF expressing cells do not usually persist under negative control for an extended period of time in vivo, and most often they differentiate soon after initiation of MRF expression. In this scenario, expansion of myogenic cell mass could be achieved if already differentiated muscle cells or other proximal tissues such as the neural tube can continuously induce MRF expression in their neighboring non-myogenic cells. At least two separate inductive events are already postulated for myotomal formation, one signal to activate My1; another, for My3. The expression of negative regulators of MRFs (e.g.: Id-1, Hox-7.1, m-twist) in neighboring sclerotomal and dermotomal cells might serve to ensure that the myogenic induction does not over-extend and take over the rib and dermis precursors. It is important to note that the two models are not mutually exclusive, and during embryogenesis both methods are probably used, according to the lineage and cell environment.

The MRFs and switch in differentiated phenotype:

The in vivo role of MRFs is clear with respect to recruiting naive, uncommitted cells to a myogenic phenotype. However, when ectopically expressed, MRFs are also

capable of converting already differentiated cells into a myogenic fate (Weintraub *et al.*, 1989; Choi *et al.*, 1990; Miner *et al.*, 1992; Blau, 1992). This "jump" in differentiation pathways is surprising, since a differentiated cell is usually considered to be a terminal phenotype. Interestingly, there is evidence for such a switch in phenotype during normal perinatal development of esophagus muscle, as these cells transdifferentiate from a functional smooth type to skeletal muscle by sequentially expressing first the MRFs, and then skeletal muscle-specific structural genes (Patapoutian *et al.*, 1995c). This is one the few examples of transdifferentiation that occurs during normal development of vertebrates, and reinforces the idea that MRFs are powerful regulators, and that evolution has used them to their potential. The details, and the significance, of transdifferentiation is further discussed in the third chapter of my thesis.

The Fly Eye and Vertebrate Muscle Development:

The remarkable similarities between the molecular mechanisms underlying fly neurogenesis and vertebrate myogenesis have been discussed previously (Jan and Jan, 1993). Both systems rely heavily on a family of positive and negative acting HLH proteins to specify the individual cell fates. In fly neurogenesis, b-HLH family proneural genes [*achaete* (*ac*), *scute* (*sc*), *atonal* (*ato*), etc.] specify neural precursor cells that is similar to the role of Myf-5 and MyoD in skeletal muscle determination. Based on more recent data from both systems, the parallels between them can now be extended. Similar to the subdomains of the myotome that express, and are dependent on, different MRFs; *ac* and *sc* are initially expressed in, and are required for, different proneural clusters of the wing disc. Also similar to the MRFs, while loss-of-function phenotypes of the *achaete-scute* (*ACS*) complex give rise to distinct phenotypes, ectopic expression of any of the four *ACS* complex genes can induce the formation of external sense organ formation, as they are able to substitute for one another.

More recent studies in the compound eye of Drosophila have confirmed and extended the parallels between fly neurogenesis and vertebrate myogenesis. The fly eye is composed of 800 ommatidia. The photoreceptors within the ommatidia differentiate as the morphogenetic furrow (MF) transverses the eye disc from posterior to anterior during the third larval instar. The first photoreceptor to differentiate within an ommatidium is the R8, which then initiates an inductive cascade for the formation and differentiation of other photoreceptors posterior to the MF. *Atonal* (*ato*), a b-HLH protein, functions as a proneural gene in the developing fly eye and is required for R8 formation (Jarman *et al.*, 1994).

Apart from the molecular similarities, the fly eye is an appropriate developmental system to compare to vertebrate myotomal formation because of some morphological parallels. Both systems develop through a wave of differentiation that is accompanied by changes in cellular morphology that result in repeated structures along the rostrocaudal axis (somites and ommatidia) . While R8 is the first photoreceptor to differentiate at the MF and is dependent on the b-HLH protein, *ato*; the first myotomal cells at the dorsomedial lip of somites differentiate at a time shortly after somites bud from the pre-segmental plate, and are dependent on another b-HLH, Myf-5. Also, while R8 is later required for inductive cues that carry out differentiation of other photoreceptors within the ommatidium, the early Myf-5-expressing cells are essential for the subsequent formation of secondary myotome, as well as rib precursor cells within the somite.

The expression pattern of HLH molecules with possible negative functions also have striking similarities in fly eye neurogenesis and vertebrate myogenesis. In Drosophila, *extramacrochaetae* (*emc*) is an HLH protein without a basic domain, similar to structure and function to Id-1. Another HLH protein, *hairy* (*h*), has a basic domain, binds DNA, but is a negative regulator of proneural genes. This is reminiscent of m-twist, which is also a b-HLH DNA binding protein and can repress the MRFs (Hebrok *et al.*, 1994; Yun *et al.*, 1995). All four negative regulators are expressed in cells immediately adjacent

to those expressing the positive b-HLHs: Id and m-twist are expressed in early non-segmented paraxial mesoderm, and later in somitic sclerotomal and dermotomal cells that neighbor the MRF containing myotomes (Wolf *et al.*, 1991; Wang *et al.*, 1992; Yun *et al.*, 1995), while in the Drosophila eye ommatidia, *emc* and *h* are expressed just anterior to the MF and *ato* expressing cells (Brown *et al.*, 1995). While either *h* or *emc* alone are not required for normal photoreceptor differentiation, $emc^{-}h^{-}$ clones in the eye disc cause ectopic neurogenic differentiation anterior to the furrow (Brown *et al.*, 1995). This suggests that the two negative regulators are partially redundant, and that they jointly repress the expression or activity of *ato*. It would be interesting to see if double mutant phenotype of m-twist and Id-1 would cause ectopic expression of the MRFs in the mouse. According to their expression pattern, and the $emc^{-}h^{-}$ phenotype, the negative regulators in both systems might be playing a crucial role to sharpen the boundaries, as well as the timing, of the decision to differentiate. The need for multiple negative regulatory proteins for the b-HLH driven developmental programs is not clear; however, given their potent capacity to dictate cell fate decisions, it is not surprising that a combinatorial negative regulation of b-HLH expression and activity is used to secure against inappropriate action of these regulators.

Finally, vertebrate neurogenesis might also be regulated by ACS homologues. MASH-1 (murine achaete scute homologue) is required for the differentiation of a subset of neurons (Guillemot *et al.*, 1993). Another vertebrate family member, NeuroD, when ectopically expressed in frog embryos can recruit epidermal cells to a neuronal phenotype (Lee *et al.*, 1995). *Nautilus*, the only MRF homologue found in Drosophila, is expressed in a subset of muscle precursors, but seems to be dispensable for myogenic specification (Michelson *et al.*, 1990).

Mechanisms of Cell Type Specification:

British embryologist Conrad Waddington, writing in 1940, used the image of a ball rolling down a landscape through pathways of branching tracks as a useful tool to describe developmental progression, and named it the "epigenetic landscape." The landscape in this image represents the cellular environment; the ball, as the developing cell; and the tracks, as the differentiation pathways. Cells rarely reverse track to an adjacent one, and with time, they proceed down the landscape and become more and more distant from each other, reflecting different patterns of gene activity. The role of Myf-5 and MyoD appears to be in recruiting multipotential cells to a myogenic phenotype, and the role of myogenin is to drive the execution of differentiation. This fits nicely in this model of progressive specification.

"Master regulatory gene" is a phrase that is often used to describe potent regulators such as the MRFs, and has come to define a gene that when ectopically expressed in a permissive environment can recruit cells to a specific phenotype. Is this a common mechanism in Development where each cell or tissue type has its own master control gene? It would be rather inefficient for evolution to draw on a separate regulatory gene family as lineage-specific differentiation factors for each and individual cell type. It is more beneficial to extract specificity by using combinations of regulators to achieve different phenotypes. Even most so-called master control genes can only carry out their differentiation program under certain conditions, and hence are context dependent. For example, *eyeless* (*ey*), a homologue of the mouse Small eye (Pax-6) gene, in an ectopic expression assay can transform imaginal discs into eye structures and hence is called a master control gene. However, it is obviously not sufficient for eye development, since *ey* is also normally expressed in cells that do not form eyes (Halder *et al.*, 1995; Quiring *et al.*, 1994). The MRFs also may not be entirely restricted to muscle progenitors. In the mouse, Myf-5 is transiently expressed in a few neuronal cells during development; however, it is now known whether Myf-5 protein is ever synthesized, or if these embryonic cells survive to adulthood (Tajbakhsh *et al.*, 1994). Regardless, judged by overall expression pattern,

overexpression capacity, and in vivo requirement, MRFs are necessary and, in most contexts, sufficient for carrying out skeletal myogenesis. However, the myogenic cell fate determination process seems to be an exception rather than the rule, since most decisions of cellular specification are under more complex combinatorial and context-dependent control of regulatory genes.

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Table 1. MRF Knockout Phenotypes

Genotype	Myf5 $^{-/-}$	MyoD$^{-/-}$	Myogenin$^{-/-}$	MRF-4$^{-/-}$			Myf5$^{-/-}$ MyoD$^{-/-}$
				Arnold	Wold	Olson	
Lethality	Death at birth	Viable	Death at birth	Death at birth	Low penetrance survival to adulthood; death at birth	Viable	Death at birth
Skeletal Phenotypes	Rib stubs	Normal	Malformed ribs; bifurcations; fusions; incomplete attachment at sternum	Rib stubs (myf5 phenocopy)	Malformed ribs; bifurcations; fusions; incomplete attachment at sternum	Malformed ribs; bifurcations; fusions; good attachment at sternum	Rib stubs
Muscle Phenotypes							
Somitic before E10	Absence of muscle-specific gene expression	Normal	Normal?	No myocytes No myogenin (myf5 phenocopy)	Reduced myotomal formation	?	Absence of muscle-specific gene expression
Somitic after E10.5	"Recovering" Myotome Normal (MyoD)	?	Normal?	"Recovering" Myotome Normal (MyoD)	"Recovering" Myotome Normal (MyoD)	Normal	same as above
Newborn	Normal	?	Severe defecit in differentiation	Deep back muscle deficiency MHCemb low	Partial deficiency in intercostals; MHCemb low	Grossly normal; MHCemb low	same as above; No muscle; No presump-tive myoblasts
Adult	—	3x increase in Myf5 RNA	—	—	?	5x increase in myogenin	—

Figure 1

Model for Myotomal Domains and MRF Expression

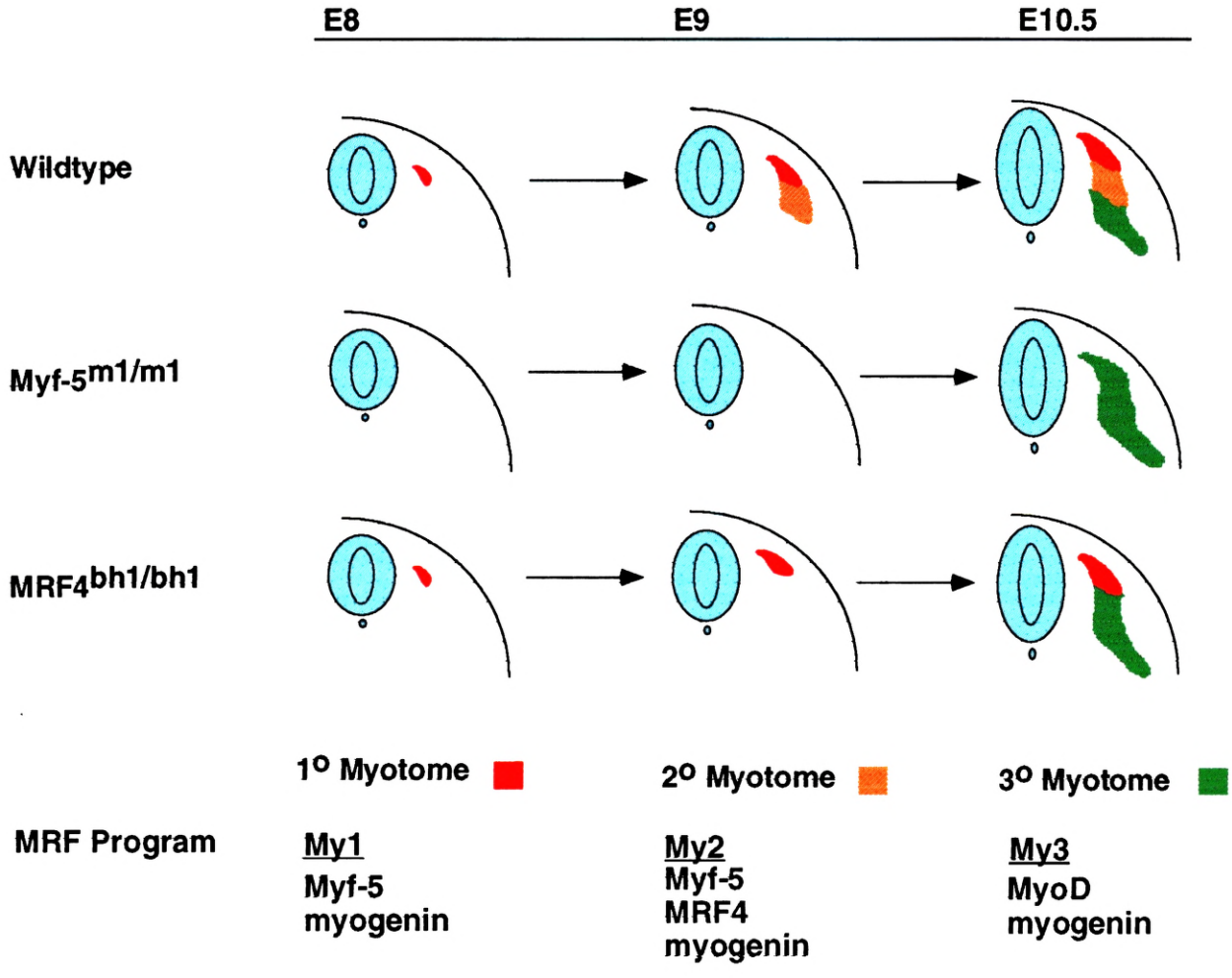
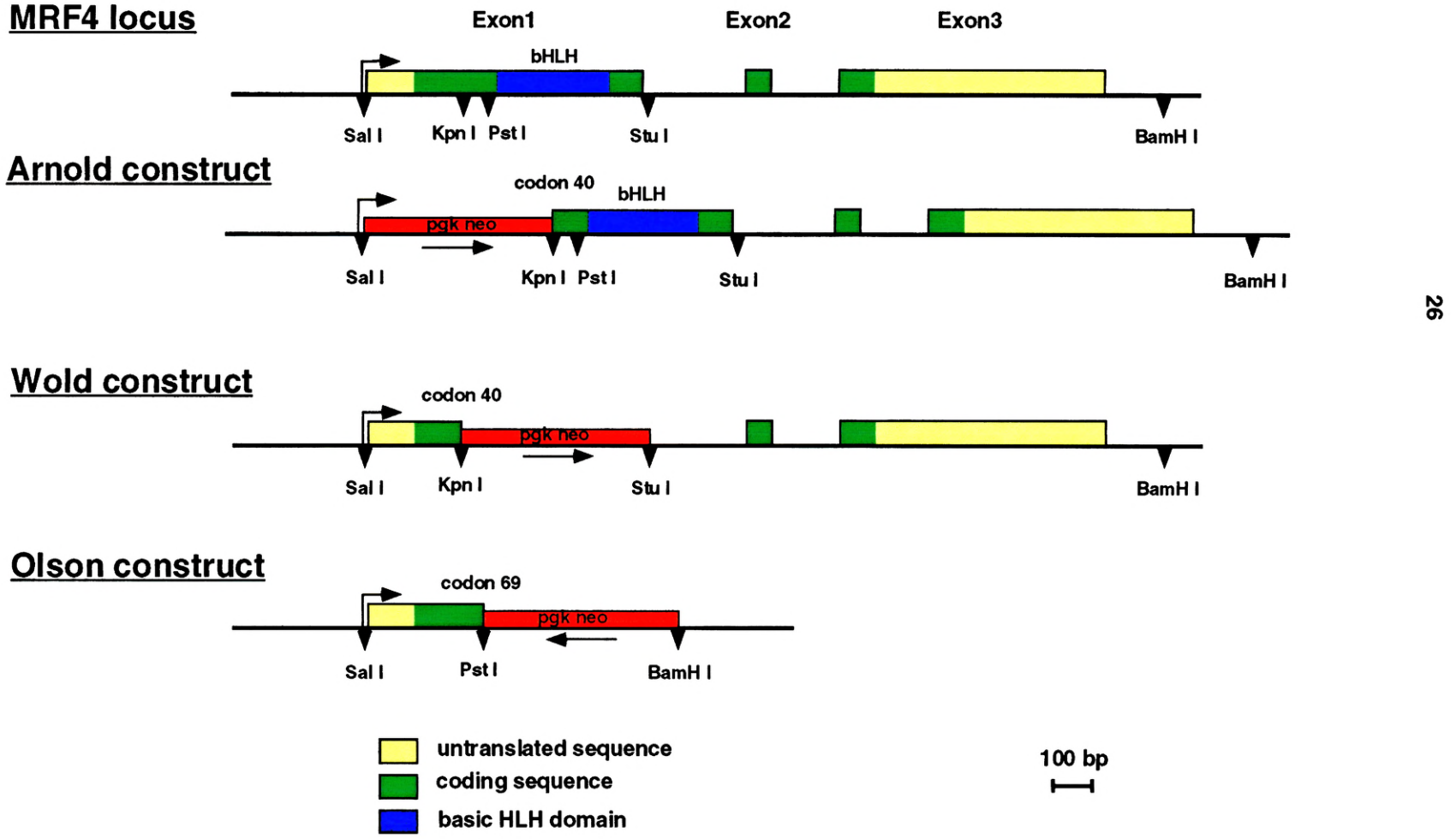


Figure 2

MRF4 Null Mutations: One Gene, Three Phenotypes



Chapter 2

Disruption of the Mouse *MRF4* Gene Identifies Multiple Waves of Myogenesis in the Myotome

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Disruption of the Mouse MRF4 Gene Identifies Multiple Waves of Myogenesis in the Myotome

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Short Title: MRF4 Disruption Causes Myotome and Rib Defects

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Summary

MRF4 (herculin/Myf-6) is one of the four member MyoD family of transcription factors identified by their ability to enforce skeletal muscle differentiation upon a wide variety of nonmuscle cell types. In this study the mouse germline *MRF4* gene was disrupted by targeted recombination. Animals homozygous for the $MRF4^{bh1}$ allele, a deletion of the functionally essential bHLH domain, displayed defective axial myogenesis and rib pattern formation, and they died at birth. Differences in somitogenesis between homozygous $MRF4^{bh1}$ embryos and their wildtype littermates provided evidence for three distinct myogenic regulatory programs (My1-My3) in the somite, which correlate temporally and spatially with three waves of cellular recruitment to the expanding myotome. The first program (My1), marked initially by *Myf-5* expression and followed by *myogenin*, began on schedule in the $MRF4^{bh1/bh1}$ embryos at day 8 post coitum (e8). A second program (My2) was highly deficient in homozygous mutant *MRF4* embryos, and normal expansion of the myotome failed. Moreover, expression of downstream muscle specific genes, including *FGF-6*, which is a candidate regulator of inductive interactions, did not occur normally. The onset of *MyoD* expression around e10.5 in wildtype embryos marks a third myotomal program (My3), the execution of which was somewhat delayed in *MRF4* mutant embryos but ultimately led to extensive myogenesis in the trunk. By e15 it appeared to have largely compensated for the defective My2 program in *MRF4* mutants. Homozygous $MRF4^{bh1}$ animals also showed improper rib pattern formation perhaps due to the absence of signals from cells expressing the My2 program. Finally, a later and relatively mild phenotype was detected in intercostal muscles of newborn animals.

Introduction

In diverse developmental pathways, including skeletal myogenesis in vertebrates, specific sets of basic helix-loop-helix (b-HLH) class transcription factors form regulatory networks important for cell fate specification and/or terminal differentiation (reviewed by Jan and Jan, 1993). In mammals, the MRF (muscle regulatory factor) group includes MyoD, myogenin, Myf-5 and MRF4/herculin/Myf-6, (reviewed by Weintraub, 1993) and together they compose the core of the myogenic bHLH net. Evidence from several lines of investigation have led to the view that these genes are individually and collectively important for muscle determination and differentiation. Both in tissue culture cells (reviewed by Olson, 1990) and transgenic animals (Hopwood and Gurdon, 1990; Miner *et al.*, 1992), dominant gain of function assays have shown that each MRF can activate muscle specific genes and, in permissive cell environments, drive wholesale conversion of the host cells to a myocyte-like phenotype. At the molecular level, MRFs are sequence specific DNA binding proteins that bind to functionally important sites in the enhancers of many muscle specific genes (Murre *et al.*, 1989; Weintraub *et al.*, 1991). In vivo, MRF expression is largely restricted to skeletal muscle precursors and mature myofibers (Buckingham, 1992), consistent with functions specific to myogenesis. Finally, germline gene disruption experiments in the mouse are now providing stringent in vivo tests of inferences from prior expression and molecular studies. So far, these experiments have shown that *MyoD* and *Myf-5* are jointly important for formation and/or survival of muscle precursor populations (Rudnicki *et al.*, 1993), while myogenin is needed for efficient and proper muscle differentiation in vivo (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993). In addition to these myogenic phenotypes, null alleles of *Myf-5* (Braun *et al.*, 1992) and *myogenin* also affected axial skeletogenesis, disrupting to different degrees distal rib formation. Here, we describe disruption of the mouse *MRF4* gene.

All known skeletal muscle in an adult vertebrate originates from cells of the dorsal prechordal and paraxial mesoderm (Wachtler and Christ, 1992). In the trunk and tail regions, this multipotential mesoderm is first segmented into somitic blocks in a rostrocaudal progression on both sides of the neural tube. Under the influence of signals from epidermis, neural tube and notochord, these somites subdivide into dermamyotome (muscle and skin precursors) and sclerotome (cartilage and bone precursors), and subsequently the dermamyotome further segregates into myotome and dermatome. Within the developing dermamyotome and myotome, there is a dynamic pattern of MRF expression such that each MRF is expressed in a distinctive spatiotemporal pattern that generally overlaps with expression of one or more other family members (reviewed, Buckingham, 1992; Smith *et al.*, 1994). The pattern for *MRF4* is different from the others in three major respects. First, its myotomal expression occurs in a discrete wave that begins at about day 9 post coitum (e9) and ends at around e11.5, while the others continue to be expressed significantly until at least e15 when the axial muscle masses derived from the myotome are quite well defined. Second, *MRF4* is not involved in early myogenesis in the head or limb musculature but the other three MRFs are. Finally, *MRF4* expression initiates a second wave at e16 and ultimately comes to dominate quantitatively over the other MRFs in adult muscle, an observation that led to the suggestion that MRF4 may be needed to maintain the differentiated skeletal phenotype (Miner and Wold, 1990).

In this work, disruption of the *MRF4* gene produced both muscle and skeletal phenotypes in the mouse embryo and fetus. Molecular and cytological analysis of homozygous mutant $MRF4^{bh1}$ embryos showed that early and late waves of myogenic commitment and differentiation in the myotome occurred similarly in mutant and wildtype myotomes, but an intermediate myotomal expansion during the period of somitic *MRF4* expression (~ e9-11) failed. These observations, considered together with patterns of MRF expression and their

respective mutant phenotypes, led us to propose a new model for myotome formation in which each of three myotomal MRF programs (My1-My3) drives a distinct wave of myotomal cellular expansion. In this model, different combinations of MRFs are required for execution of each wave of commitment and differentiation. The effects of the *MRF4*^{bh1} mutation on rib morphogenesis are contrasted with those of mutations in *myogenin* and *Myf-5*; myotomal cellular domains and specific signaling molecules produced from them are considered as candidates for intramyotomal and myotome/sclerotome interactions.

Materials and Methods

MRF4 inactivation in ES cells

The linearized targeting plasmid was electroporated into CJ7 embryonic stem (ES) cells followed by selection in the presence of G418 and Gancyclovir. Selection of homologous recombination events over other integration sites was facilitated by the presence of two HSV-TK expression cassettes, one flanking each segment of *MRF4* homology. 423 independent clones were isolated, and the clones containing a single, correct homologous recombination on *MRF4* locus were identified by both Southern hybridization and PCR. From 23 positive clones, six were selected for injection into mouse blastocysts. Coat chimeras ranging from 5% to greater than 90% were generated from six lines, of which two were transmitted to the germline. Heterozygous progeny were crossed with C57B6 mice. Most developmental timepoints assayed in this work were examined in both independent lines and no differences in phenotype were detected.

Genotyping of Progeny.

Genomic DNA was isolated from either yolk sac or tail biopsies. For Southern hybridization, 10μg of DNA was digested with either BamHI and KpnI restriction enzymes, or BamHI and StuI at 37°C. DNA was fractionated on 0.8% agarose gel with Tris-Acetate-EDTA buffer, and then transferred to Hybond-N filter with 10XSSPE. Probe for hybridization was labeled with [α^{32}P]-dCTP using a random-primed labeling kit (Boehringer Mannheim) according to the manufacturer's instruction. Hybridization was performed at 68°C overnight according to Sambrook et al. (1989). For genomic PCR, 1μg of DNA was used in 29 cycles with the following three primers: MRF4-forward, GGGAGACTGATGCTCCATGACAGC (from *MRF4* promoter); MRF4-reverse, GTGTTCCTCTCCACTGCTGTCGCT (from *MRF4* exon 1); PGK-reverse, GCGCTACCGGTGGATGTGGAATG (from PGK promoter).

RNA isolation and RT-PCR

RNAs were prepared by the method of Chomczynski and Sacchi (1987) from the trunk (without limb) region of embryos from different developmental timepoints. Quantitative RT-PCR was carried out according to the method of Robinson and Simon (1991). Both reverse transcription and PCR were performed in the same tube in a single buffer with specific primers. AMV-RT (Promega) was used instead of MMLV-RT, and Taq antibody (Clontech) was also added to block Taq activity at lower temperatures. 22-28 cycles were used for different primer sets as shown in Table I. An initial titration was carried out to assure that amplifications at high cycle numbers were still in linear range and quantitative (AP and BW, manuscript in preparation). All primer sets were designed to span at least one intron to distinguish RNA from DNA contamination, and the sizes of the products were between 200 and 500 bases. For the e18.5 samples, reverse transcription was carried out separately with random hexamer primers (Pharmacia).

Whole-mount skeletal staining.

Newborn mice were skinned and eviscerated prior to fixation. Embryos were fixed directly in 100% EtOH. After fixation for 3 days, carcasses were incubated in acetone for 3 days. Bone and cartilage of mice or embryos were stained for 3 days at 37^{o}C with a solution containing 0.005% alizirin red S, 0.015% alcian blue 8GX, 5% acetic acid and 70% EtOH. The samples were incubated in 20% glycerol - 1% KOH solution for 6hrs at 37^{o}C and then kept at room temperature until the skeleton was clearly visible through the surrounding tissue.

Frozen sections and antibody staining

Embryos were fixed in 4% paraformaldehyde at 4^{o}C overnight. Embryos younger than e11 were sunk into 15% sucrose & 7.5% gelatin in PBS solution and then frozen in OCT

(Tissue-Tek). Older embryos were immediately frozen in OCT. Sections of 10-20μm thickness were obtained using a cryostat, and were blocked for 20 minutes in 10% goat serum and 3% BSA in PBS before applying the primary antibody for 1-3 hours at room temperature. The secondary antibody solution was applied for 1 hour. Antibodies against myosin heavy chain, (MF20; 1:10 dilution; mouse IgG2b; Developmental Studies Hybridoma Bank), striated muscle specific α actinin (1:400 dilution; mouse IgG1; Sigma), myogenin (F5D; 1:5 dilution; mouse IgG1; courtesy of W. Wright), and MyoD (1:10 dilution; mouse IgG1; Novocastra Lab. Ltd.) were used. Secondary antibodies were conjugated to Fluorescein and specific to mouse IgG isotypes and used as 1:100 dilution in 3% BSA in PBS (Southern Biotechnology Associates). Images were captured digitally on a confocal microscope (BioRad).

Histology.

Newborn mice were fixed with 4% paraformaldehyde, dehydrated gradually with EtOH, and embedded in paraffin. 16μm sections were stained with both hematoxylin and eosin. Embryos were frozen in OCT after fixation with 4% paraformaldehyde, sectioned at 20μm thickness, and stained with both hematoxylin and eosin.

Results

Construction of *MRF4^{bh1}* mutant mouse strains.

Two independent strains of mice were generated in which the functionally essential bHLH domain of the *MRF4* gene was deleted from the chromosome via targeted homologous recombination in embryonic stem cells and subsequent transmission into the mouse germline. The targeting plasmid was designed to create a null allele of *MRF4* by replacing codons 40 to 173 of *MRF4* (Miner and Wold, 1990) with a PGK-neo selection cassette (Fig. 1a). This eliminates the entire basic-helix-loop-helix domain which is required for MRF protein dimerization and DNA binding in biochemical assays (Lassar *et al.*, 1989; Lassar *et al.*, 1991) and is also required for myogenic activity in transfection assays (Tapscott *et al.*, 1988; Yutzey *et al.*, 1990). We call this the *MRF4^{bh1}* allele. Blastocyst injections produced chimeras from six different targeted embryonic stem cell lines; two independent cell lines from different electroporation experiments were ultimately established in the germ line. Heterozygous males and females appeared grossly normal and were fertile. Genotypes of progeny were determined by Southern hybridization and by PCR of DNA from tail or yolk sac. Southern blot analysis showed that the map of the targeted locus is as predicted if homologous recombination had occurred between the vector and the host chromosome, as indicated (Fig. 1B). Additional Southern blot analysis showed the related downstream *Myf-5* locus to be intact and unaffected (data not shown). Progeny from crosses of *MRF4^{bh1}* heterozygotes harvested during gestation yielded 47 (29%) wildtype, 85 (53%) heterozygous and 28 (18%) homozygous embryos, results that are within 95% confidence limits for 1:2:1 Mendelian ratios. Homozygous animals died shortly after birth with 100% penetrance. They showed respiratory distress which may be the proximal cause of lethality.

MRF4 is an important regulator of early somitogenesis

Quantitative RT-PCR and immunocytological assays were used to compare littermates of wildtype, heterozygous, and homozygous genotypes . The earliest marker of myotomal commitment presently known is *Myf-5*. Previous in-situ hybridizations have shown that *Myf-5* expression in somites is first detectable at e8, when the first four somites have just formed (Buckingham, 1992). The RT-PCR analysis performed here also showed no expression of *Myf-5* in wildtype e7.5 embryos, but by e8 (6-7 somites) wildtype (data not shown), heterozygous, and homozygous $MRF4^{bh1}$ embryos all showed comparable levels of *Myf-5* expression (Fig. 2A). As expected, the other MRFs were not yet expressed at this time in heterozygous or homozygous embryos. Proper *Myf-5* initiation argues that the *MRF4* knockout allele we have constructed does not exert detectable cis-effects on the *Myf-5* gene which is located 7kb downstream (Miner and Wold, 1990). By e9 (13-14 somites) myogenin protein expression (Fig. 2b) was detected by immunostaining in heterozygous and homozygous *MRF4bh1* embryos. We conclude that the earliest myotome formation is largely unaffected by this *MRF4* mutation, and this is consistent with the fact that *MRF4* is not expressed detectably until after e9 (Bober *et al.*, 1991; Hinterberger *et al.*, 1991; Smith *et al.*, 1994).

The earliest phenotype we detected in *MRF4bh1* animals was a deficit in myotome development that corresponded in time with the first wave of *MRF4* expression, beginning around e9 and ending around e11. Quantitative RT-PCR was used to measure expression of an expanded set of genes normally expressed in somites at this time. For all studies at e10 and later, trunk regions were dissected and analyzed without head and limbs. The intent was to eliminate interference from MRF4-independent myogenic programs that are active in head and limb. By e10, expression of *Myf-5*, *myogenin*, and *MyoD* in the trunk were significantly reduced relative to their levels in wildtype littermates (Fig. 3). When normalized to *GAPDH* levels, quantitation of band intensities showed that *Myf-5*,

myogenin, and *MyoD* RNA levels in homozygotes were 25%, 8%, and 10% of wildtype levels, respectively. Interestingly, *MRF4*, *Myf-5*, and *MyoD* levels were reproducibly reduced in the heterozygotes, suggesting that haploinsufficiency in *MRF4* is not compensated by upregulation of other family members and, moreover, that haploinsuffiency in *MRF4* radiates through the MRF network to include other members. Among downstream muscle specific differentiation genes surveyed, *MRF4bh1* homozygotes showed very substantial deficits, although different genes were affected to different extents. For example, *FGF-6* and *M-Cadherin*, genes that are interesting for their potential effects on cell-cell interactions, were expressed at markedly reduced but still detectable levels (8 and 25 % of wildtype, respectively). However, the effect on the embryonic myosin heavy chain (*MHC-emb.*) was more dramatic, as its RNA appears to be entirely absent from mutant embryos at e10, even though in wildtype embryos it has accumulated at high levels. Since defects in rib morphogenesis become evident later in development and might arise from earlier sclerotomal defects, an array of sclerotomal markers including *Pax-1*, *M-Twist, Id-1*, *Gli-1*, and *Urokinase* were also surveyed. No noticeable effects were detected in any of the sclerotomal markers tested (Figure 3).

In wildtype animals MyoD expression begins gradually at e9.5 (Smith *et al.,* 1994), and it accumulates to significant levels by e11 (Sassoon *et al.*, 1989). This is an informative time to evaluate the impact of *MRF4bh1*, because MRF4 is concurrently disappearing from wildtype myotomes. At e11, MRF members were still expressed at reduced levels in *MRF4*$^{bh1/bh1}$ embryos compared to wildtype. However, the reduction was less dramatic than that observed one day earlier. Quantitation of band intensities normalized to *GAPDH* showed *Myf-5*, *myogenin*, and *MyoD* RNA levels in homozygotes were 25%, 21%, and 32% of wildtype levels, respectively. The picture was similar for muscle specific differentiation markers. We conclude that the severe deficit in myotomal myogenesis

observed at e10 is moderated by e11, and this was observed in both knockout lines (Fig. 3 and data not shown).

An important issue is how the gene expression observed in whole trunk RNA specimens is distributed among cells of the developing somite. At one extreme, RNA measurements of Figure 3 could reflect differences in levels of gene expression distributed over identical cellular domains in the mutant and wildtype; at the other extreme, all RNA could be accounted for by changes in the number and/or type of myotomal cells in mutant versus wildtype. To help discriminate these possibilities, e11 wildtype and homozygous *MRF4bh1* littermates were sectioned and probed with antibodies to relevant muscle specific regulators and structural proteins including myogenin, MyoD, α actinin (Fig. 4), and myosin heavy chain (data not shown). The myotomes of mutant animals were greatly reduced in size and cell number. Differentiated myocytes could be identified by their expression of α actinin (Fig 4A, B, G, H). In the homozygotes, these myocytes were confined mainly to the dorsal myotomal domain. This was most obvious in caudal hindlimb level somites which lag developmentally behind the more mature rostral forelimb somites. By e11, MyoD expression in wildtype and in mutants has begun to accumulate (Fig. 4E, F, K, L). At the cellular level, MyoD protein expression outlines a new and much larger presumptive myotomal domain in the mutants than that shown for with myogenin or α actinin, and the MyoD domain was concentrated more ventrolaterally.

Axial myogenesis in *MRF4bh1* homozygotes is grossly normal by e14

By e14, no *MRF4* expression in wildtype embryos can be detected, and it will not reappear until about e16. However, the period of myotomal myogenesis coincident with the expression of *MyoD*, which begins around e10.5, has been active for several days, and during this time axial muscle mass has expanded significantly. At e14 the RT-PCR analysis was again focused on axial musculature. Comparable expression levels were

found for most muscle regulators and structural genes tested in wildtype, heterozygous and homozygous *MRF4bh1* fetuses from both knockout strains (Fig. 5 and data not shown) . Exceptions were *Myf-5* and *M-Cadherin*, both expressed at reproducibly reduced levels in mutant animals. At the cellular level, we observed grossly normal muscle mass patterns by histological staining of e15 embryos (Fig 6a and b) and by muscle specific antibody staining of both limb muscles and intercostal muscles (data not shown).

MRF4 has subtle effects on intercostal muscles in the newborn.

To examine the effects of the second wave of *MRF4* expression, which starts around e16 and correlates with the timing of widespread secondary differentiation of muscle, we examined RNA from ribcage region of e18.5 and newborn animals by RT-PCR. *MRF4*$^{bh1/bh1}$ animals expressed largely normal levels of most muscle specific markers, although a modest reduction in *Myf-5* and some muscle specific structural genes was observed (Fig 5). Similar results were obtained for RNA isolated from limbs (data not shown). However, examination of sections from e18.5 and newborn mice revealed that some intercostal muscles associated with ribs 3-5 were either disorganized or significantly reduced in fiber number in *MRF4*$^{bh1/bh1}$ animals. This pheontype varied in intensity among different homozygous animals, and it remains to be determined whether genetic background is playing a significant role. In animals displaying the most intense intercostal phenotype, substantial numbers of mononuclear cells were present in homozygous animals (Fig 6c and d). Antibody staining for myosin heavy chain (data not shown) and α actinin showed that these cells were not differentiated myocytes (Fig 6e and f). This effect on myogenesis was not evident prior to the period of fetal *MRF4* expression (Fig 6a and b).

Rib defects in *MRF4* null mutant mice.

Homozygous mice showed obvious respiratory distress and died shortly after birth. Rib defects have been observed previously in *Myf-5*- and *myogenin*-null mutant mice (Braun

et al., 1992; Hasty *et al.*, 1993; Nabeshima *et al.*, 1993). We therefore examined whether *MRF4*-null mutant mice exhibit skeletal abnormalities by staining with alizirin red S and alcian blue to detect bone and cartilage, respectively . In newborn mice homozygous for *MRF4bh1*, rib development is severely disturbed (Fig. 7). The abnormalities include rib bifurcation, fusion of rib cartilage from adjacent ribs, truncated ribs that fail to attach to the sternum, and disorganized ossification in the sternum. Rib foreshortening in ribs 2-12 was usually less than 20% of total length, and this led to variable failure to join the sternum. Interestingly, the first and last (13th) ribs were more dramatically shortened compared with the others. Some ribs also displayed abnormal angles of extension (Fig. 7 g-i). Finally, the tuberculum anterior that is normally present on the 6th cervical vertebrae was absent in most $MRF4^{bh1/bh1}$ mice (83% penetrance). Other skeletal elements, including proximal regions of ribs and vertebrae appeared to be normal (data not shown).

To examine when rib defects develop relative to chondrogenesis and ossification, e14 embryos were stained with both alizirin red S and alcian blue. Normally, rib cartilage cytodifferentiation in mice begins at e13, and ossification begins at e14 (Rugh, 1968). In all genotypes, the skeleton at e14 was entirely cartilaginous except for the clavicle and the earliest ossification centers of facial bones. However, in homozygous *MRF4bh1* mutants, the rib bifurcations and truncated rib forms were already clearly visible (Fig. 7j, k). Thus, cytodifferentiation of most rib cartilage occurred at the normal time by this measure, and major rib pattern formation defects were clearly visible as soon as chondrogenesis rendered rib anlagen histochemically visible. These events occur well before the second period of *MRF4* expression that begins at e16.

Discussion

In this study the mouse *MRF4* (*herculin/Myf-6*) gene was disrupted by targeted recombination. Animals homozygous for this *MRF4bh1* allele, which deletes the bHLH domain, displayed phenotypic effects on axial myogenesis and on rib pattern formation. They ultimately died at birth of apparent respiratory insufficiency. A substantial failure in myotomal development of homozygous mutants was observed, and it corresponded with the early somitic wave of *MRF4* expression (e9 to 11). At the molecular level this generated a gross but transient deficiency in expression of other MRFs and some muscle specific differentiation genes, including *FGF-6* and *M-Cadherin*, which may be important in pattern formation and inductive interactions with the sclerotome (see below). The early myotomal deficit was largely overcome by subsequent myotomal expansion after the end of the *MRF4* expression period in the myotome. However, perhaps as a consequence of the earlier myotomal phenotype, rib pattern formation was disrupted, and may be the primary cause of lethality. A late fetal muscle phenotype was also detected in some intercostal muscles of newborn mice, suggesting a possible effect from the lack of *MRF4* expression that normally begins in muscles at e16; this might also contribute to lethality in the mutants.

MRF4bh1 reveals three waves of myotomal expansion, each dependent on a different set of MRFs

A striking aspect of myotome formation in *MRF4bh1* homozygotes was the observation that an entire cellular myotomal domain appeared to be missing. Comparison of mutant and wildtype embryos before, during and after the window of *MRF4* somitic expression leads us to propose the model shown in Figure 8 for myotome biogenesis in the mouse. It identifies three different myotomal MRF programs (My1, My2 and My3) with three waves of myotomal cellular commitment and differentiation that result in production at the cellular

level of primary, secondary, and tertiary myotomes. This nomenclature refers to different spatiotemporal phases of myotome formation, and should not be confused with primary and secondary myocytes that refer to embryonic and fetal myogenic differentiation, respectively. The My1 program is first and begins at e8; it uses Myf-5 and myogenin. By e9, this program has produced differentiated myocytes that are expressing myogenin and structural markers such as cardiac and skeletal α actin (Buckingham, 1992) in a small domain that typically includes less than 20 cells per somite at the future forelimb level. These cells are concentrated dorsally in the somite. The expression of Myf-5 protein in a domain of this size and position was recently described in a detailed study by Smith and Miller (Smith *et al.*, 1994). Previous studies of a *Myf-5* gene disruption (*Myf-5^{ml}*) (Braun *et al.*, 1992; Braun *et al.*, 1994) have shown that there is no detectable myocyte differentiation in *Myf-5* deficient homozygotes until e10.5. This suggests that *Myf-5* function is essential for the My1 program, and is consistent with expression patterns. Whether myogenin is also essential for execution of this MRF program is not certain, as phenotypic data is not yet available for myogenin null mutants at the early times (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993). The My1 program is initiated on schedule by both molecular and cellular assays, in the *MRF4bh1* homozygotes (Figure 2).

The phenotype of *MRF4bh1* homozygotes suggests the existence of a second MRF program (My2) that is needed to execute a new wave of myogenic recruitment in the myotome. This myotomal expansion normally occurs during the period of *MRF4* expression beginning at about e9 in rostral somites, and involves an increase of at least 15-fold in the number of cells expressing myogenin or other skeletal muscle markers (Smith *et al.*, 1994). The most straightforward interpretation of data from our *MRF4* gene disruption is that the proposed secondary wave of myotomal expansion requires MRF4 function. Myf-5 is also required, because *Myf-5^{ml}* homozygotes form neither the proposed primary or secondary myotomes, nor do they turn on *MRF4* or other muscle

specific genes during this time (Braun *et al.,* 1994). It is not clear, however, whether the dependence of secondary myotome formation on Myf-5 is cell autonomous or not. Thus in one scenario, Myf-5 expressing myoblasts within the primary myotome would migrate, change their MRF expression pattern (including initiation of *MRF4* expression) and then form the secondary myotome. In a nonautonomous mechanism, mesodermal precursor cells neighboring the primary myotome could be directly induced to express MRF4 and myogenin and thus commence formation of the secondary myotome. In either case, *MyoD* is not widely expressed until later times, and does not significantly compensate. It is interesting that *myogenin* null embryos apparently show normal somitic and myotomal morphology during the My2 program, and it will now be useful to examine muscle specific gene expression between e8.5 and e10.5 in *myogenin* knockout mice to see whether the secondary wave of muscle differentiation proposed here occurs normally.

The proposed My3 program is marked by the onset of MyoD expression, and prominent myogenin expression. Together, they appear sufficient to direct a third major cellular expansion that produces the tertiary myotome of Figure 8 between e11.5 and 15. Thus, in *Myf-5^{nl}* null embryos, there is no detectable myotome formation until the onset of MyoD expression around e10.5, but that following onset of its expression a large myotome quickly forms, and subsequent axial myogenesis appears surprisingly normal (Braun *et al.*, 1994). This led those investigators to propose the regulatory and functional independence of a MyoD driven myotome, and data presented here lead us to identify it as the third of three waves of myogenesis in the mouse myotome. *MRF4* is not detectably expressed during most of the My3 period, nor is there any evidence that it can be accessed to compensate for MyoD when *MyoD* has been mutated (Rudnicki *et al.*, 1992). However, in the *MRF4bh1* homozygotes, the timing of the MyoD dependent myotome expansion was somewhat delayed, suggesting the possibility of positive inductive interaction between My2 and My3. *Myf-5* expression normally declines between e11 and 16, and its domain of

expression does not appear to fully overlap the expanded area in which MyoD and myogenin are prominent (Smith *et al.*, 1994). However, in *MyoD* knockout mice, Myf-5 appears to compensate (Rudnicki *et al.*, 1992); it is expressed at relatively elevated levels in these animals for an extended period. They ultimately appear to have normal axial musculature. This is one of several situations in which it is unclear whether compensation is at the level of cells or at the level of MRF molecules. Thus, compensation might represent the expansion of a pool of cells that express Myf-5 and myogenin or might instead reflect crosstalk among MyoD family regulators within a cell.

Rib pattern formation phenotypes in *MRF4$^{bh1/bh1}$* mice.

The rib morphogenesis phenotypes in the *MRF4bh1* homozygotes are quite different from those of the *Myf-5^{ml}* homozygotes and are more similar to the *myogenin* null rib defects. The *Myf-5* mutant has only rudimentary rib stumps at all vertebrae, but the *MRF4bh1* homozygotes have extensive rib formation that is mainly disrupted at distal locations close to the sternum. This argues that the basis for the pattern formation disturbances in these animals are mainly interactions between myotome and sclerotome that depend on cells and signals produced in the My2 myotome of the model, while the more dramatic reduction in rib formation found in the *Myf-5* mutant might be attributable to failure of the My1 program. Christ and colleagues (Huang *et al.*, 1994) have recently reported that in the chick-quail system, cells from the somitocoele of early epithelial somites are rib progenitors. Moreover, homotypic somitic transplantation experiments revealed that rib progenitors from one somite can later be found in two ribs, the somite transplanted and the next most rostral rib. At slightly later developmental times, cytological studies have noted the close apposition of lateral sclerotomal cells with the emerging myotome. It is interesting to speculate that cells of the secondary (and perhaps primary) myotome of Figure 8 provide signals and cellular scaffolding as these cells elongate in the rostral caudal

axis; and if these signals or cellular scaffolding are underdeveloped, migration of rib progenitors may be obstructed, and later reflected as the bifurcations and spurs (Fig. 4).

A candidate signaling molecule that could mediate inductive interactions within the expanding myotome or between myotome and sclerotome was suggested by results presented here. *FGF-6* is specifically expressed in myotomes starting at e9.5, about the same time that *MRF4* starts to accumulate (Han and Martin, 1993; deLapeyriere *et al.*, 1993). FGF-6 belongs to the family of fibroblast growth factors, and here we demonstrate that its levels are strongly downregulated in myotomes of *MRF4* null mice (8% of wildtype). Although the specific functions of FGF-6 are not presently known, it is an attractive candidate for intercellular signaling between cells of the myotome and the pool of proliferating precursors that are being recruited to the expanding myotome throughout its maturation. A second plausible interaction is with adjacent sclerotomal cells that are thought to include rib progenitors. Therefore, it is potentially relevant that FGF family receptors such as FGFR-1 and FGFR-2 are expressed in sclerotomal cells (Peters *et al.*, 1992; Yamaguchi *et al.*, 1992), but no known candidate ligands have been identified within the sclerotome itself. This raises the possibility that FGF-6 secreted from myotomes might act as the ligand for these receptors. The possibility of involvement of the FGF family in rib development is also suggested by recent findings of several inherited human skeletal disorders that correlate with mutations in FGF receptors, including FGFR1 and 2 (reviewed by Eriebacher *et al.*, 1995).

The skeletal and myogenic phenotypes of $MRF4^{bh1/bh1}$ mice resemble phenotypes of mutations in two genes involved in cell-cell inductive interactions. Follistatin interacts with activin/inhibin, and modulates its function (reviewed by DePaolo *et al.*, 1991). In *follistatin* null mutant mice the 13th rib is absent and, in some genetic backgrounds, the 7th rib fails to attach to the sternum (Matzuk *et al.*, 1995). In addition, intercostal muscles of

newborn *follistatin* null mice showed sparse and somewhat disorganized muscle fibers, similar to *MRF4* null mice. The expression pattern of *follistatin* is also suggestive. It appears in somites prior to *MRF4* expression (Albano *et al.*, 1994; Feijen *et al.*, 1994), opening the possibility that it acts upstream of *MRF4*; it will be informative to study how *MRF4* and *Myf-5* are expressed in somites of *follistatin* null mice. BMP5 is one of the large family of bone morphogenetic factors related to TGFβ. In *BMP5* null mice, the 13th rib and the tuberculum anterior of the sixth cervical vertebra are absent (Kingsley *et al.*, 1992), two features also found in $MRF4^{bh1/bh1}$ animals. *BMP5* is expressed widely in skeletal precursors (King *et al.*, 1994), and it remains to be determined whether its expression is altered in *MRF4* null mutants.

Myf-5 and *MRF4* are located only 7kb apart on the chromosome (Miner and Wold, 1990), while *myogenin* and *MyoD* are unlinked. This raises the possibility that changes at the *MRF4* locus, which in this study included deletion of protein coding and first intron sequences as well as insertion of a neo selection cassette, might exert effects on *Myf-5* in cis. A recent study by Arnold and colleagues provides evidence for such complex cis interactions (Braun and Arnold, 1995). They found that a disruption designed to remove a segment of *MRF4* that is entirely upstream (5') of that deleted in *MRF4bh1* allele unexpectedly eliminated virtually all *Myf-5* expression and function. This led to a phenocopy of the $Myf\text{-}5^{m1/m1}$ rib and myotome defects. It is clear that early *Myf-5* expression, which defines the start of the My1 program in our model, was indistinguishable from wildtype in the *MRF4bh1* disruption (Figure 2). Moreover, the bh1 rib defects were clearly not a phenocopy of the more extreme *Myf-5* deficiency. In addition, we observe significant expression of *Myf-5* at later developmental times, which further distinguishes it from the allele of Braun and Arnold (1995). We conclude that the *MRF4bh1* allele does not generate a wholesale cis disruption of *Myf-5* expression. However, cis effects could be a part of the myotomal $MRF4^{bh1/bh1}$ phenotype, if such cis

effects operate on a specific subset of *Myf-5* expression that mainly overlaps spatiotemporally with *MRF4* expression in the wildtype. Such cis effects on *Myf-5* could also explain the RT-PCR data from e14 embryo trunks where, unlike *MyoD* and *myogenin*, *Myf-5* expression remains at lower levels in *MRF4*$^{bh1/bh1}$ embryos than in wildtype. However, this remains only one interpretation, as at this late embryonic stage, *Myf-5* expression in wildtype is dropping relative to other MRFs and may therefore simply not play a significant part in the MyoD directed My3 program. A third *MRF4* allele has been generated concurrently with these (Zhang *et al*, 1995). This allele removed a larger segment of protein coding sequence than in the bh1 allele, including some 3' flanking sequences, and it also left a PGK-neo cassette behind, though in different orientation. It displayed a rib phenotype similar in pattern formation character to *MRF4*$^{bh1/bh1}$ but with a far milder effect at the point of joining to the sternum. Apparently owing to the milder rib phenotype, homozygotes of this mutation were viable and this permitted studies of adult muscle where a five-fold relative upregulation of *myogenin* RNA was observed, suggesting compensation for the deficit in *MRF4*. It will now be interesting to compare early somitic myogenesis in the two alleles prior to e11.5. Also, given the uncertainties attached to both positive and negative regulatory influences originating from the selection cassette enhancer/promoter residue present in all three *MRF4* mutations and in the *Myf-5*^{m1} allele, analysis of this locus will benefit from new methods that allow for nearly complete excision of targeting vector residue (reviewed by Sauer 1993).

Additional questions concerning the lineage, fate, and function of cells of the embryonic myotome are raised by our results. With respect to the fate of cells from the 1^{o} or 2^{o} myotome in later development, no cell tracing experiments have yet been reported that could tell us how long they live, whether they expand their domain, nor where these cells might be located in the mature musculature. The cells appear to be mainly mononucleate myocytes and, by their cell number alone, they cannot make a major contribution to the

mature axial musculature. The observation that rib defects occur in knockouts of any of the three MRFs that are expressed in the primary or secondary myotomes, together with the capacity of the tertiary myotome to compensate for lack of the previous two in later muscle formation, suggests that the early myotome may be mainly important as an inductive regulator and pattern formation guide for rib anlagen.

In a broader context, analysis of the *MRF4bh1* mutant underscores the dynamic quality of the somite. We have proposed that different combinations of *MyoD* family genes are needed to support distinct waves of cellular commitment and muscle differentiation, but why is the myotome built up in this apparently piecemeal fashion? One reason could be to limit muscle differentiation to specific subsets of precursors at early times, while others in the same signaling microenvironment are permitted to proliferate further. Also, at different times in the growth and maturation of the myotome, the sources of inductive and inhibitory interactions driving muscle determination and differentiation such as neural tube, sclerotome, earlier myotome or dermatome are themselves changing rapidly and are likely to be expressing different signals. Thus the multiplicity of MRFs may be most important, at least in the embryo, because they provide for myogenic responses to separate signaling pathways. The complexity and developmental diversity of cis regulatory elements currently being identified in MRF genes provides indirect support for this view (Patapoutian *et al.*, 1993; Cheng *et al.*, 1993; Yee and Rigby, 1993; Goldhammer *et al.* 1995), while direct tests may require gene transplacement experiments in which one MRF protein coding sequence replaces another in the mouse germline.

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Table I: Primers used in RT-PCR.

Gene	Forward Primer	Reverse Primer	Cycle #
MRF4	CTACATTGAGCGTCTACAGGACC	CTGAAGACTGCTGGAGGCTG	27
Myf-5	TGAATGTAACAGCCCTGTCTGGTC	CGTGATAGATAAGTCTGGAGCTGG	26
MyoD[a]	AGGCTCTGCTGCGCGACC	TGCAGTCGATCTCTCAAAGCACC	26
myogenin[a]	GAGCGCGATCTCCGCTACAGAGG	CTGGCTTGTGGCAGCCCAGG	28
FGF6	GTGCTCTCTTCATTGCCATGAACAG	CCCCGTGAGCCTTCATCC	28
MEF2D[c]	CAAGCTGTTCCAGTATGCCAG	AAGGGATGATGTCACCAGGG	26
M-cadherin	CAGGTTCACCATCCTTGAAGGT	TGGGTCGTAGTCTTTGGAGTAGC	26
ACHR-γ	CAGCGCAATGGATTAGTGCAGG	GTCAGGCACTTGGTTGTAGTGGG	27
NCAM (MSD)	TCCTCCACAGGCTCCTGCTAAC	CGCTCTGTACTTGACCAGATAGTG	26
MLC1F	AAAGACGTGAAGAAGCCCGCTG	ATAACCTCCCTGGTCCTTGTTG	23
α skeletal actin	TTATCGGTATGGAGTCTGCGGG	CACAGCACGATTGTCGATTGTGG	22
MCK	TTCGGCAACACCCACAACAAGTTC	ACATAGTTGGGGTCCAGGTCGTC	22
MHC-embryonic	GCAAAGACCCGTGACTTCACCTCTAG	GCATGTGGAAAAGTGATACGTGG	23
MHC-perinatal	GAAGACCGCAAGAATGTGCTCC	CCTCCTGTGCTTTCCTTCAGCC	22
PAX-1[c]	CACATTCAGTCAGCAACATCCTG	TGTATACTCCCTGCTGGTTGGAA	25
M-TWIST	AGCGGGTCATGGCTAACGTGCGGGA	GGAGCCGGTCCTTACCTAGG	26
ID-1	CTGGAGCTGAACTCGGAGTCTG	CTGAAAGGTGGAGAGGGTGAGG	23
Gli[d]	CTGATTTCAGGGAAGAGAGCAGACTGA	ACAAGCTTATGCAGCTGATCCAGCCTA	26
Urokinase	GTCTGTAGACCAACAAGGCTTCC	GGATTATAGGAGCTCTCCTTCGAC	27
GAPDH	GTGGCAAAGTGGAGATTGTTGCC	GATGATGACCCGTTTGGCTCC	22

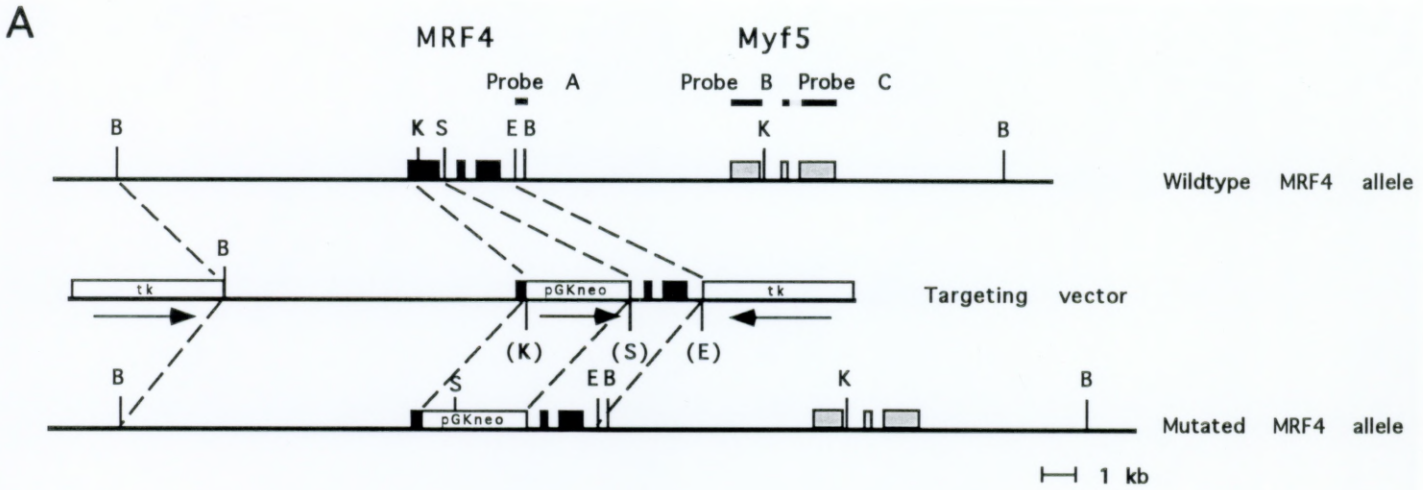
[a] Hannon et al. 1992; [b]Martin et al. 1994; [c]Fan and Tessier-Lavigne, 1994; [d]Walterhouse et al. 1993 .

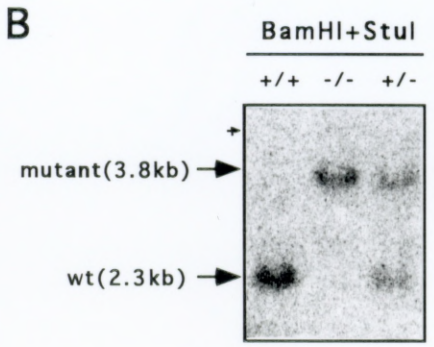
Figure 1. Targeted disruption of the *MRF4* locus by homologous recombination.

(A) Structure of the wildtype and mutated *MRF4* loci. For the targeting vector, the KpnI-StuI fragment of the *MRF4* gene, which contains all coding sequences for the basic HLH domain, was replaced with a PGK-neo cassette for positive selection in G418. HSV-tk cassettes flanked the targeting vector's homologous sequences to maximize the efficiency of negative selection with gancyclovir. Direction of arrows in the targeting vector represents the direction of transcription units. Exons of *MRF4* and *Myf-5* genes are shown as black and gray rectangles, respectively. The transcriptional direction of both *MRF4* and *Myf-5* genes is from left to right. Abbreviations for restriction enzymes are B, BamHI; E, EcoRI; K, KpnI; and S, StuI. Restriction enzyme sites in parentheses were lost in the cloning process.

(B) Southern hybridization of genomic DNA. Genomic DNA isolated from tail was digested with BamHI and StuI, fractionated on a 0.8% agarose gel, and transferred to Hybond-N. Using probe A (see panel A), 2.3kb and 3.8 kb bands that represent wildtype and mutated *MRF4* alleles, respectively, were detected.

(C) Genomic DNA PCR. DNA was amplified with locus specific primers (see materials and methods) for 29 cycles and analyzed on an agarose gel. 1kb ladder was used as a marker (M).


A
MRF4
Myf5
Probe A
Probe B
Probe C
B
K S
E B
K
B
Wildtype MRF4 allele
B
tk
pGKneo
tk
Targeting vector
(K)
(S)
(E)
B
S
E B
K
B
pGKneo
Mutated MRF4 allele
1 kb


B
BamHI+StuI
+/+ -/- +/-
mutant(3.8kb)
wt(2.3kb)

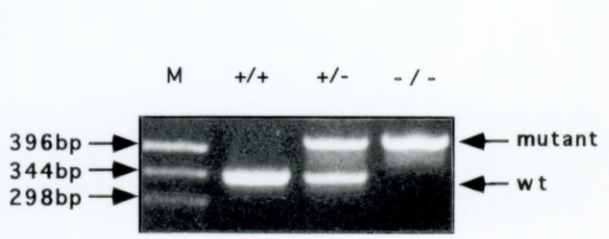
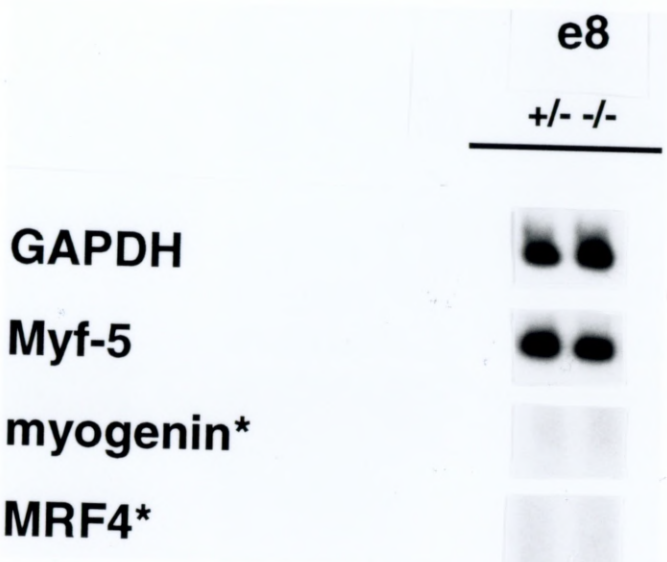

C
M +/+ +/- - / -
396bp
344bp
298bp
mutant
wt

Figure 2. Expression of MRFs in mice lacking *MRF4* during initial myotome formation.
(A) Quantitative RT-PCR on *MRF4bh1* homozygote and control littermates from whole e8 embryos. 6-7 somite stage homozygote and heterozygote animals show comparable *Myf-5* and control *GAPDH* levels and no detectable *myogenin* and *MRF4* expression (*).
(B) Transverse sections of heterozygote and homozygote e9 (13 somite) embryos showing myogenin protein expression in caudal (young) somites.


e8
+/- -/-
GAPDH
Myf-5
myogenin*
MRF4*

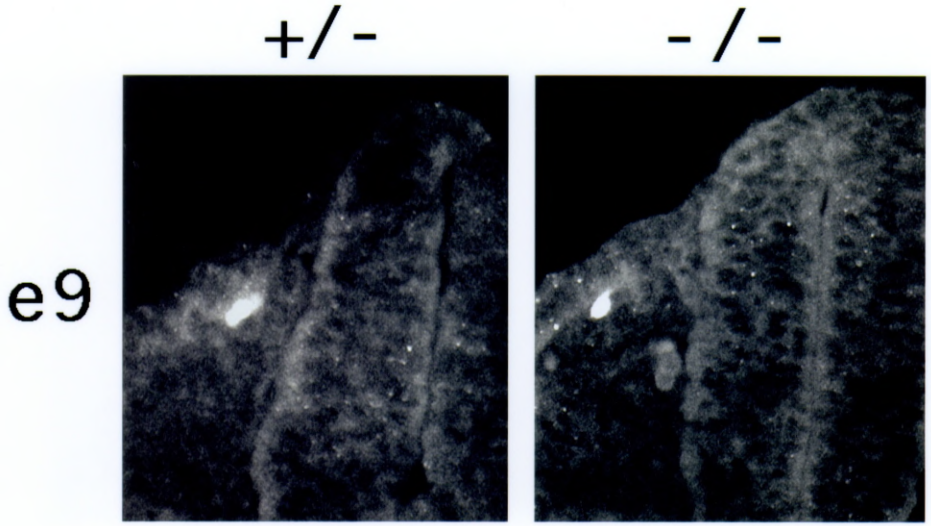

+/-
-/-
e9

Figure 3. Early *MRF4* expression is required for muscle specific gene expression in myotomes.

Quantitative RT-PCR on *MRF4bh1* homozygote and control littermates from e10 and e11 trunk (without head and limb) embryos with muscle specific and other non myotomal somitic genes. At e10 all muscle specific genes examined are expressed at lower levels in homozygous animals. Heterozygous animals also show some downregulation compared to wildtype littermates. Non-myotomal somitic genes and control *GAPDH* are expressed at normal levels in *MRF4* knockouts. At e11, muscle specific genes are still expressed at reduced levels in *MRF4* knockouts, however, the downregulation of some genes, such as *MyoD*, is less dramatic compared to that seen at e10.

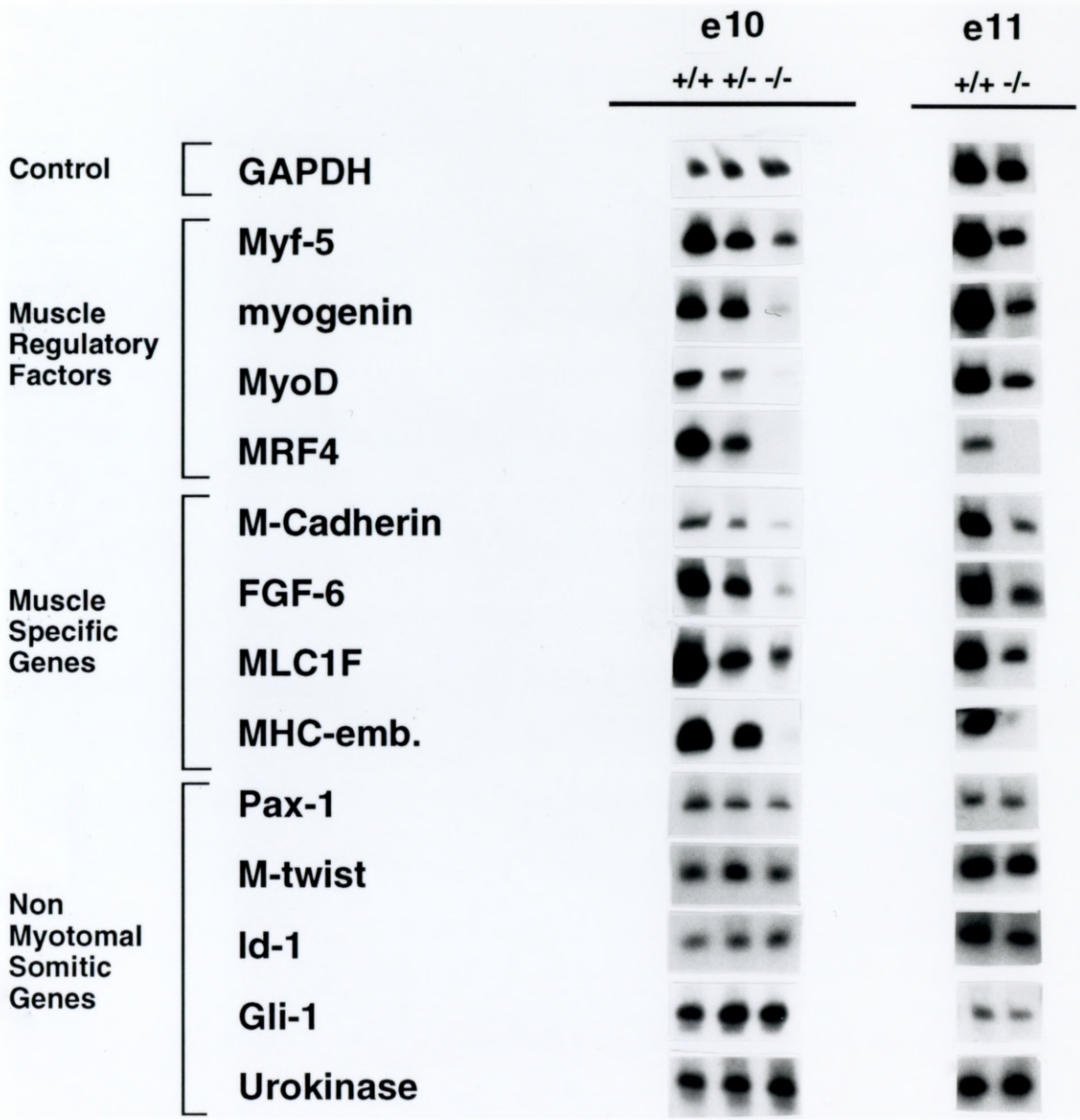

e10
+/+ +/- -/-
e11
+/+ -/-
Control
GAPDH
Muscle Regulatory Factors
Myf-5
myogenin
MyoD
MRF4
Muscle Specific Genes
M-Cadherin
FGF-6
MLC1F
MHC-emb.
Non Myotomal Somitic Genes
Pax-1
M-twist
Id-1
Gli-1
Urokinase

Figure 4. Effects of *MRF4* knockout on myotomal domains at e11.

Transverse sections of wildtype and *MRF4bh1* homozygous e11 littermates stained with antibodies against α actinin (A, B, G, H), myogenin (C, D, I, J), and MyoD (E, F, K, L). All panels show dorsal region of transverse sections. The ventral side of embryo is facing downwards, and the neural tube is on the upper left hand corner. Forelimb level somites (A-F) represent more mature myotomes than do hindlimb level somites (G-L). The number of cells expressing muscle specific markers is strongly reduced in homozygote animals. α actinin expression in both forelimb (A, B) and hindlimb (G, H) level myotomes is reduced to the dorsal, earlier forming myogenic cells in *MRF4* knockouts. Myogenin expression in the younger hindlimb level somites (I, J) is restricted to a few cells of the dorsal myotome in the $MRF4^{bh1/bh1}$ animals. In the forelimb region (C, D), myogenin expression in the *MRF4* knockout starts expanding ventrally; however, it is still in a very restricted region compared to the wildtype littermate. MyoD expression (E, F, K, L) is the least affected in homozygous animals. Although fewer cells express MyoD in the myotomal region of $MRF4^{bh1/bh1}$ animals, the domains of expression are comparable in wildtype and homozygote animals at both limb levels.

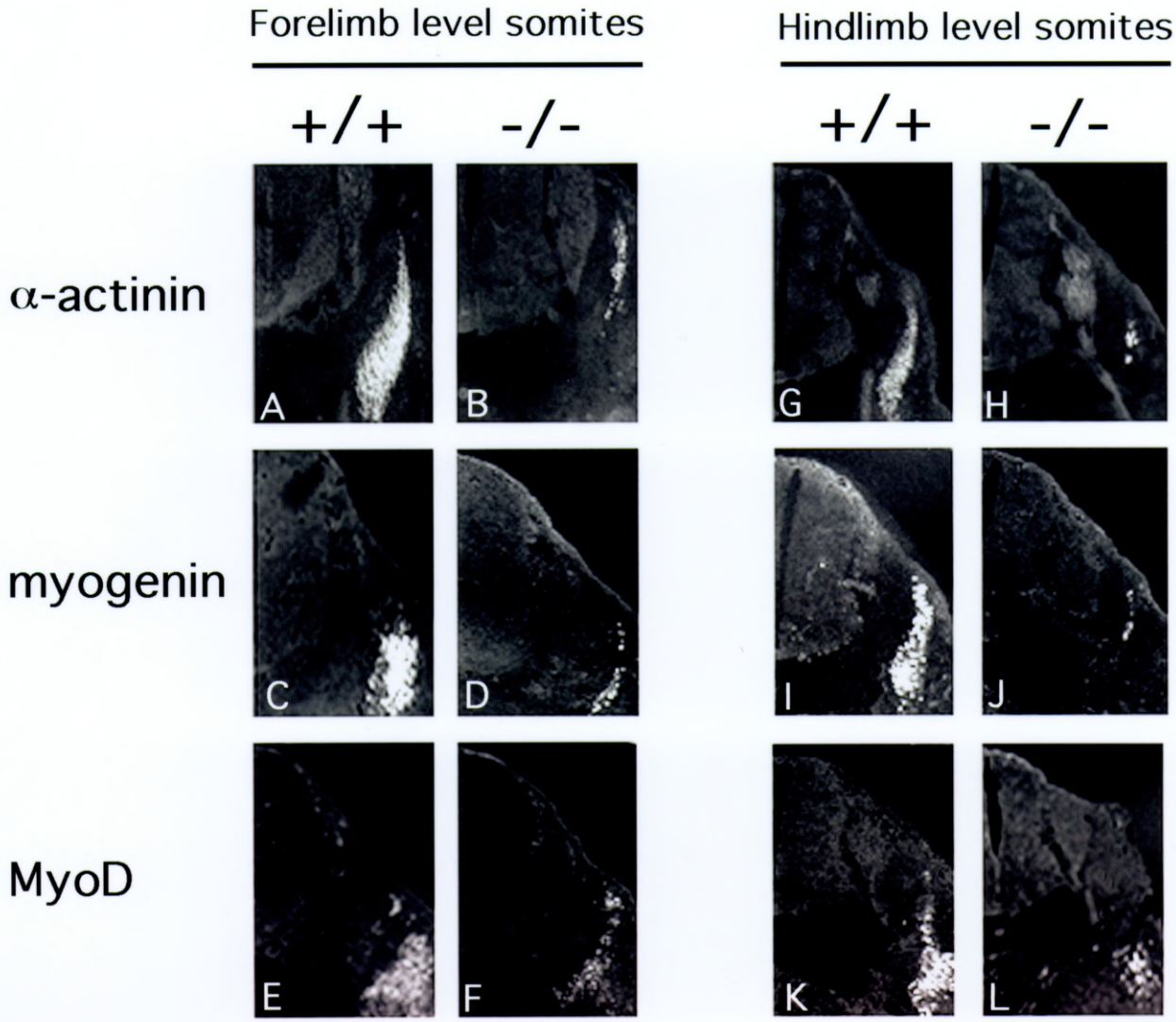

Forelimb level somites
Hindlimb level somites
+/+
-/-
+/+
-/-
α-actinin
myogenin
MyoD
A
B
C
D
E
F
G
H
I
J
K
L

Figure 5. Expression of muscle specific genes in mice lacking *MRF4* during late embryogenesis.

Quantitative RT-PCR on *MRF4* knockout and control littermates from e14 and e18.5 embryos. RNA was isolated from ribcage region of animals, avoiding internal organs such as heart. At both timepoints, most muscle specific genes and control *GAPDH* show normal levels of expression in homozygote animals. *Myf-5* levels are lower in $MRF4^{bh1/bh1}$ mice , and no *MRF4* expression is observed. *M-Cadherin* is downregulated in homozygote animals at e14, while *MLC1F* and *MHC-embryonic* are slightly downregulated at e18.5.

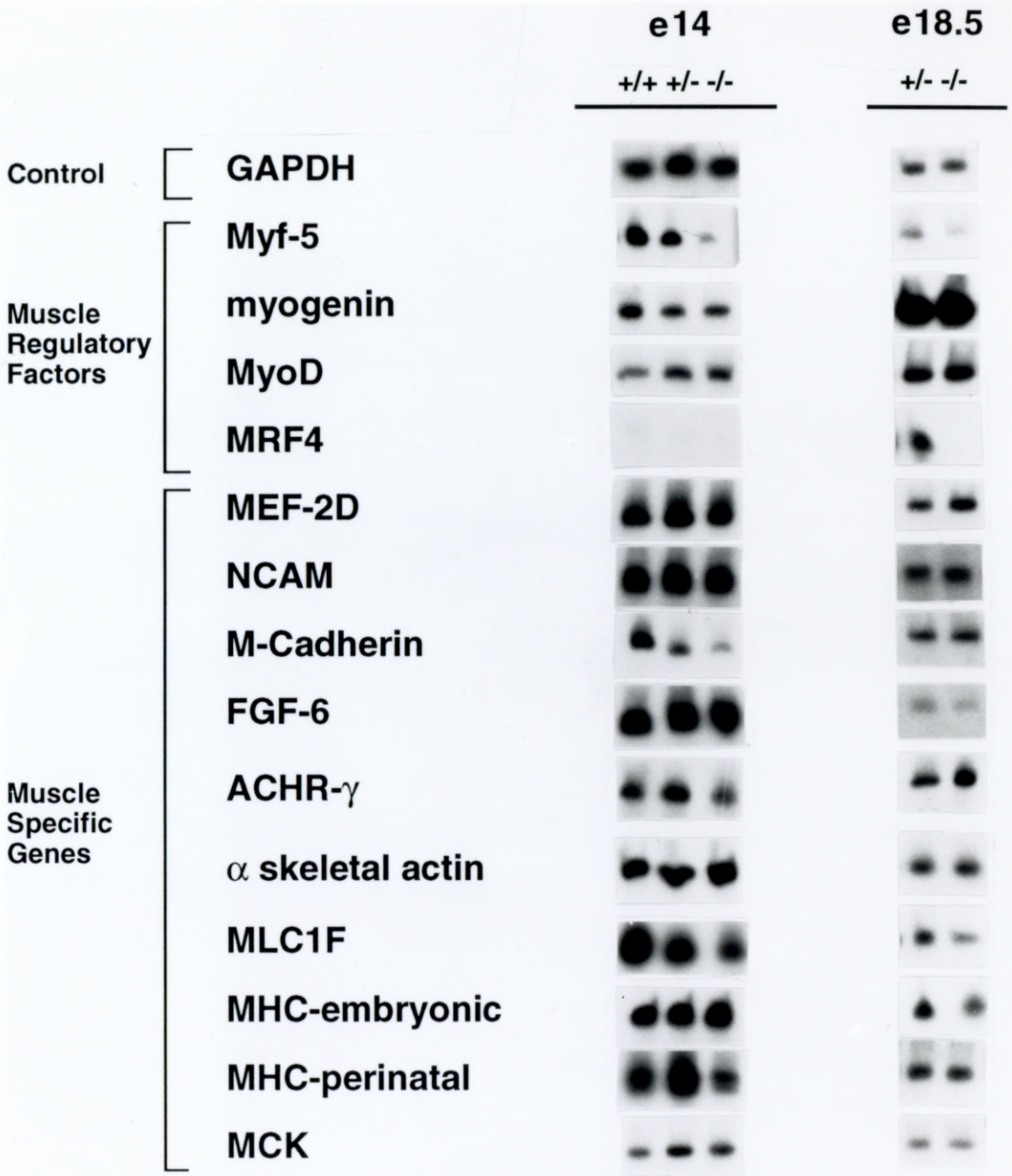

e14
+/+ +/- -/-
e18.5
+/- -/-
Control
GAPDH
Muscle Regulatory Factors
Myf-5
myogenin
MyoD
MRF4
Muscle Specific Genes
MEF-2D
NCAM
M-Cadherin
FGF-6
ACHR-γ
α skeletal actin
MLC1F
MHC-embryonic
MHC-perinatal
MCK

Figure 6. Abnormal intercostal muscle formation at e18.5 and newborn in *MRF4*$^{bhl/bhl}$ mice.

Frozen sections of e15.0 day embryos (A and B) or sections of paraffin embedded newborn mice (C and D) were stained with hematoxylin/eosin. Unlike the wildtype mice (C), disorganized muscle fibers and an increased number of mononuclear cells are seen in intercostal muscle of newborn *MRF4*$^{bhl/bhl}$ mice (D). However, intercostal muscle looks similar in both wildtype (A) and *MRF4*$^{bhl/bhl}$ (B) embryos at e15. Immunostaining of intercostal muscles with α actinin antibody shows *MRF4*$^{bhl/bhl}$ mice (F) have abnormal muscle formation between ribs compared to wildtype littermate (E) at e18.5 (r, rib).

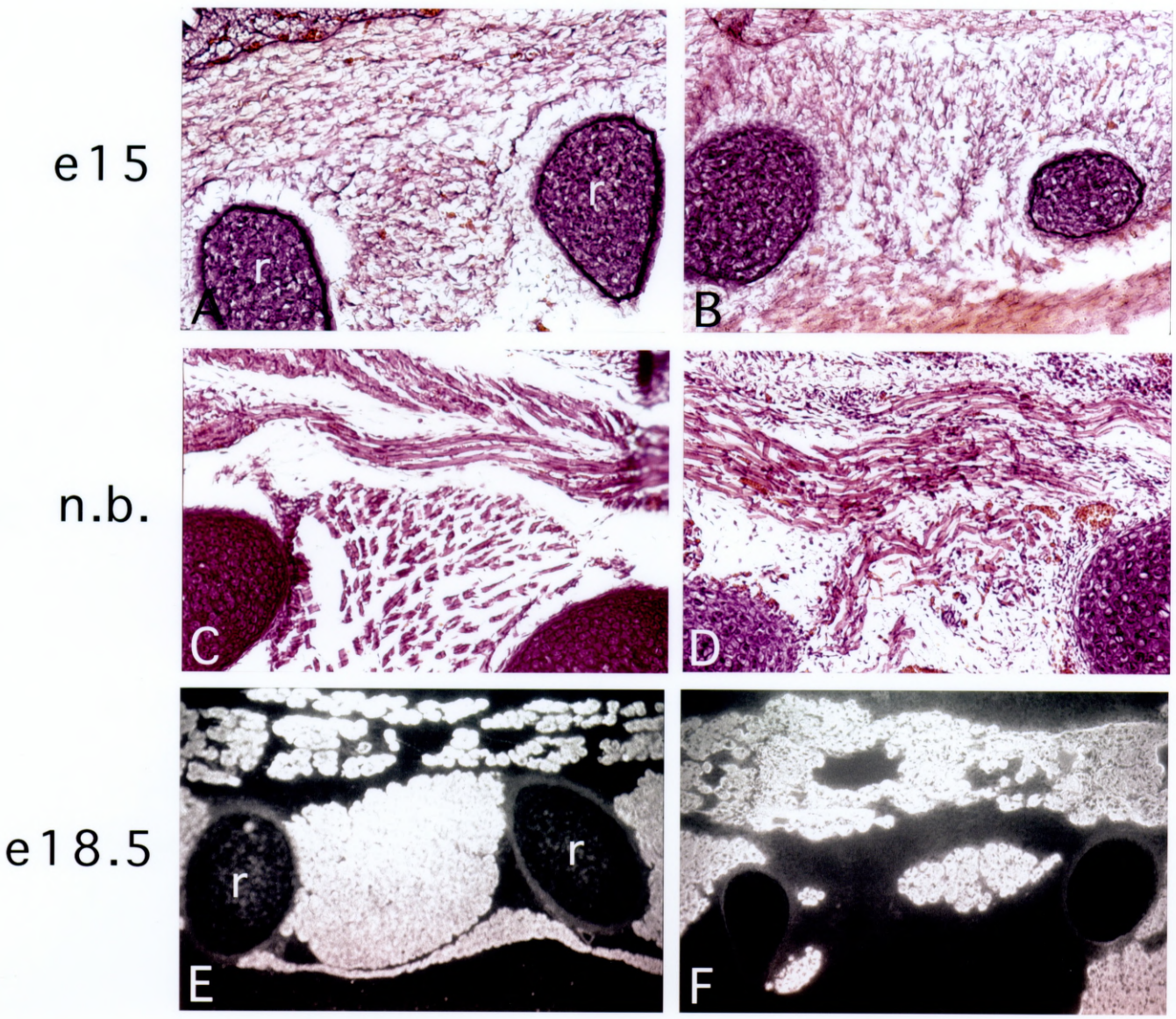

+/+
-/-
e15
n.b.
e18.5
r
r
A
B
C
D
r
r
E
F

Figure 7. Skeletal defects in *MRF4*$^{bh1/bh1}$ mice.

Skeletons of wildtype and *MRF4*$^{bh1/bh1}$ mice were stained with alizirin red S and alcian blue. (A and B) Ventral view of thoracic region of wildtype (A) and *MRF4*$^{bh1/bh1}$ (B) newborn mice. While the first seven ribs are attached to the sternum in wildtype mice, only few ribs are connected in *MRF4*$^{bh1/bh1}$. Anomaly of xiphoid process and severe truncation of first rib is also seen in *MRF4*$^{bh1/bh1}$ mice (arrows). (C and D) Ventral view of lower thoracic vertebrae of newborn mice. Unlike wildtype (C), the 13th rib appears as an anlage in *MRF4*$^{bh1/bh1}$ (D, arrow). (E and F) Lateral view of cervical vertebrae of newborn mice. In *MRF4*$^{bh1/bh1}$ (F), the tuberculum anterior on the 6th cervical vertebra is missing, and a very truncated first rib is observed (arrows). (G-I) Lateral view of thoracic region of newborn *MRF4*$^{bh1/bh1}$ mice. *MRF4*$^{bh1/bh1}$ skeletons show a bifurcation, a fusion, and abnormal angle of rib extension (arrows). (J and K) Lateral view of thoracic region of e14.0 day embryo. Rib defect is already obvious at e14 day of *MRF4*$^{bh1/bh1}$ embryo (K, arrows). Abbreviations are xp, xiphoid process; r, rib; t, thoracic vertebrae; c, cervical vertebrae.

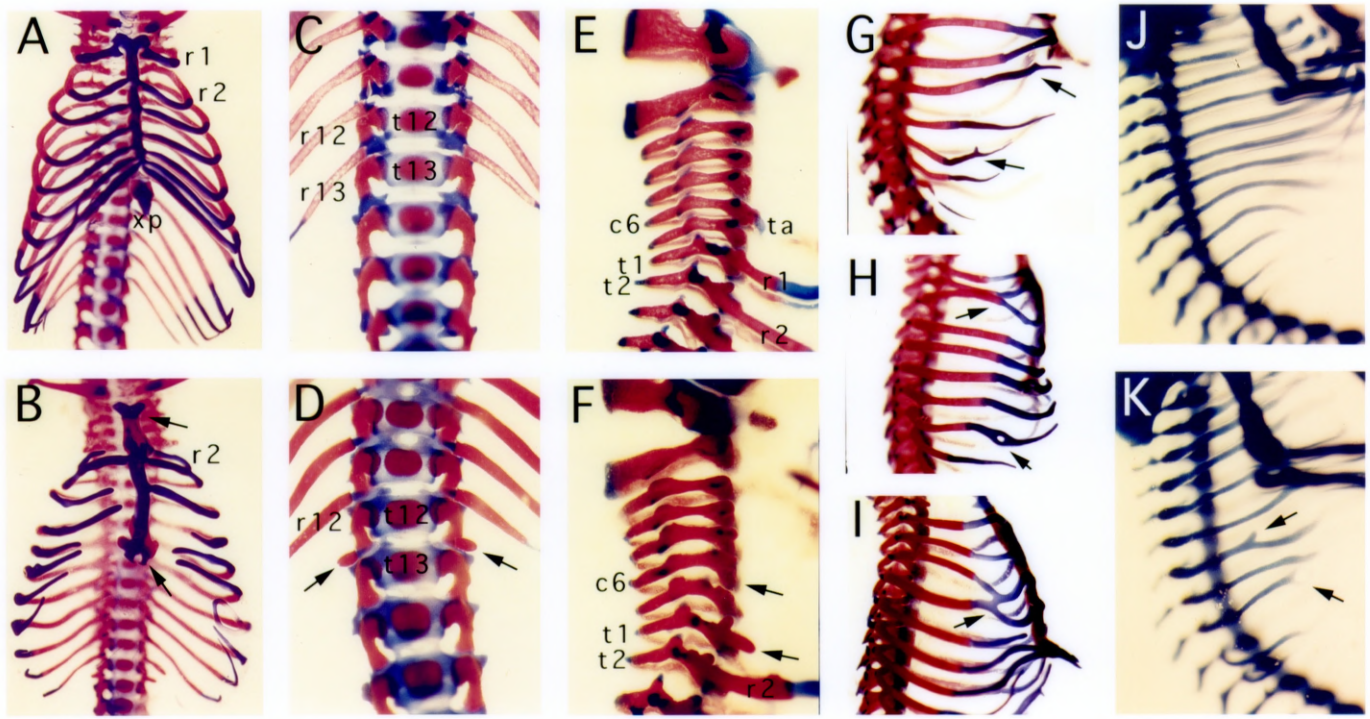
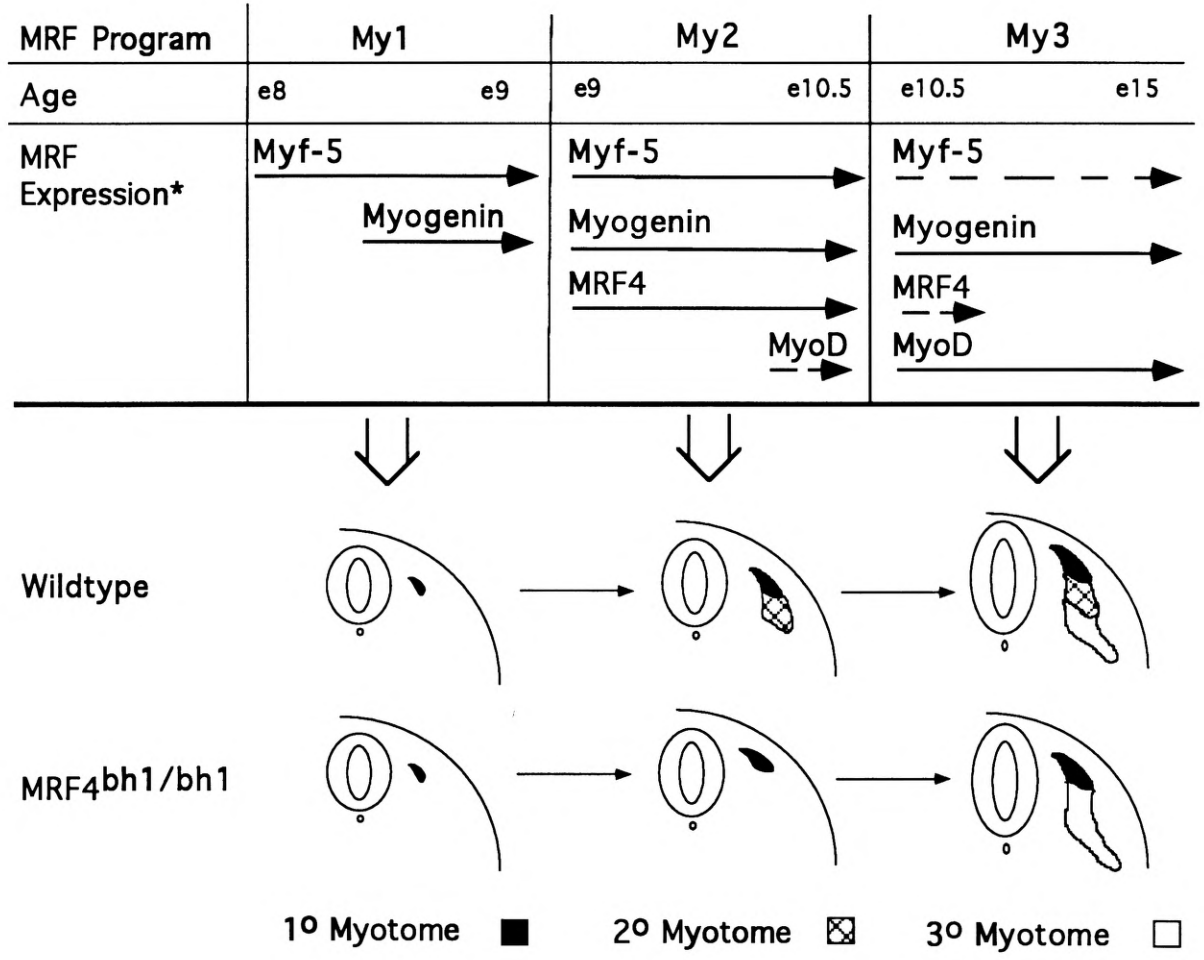

A
r1
r2
xp
B
r2
C
r12
t12
r13
t13
D
r12
t12
t13
E
c6
ta
t1
t2
r1
r2
F
c6
t1
t2
r2
G
H
I
J
K

Figure 8. A summary model is proposed for myotome formation in the mouse and the role of MRF regulators in the process. It is based on the molecular and histological phenotypes of the various MRF knockouts and on the expression patterns of the MRFs according to previous in-situ hybridizations studies and immunohistochemical staining (reviewed, Buckingham, 1992; Smith *et al.*, 1994). See the discussion for details and explanation.

Model for MRF Regulatory Programs in Myotome Biogenesis



* MRF expression and myotomal domains represent forelimb level somites

Chapter 3

Evidence for Developmentally Programmed Transdifferentiation of Smooth Muscle to Skeletal Muscle in Mouse Esophagus.

Submitted to *Science*, July 1995

Evidence for Developmentally Programmed Transdifferentiation of Smooth Muscle to Skeletal Muscle in the Mouse Esophagus

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Abstract

Transdifferentiation is a relatively rare phenomenon in which cells of one differentiated type and function switch to a second discrete identity. Smooth muscle and skeletal muscle are distinct tissues that arise in vertebrate embryos via separate developmental pathways. We found that the musculature of the mouse esophagus is exceptional because it begins as smooth muscle in the fetus but later undergoes a conversion to skeletal muscle during early postnatal development. Surprisingly, the switch from smooth to skeletal muscle features the transitory appearance of individual cells expressing both phenotypes, suggesting that this conversion occurs by programmed transdifferentiation.

Text

Skeletal and smooth muscles of vertebrates are distinct tissues that differ with respect to structure, innervation, and function (*1*). For example, at the cellular level, skeletal muscle is composed of fused multinucleate myotubes containing striated fibers, while smooth muscle is composed entirely of mononucleate, nonstriated cells. At the molecular level, these differences arise from selective expression of genes specific for each muscle type. For example, the MyoD family of myogenic regulatory factors (MRFs: MyoD, myogenin, Myf-5 and MRF4) are transcription factors required for the formation and proper differentiation of skeletal muscle in vertebrates and their expression is specific to the skeletal muscle lineage (*2*).

The musculature of the mammalian stomach and intestine is composed exclusively of smooth muscle type, but the esophagus differs because it also contains skeletal muscle. While the developmental origin of esophageal skeletal muscle has not been studied previously, all known vertebrate skeletal muscles are derived from dorsal prechordal and paraxial mesoderm, and smooth muscles of the gut are thought to arise from lateral splanchnic mesoderm. During embryogenesis, these mesodermal anlagen are separated

from each other long before the first smooth or skeletal muscle cells are specified or differentiated.

By monitoring the expression of genes specific for smooth or skeletal muscle, we found that during early development, mouse esophageal musculature consists of solely differentiated, functional smooth muscle. Subsequently, esophageal expression of smooth muscle genes declines and is replaced by skeletal muscle-specific genes. This transition from smooth to skeletal muscle types occurred in a rostrocaudal progression beginning in late fetal development and continuing through the first two weeks of postnatal development. Antibodies to skeletal fast myosin heavy chain (MHC) and smooth myosin light chain kinase (MLCK) were selected for this study, since these markers were entirely specific for their respective tissue types: skeletal MHC began to be expressed in myotomes at embryonic day 9.5 (E9.5), while smooth MLCK was first detected in visceral smooth muscle starting at E12.5 (*3*, *4*). Transverse sections through diaphragm-level (mid-esophagus) mouse embryos from several developmental timepoints were double labeled with these antibodies. The muscularis layer initially stained for only smooth MLCK at E15 (Fig. 1A) and at P0 (newborn) (Fig 1B), but by postnatal day 3 (P3) it expressed both skeletal and smooth muscle proteins (Fig. 1C). Surprisingly, higher magnification revealed co-expression of both smooth MLCK and skeletal MHC in the cytoplasm of individual cells (Fig. 1D). At P7 (Fig. 1E), smooth MLCK began to be downregulated, and by P14 (Fig. 1F), and adult (*3*), the muscularis layer of esophagus had completely switched to expressing the skeletal muscle marker only. As expected, the muscularis mucosae (the inner thin layer of muscle in the esophagus) expressed only smooth MLCK at all times, while the nearby diaphragm exclusively expressed skeletal MHC. Feeding, which requires esophageal peristalsis, begins immediately following birth; and since only smooth MLCK is expressed at this level in the newborn esophagus muscularis, we conclude that smooth muscle is functionally sufficient for nursing. Smooth muscle myosin

heavy chain, another smooth muscle-specific gene, was also expressed in the esophageal musculature at prenatal timepoints, confirming the smooth muscle phenotype of these cells (*5*) . Using the same antibodies, we also found that the transition from smooth to skeletal muscle in esophagus occurred in a rostrocaudal wave, such that the upper esophagus acquired a skeletal phenotype earlier than the lower esophagus (Fig 1, G and H) (*3*). Expression of another skeletal muscle-specific protein, alpha actinin, showed the same pattern as skeletal MHC for all developmental timepoints (*3*).

The unanticipated co-expression of smooth and skeletal differentiation markers within individual cells was observed from E16 (rostral sections) to P8 (caudal sections) (Fig 1D) (*3*). To confirm the co-expression, P3 esophagus tissue was partially dissociated to yield single-cell smears. This permitted us to visualize single cells and confirmed co-expression of markers within single cells (Fig. 1, I to L). The overall developmental progression from smooth to skeletal tissue types, together with co-expression of smooth and skeletal markers in individual cells, suggests that functional, differentiated smooth muscle cells are switching directly to differentiated skeletal myocytes. The presence of syncytial myofibers in the esophageal dissociation preparations provided additional cytological evidence for the skeletal character of the final muscle phenotype (*3*).

The expression of the MRFs is specific to skeletal muscle and its progenitors, and at least one of the MRFs is expressed prior to transcription of any muscle-specific structural gene (*6*). The pattern and timing of MRF expression in the esophagus is relevant to the phenotypic switch, since the expression of MyoD family regulators mark commitment to skeletal muscle phenotype. At E15 (Fig. 2, A and B) and at birth (*3*), diaphragm-level esophageal musculature did not stain with MyoD or myogenin antibodies, while smooth muscle-specific MLCK was highly expressed. *In situ* hybridization studies also showed that Myf-5 and MRF4 RNA were not expressed in the muscularis layer of diaphragm-level

esophagus at E15 (*3*). Shortly after birth, the diaphragm-level esophageal musculature expressed myogenin (Fig. 2C) and MyoD (*3*) as well as MLCK. Single-cell resolution microscopy showed that nuclear myogenin was co-localized within the same cells that expressed cytoplasmic smooth MLCK (Fig. 2D), and this was verified by examination of dissociated esophagus tissue (*3*). We conclude that expression of smooth muscle differentiation genes precedes the expression of all four MRFs, and this supports the idea that esophagus begins as differentiated smooth muscle before it commits to a skeletal muscle phenotype.

The mixed cell phenotype suggests that the tissue-level conversion from smooth to skeletal muscle occurs by transdifferentiation. However, only a small percentage of cells expressed this mixed phenotype at any given time. While this would be expected if the transition is quite rapid, it also raised an interesting alternative to direct conversion: the switching cells are fated to die or, give rise to a minor population of the mature skeletal muscle, while the main body of skeletal muscle will come from a population of precursor cells that had never assumed a smooth muscle phenotype. Initial attempts to discriminate between these possibilities by marking individual smooth muscle cells of the early esophagus to trace their fate at later times failed due to inaccessibility of the esophagus to surgical manipulation. However, at various rostrocaudal levels and developmental timepoints, apoptosis was assayed by propidium idodide staining. No pronounced cell death was observed in the esophageal tissue. Of the rare pyknotic nuclei found, many were observed in the stratified squamous epithelium, a non-muscle tissue. The few cells in the muscularis layer of esophagus that stained strongly with propidium idodide did not express skeletal MHC or smooth MLCK, arguing against massive cell death in the muscle cells of esophagus at this time (Fig. 3, A and B) (*3*). Furthermore, 5-bromo-2'-deoxyuridine (BrdU) labeling of E18 and P5 animals showed only a moderate level of dividing cells in the muscularis layer of esophagus, and most of the dividing cells were in the smooth muscle population (Fig 3,

C to F) (*3*), arguing against the presence of a major population of skeletal-muscle precursor cells that could repopulate the muscularis layer of esophagus. Taken together, these data suggest a model in which skeletal muscle in esophagus derives mainly from smooth muscle, and are consistent with direct transdifferentiation as the principle mechanism of conversion.

Transdifferentiation is thought to be a relatively rare phenomenon, and most known examples in vertebrates differ in substantial ways from the smooth to skeletal muscle transition in the mouse esophagus (*7*). First, prominent examples such as amphibian limb regeneration and chick retina regeneration occur in response to injury (*8*, *9*), while conversion of neural crest derived adrenal chromaffin cells into sympathetic neurons occurs under experimental manipulations (*10, 11*). In contrast, the esophageal smooth to skeletal muscle conversion is part of the normal developmental program. Other cases typically involve a discrete "dedifferentiation" step in which the initial differentiated phenotype is downregulated, a relatively undifferentiated intermediate is established, and finally differentiation to an alternative "terminal" phenotype is executed. In this work, we observed coincident expression of genes that define the two terminal phenotypes involved, suggesting a direct switch without passage through an intermediate. However, the transdifferentiation in esophagus is not the only example of a direct phenotypic switch during unperturbed vertebrate development; Sympathetic neurons that innervate sweat glands undergo a perinatal change in neurotransmitter phenotype from noradrenergic to cholinergic (*12*).

A major theme in vertebrate development is that differentiated cells of mature, functioning tissues arrive at their respective identities via a series of stepwise changes in cellular phenotype. These changes lead from relatively unspecified progenitor cells of wide developmental potential to increasingly specified cells of limited potential, a pathway that

reflects sequential changes in the repertoire of genes expressed at each step. The perceived universality of such developmental progressions makes an apparent "jump" from the end product of one pathway directly to the end product of a different pathway a surprising phenomenon, and immediately raises questions about the underlying mechanism. The regulatory properties of the MyoD family skeletal muscle regulators (MRFs) suggest a candidate mechanism. Experimentally induced ectopic expression of the MRFs can dominantly co-opt various terminally differentiated cells (including primary smooth muscle cells in culture) to a skeletal muscle phenotype (*13*, *14*, *15*, *16*). In the esophageal muscle pathway, evolution may have capitalized on the potential of MRFs to drive a direct transition to skeletal muscle. And because smooth and skeletal muscle share a general contractile function, a direct shift from one to the other that preserves function during the transition might be an attractive option. In support of this idea, there is a second candidate case for a muscle conversion in the avian eye. In mammals, the iris muscles of the eye are entirely smooth in type, but in the chick they are of skeletal type and there is evidence that they express smooth muscle specific genes as a transition state to a mature skeletal muscle. This transition occurs before birth and eye function, and it is not clear whether the starting point is functional smooth muscle (*17*). There is presently no information on MRF expression in the chick iris muscle, but if the process is similar to that in the esophagus, we would expect the MRFs to be involved.

This initial characterization of a smooth to skeletal transition and its relationship to current knowledge of molecular and developmental determinants of myogenesis leaves many questions unanswered. While activation of MyoD family regulators may drive the transition, it is unclear what triggers them to be expressed or what events lead to the down-regulation of smooth muscle genes are unclear. Similarly, the origin and nature of the inductive interactions that regulate the slow rostrocaudal wave of skeletal differentiation are unknown, though one attractive explanation would be an initial induction, followed by new

conversion of more caudal cells by contact with more rostral skeletal muscle. The origin of muscle cells in the esophagus has previously received little attention, but in light of these findings, lineage studies to probe contributions to the esophagus from somitic versus lateral mesoderm should be of interest.

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18. 7-20μm frozen sections of embryos, or dissociated esophageal cells attached to polylysine treated slides, were fixed in 4% paraformaldehyde, blocked for 10-20 minutes in 10% goat serum and 3% BSA in PBS before applying the primary antibody in 3% BSA

in PBS for 1-3 hours at room temperature. The secondary antibody solution was applied for 1 hour. Antibodies against myogenin (F5D; 1:5 dilution; mouse IgG1; courtesy of W. Wright), MyoD (NCL-MyoD1; 1:10 dilution; mouse IgG1; Novocastra Lab. Ltd.), skeletal fast myosin heavy chain (M-4276; 1:400 dilution; mouse IgG1; Sigma), alpha actinin (A-7811; 1:400 dilution; mouse IgG1; Sigma), smooth-specific myosin light chain kinase (M-7905; 1:1000 dilution; mouse IgG2b; Sigma), and BrdU (MAS 250; 1:10 dilution; rat IgG2a; Sera-Lab) were used. Secondary antibodies against mouse IgG1, IgG2b or rat IgG were conjugated to Fluorescein (FITC) or Rhodamine (TRITC) and used as 1:100 dilution in 3% BSA in PBS (Southern Biotechnology Associates). When BrdU antibodies were used, the sections were treated for 15 minutes in 4M HCl before the blocking step. Images were captured digitally on a Biorad confocal microscope and figures were made using Adobe Photoshop software.

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Fig. 1. Developmental and rostrocaudal progression from smooth to skeletal muscle in muscularis layer of mouse esophagus. Confocal microscope images of esophageal tissue sections and dissociated cells labeled with antibodies to smooth-muscle myosin light chain kinase (MLCK) shown in green and skeletal-muscle fast myosin heavy chain (MHC) shown in red (*18*). Co-localization of the two proteins appears as yellow. (**A** to **F**) Diaphragm-level (middle) sections of embryonic day 15 (E15) to postnatal day 14 (P14) esophagus. (**D**) is a higher magnification of the image shown in (**C**); the arrow points to a cell coexpressing both markers. (**G** and **H**) Upper and lower esophagus sections of P3 animals, compare to middle section in panel (**C**). (**I**) Phase image of a group of P3 dissociated esophageal cells, and (**J** to **L**) fluorescent images of this field of cells that show expression of the designated marker(s). Arrows in (**I** to **L**) point to a cell that is expressing both skeletal and smooth muscle marker. Dissociation experiments were carried out by gentle mechanical disruption of tissue as well as a collagenase and dispase treatment for 15' at 37^{o}C. The objective of this treatment was to dissociate the cells to a level that insures observing single-cell populations but at the same time to avoid massive cell death due to excessive insult. Abbreviations: d, diaphragm; e, esophagus; m.m., muscularis mucosae; s, stomach; NB, newborn. The size bars in (**A** to **C,** and **E** to **H**) represent 100μm, while in (**D**, and **I** to **L**) they represent 5μm.

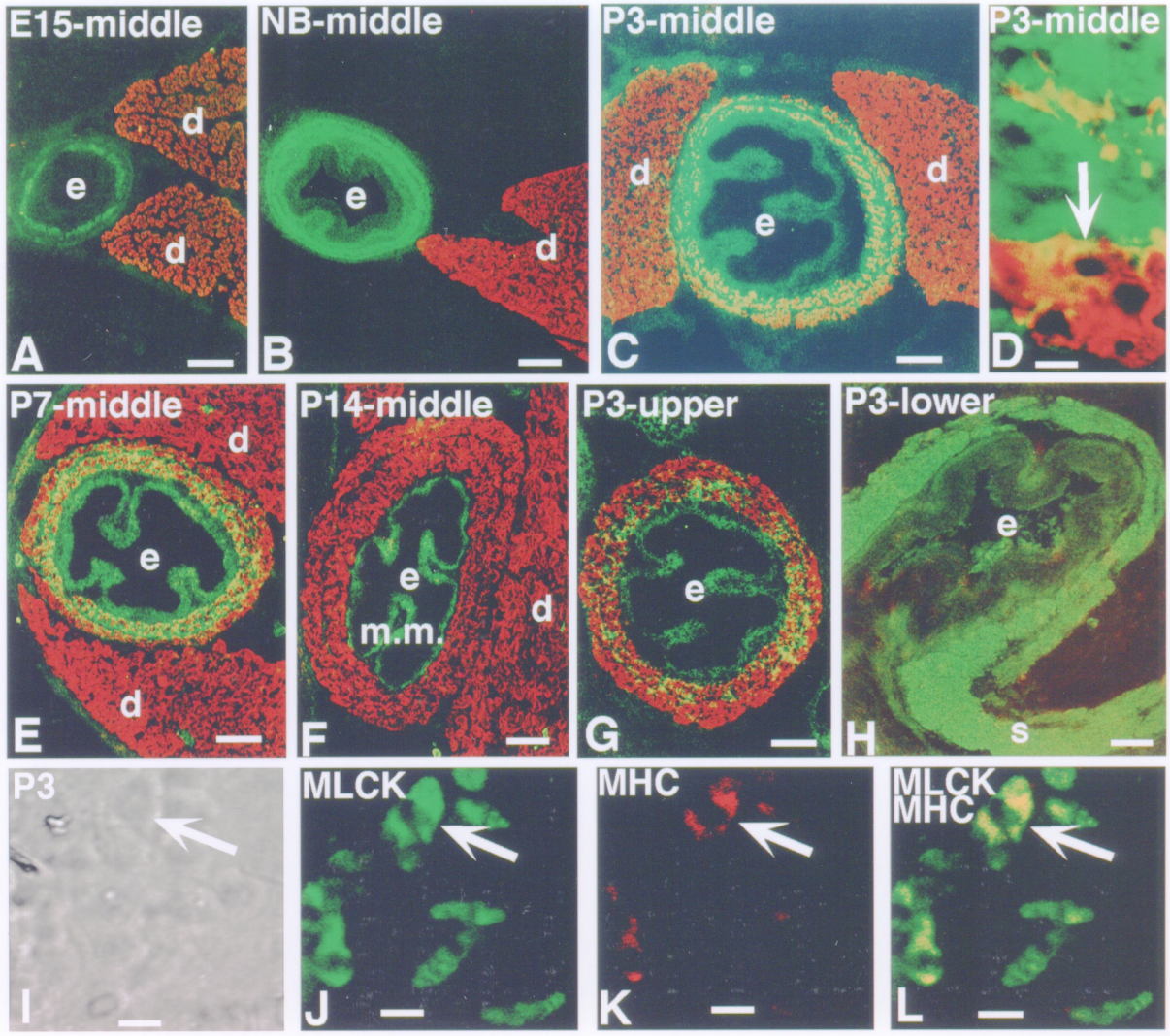

E15-middle
d
e
d
A
NB-middle
e
d
B
P3-middle
d
e
d
C
P3-middle
D
P7-middle
d
e
d
E
P14-middle
e
m.m.
d
F
P3-upper
e
G
P3-lower
e
s
H
P3
I
MLCK
J
MHC
K
MLCK
MHC
L

Fig. 2. Expression of smooth muscle-specific marker precedes expression of the myogenic regulatory factors (MRFs) during development of esophagus muscularis tissue. Transverse sections of E15 and P3 embryos through diaphragm-level (middle) esophagus are colabeled with myogenin and smooth MLCK (**A**, **C** and **D**) or MyoD and smooth MLCK (**B**). (**D**) is a higher magnification of (**C**) and shows cells that co-express nuclear myogenin and cytoplasmic MLCK (arrows). Abbreviations: d, diaphragm, e, esophagus. The size bars represent 50μm.

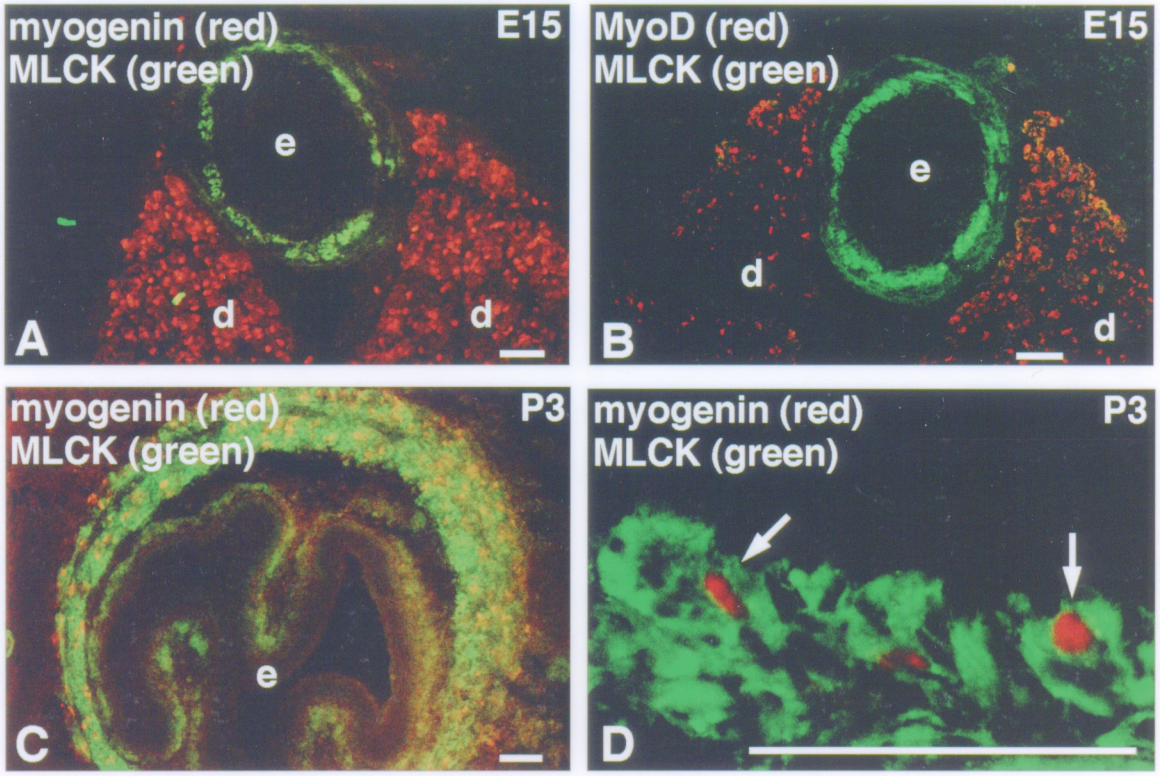
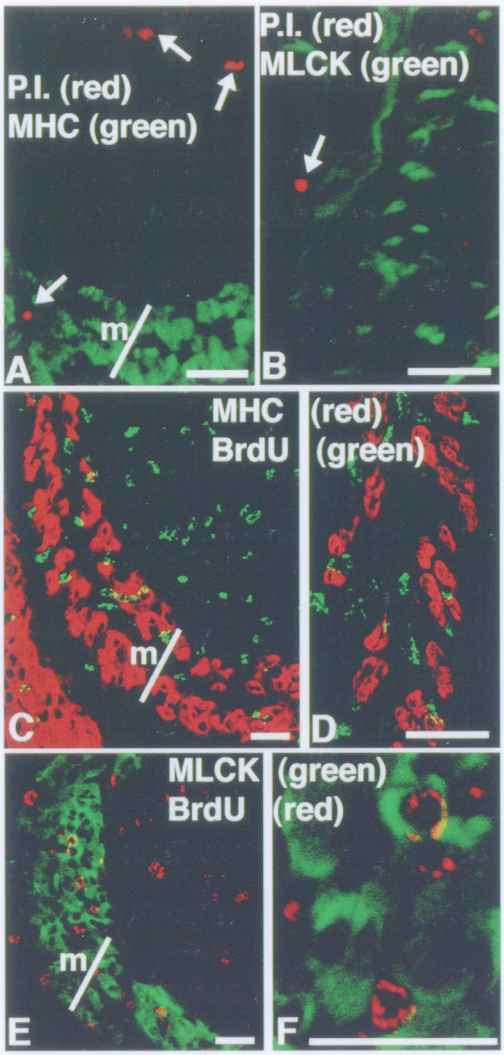

myogenin (red)
MLCK (green)
E15
e
d
d
A
MyoD (red)
MLCK (green)
E15
e
d
B
d
myogenin (red)
MLCK (green)
P3
e
C
myogenin (red)
MLCK (green)
P3
D

Fig. 3. The state of cell death and cell proliferation during perinatal development of mouse esophagus. (**A** and **B**) Diaphragm-level (middle) esophagus sections through a P6 embryo were colabeled with propidium iodide (P.I.) and skeletal MHC (**A**) or smooth MLCK (**B**). Pyknotic nuclei in propidium iodide labeled sections were readily recognized by fluorescence microscopy: they were more brightly stained than normal nuclei, and are marked here with arrows. (**A**) The two top arrows show pyknotic nuclei in the non-muscle layer of squamous epithelium of esophagus; the arrow at the bottom points to an apoptosing cell in the muscularis layer (m) that does not stain for skeletal MHC. (**B**) The pyknotic cell (arrow) in the muscularis layer does not express smooth MLCK. Propidium iodide staining was achieved as described previously (19), and visualized with TRITC optics. In brief, tissues were fixed in 4% paraformaldehyde, sectioned, stained for muscle-specific antibodies, and then incubated with 4µg of propidium iodide (Sigma) and 100µg/ml RNase (Sigma; DNase free) in PBS for 30 minutes at 37°C. (**C** to **F**) Upper esophagus sections from E18 embryos in-vivo labeled with 5-bromo-2'-deoxy-uridine (BrdU) were costained with BrdU antibody together with skeletal MHC (**C** and **D**) or smooth MLCK (**E** and **F**) antibodies. (**D**) and (**F**) are higher magnifications of the images shown in (**C**) and (**F**), respectively. BrdU labeling was achieved by injecting a female at E18 gestation with 20µl of 10mM BrdU per gram of body weight (Boehringer Mannheim, #1299964). After 4 hours, the animal was sacrificed, and the embryos were frozen and sectioned. The size bars represent 25µm.


P.I. (red)
MHC (green)
m
A
P.I. (red)
MLCK (green)
B
MHC
BrdU
m
C
(red)
(green)
D
MLCK
BrdU
m
E
(green)
(red)
F

Chapter 4

Isolated Sequences from the Linked *Myf-5* and *MRF4* Genes Drive Distinct Patterns of Muscle-Specific Expression in Transgenic Mice

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Isolated Sequences from the linked *Myf-5* and *MRF4* Genes Drive Distinct Patterns of Muscle-Specific Expression in Transgenic Mice

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Key words: Myogenesis, Myf-5, MRF4, transgenic mice,
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SUMMARY

In developing mouse embryos *MyoD* family regulatory genes are expressed specifically in muscle precursors and mature myofibers. This pattern, taken together with the well-established ability of *MyoD* family members to convert a variety of cell types to skeletal muscle, suggests a significant role for these genes in regulating skeletal myogenesis. The possibility that expression of these genes may be causally associated with segregation of the myogenic lineage from other mesodermal derivatives, or with the subsequent maintenance of muscle phenotypes at later times, raises the issue of how *MyoD* family genes are themselves regulated during development. In this work, we have initiated studies to identify DNA sequences that govern *Myf-5* and *MRF4* (*herculin*, *myf-6*) transcription. Myf-5 is the first of the MyoD family to be expressed in the developing mouse embryo, while MRF4 is the most abundantly expressed myogenic factor in postnatal animals. In spite of their strikingly divergent patterns of expression, *Myf-5* and *MRF4* are tightly linked in the mouse genome; their translational start codons are only 8.5 kilobases apart. Here, the 5' flanking regions of the mouse *Myf-5* and *MRF4* genes were separately linked to a bacterial *β-galactosidase* (*lacZ*) gene, and these constructs were each used to produce several lines of transgenic mice. Transgene expression was monitored by X-gal staining of whole embryos and by in situ hybridization of embryo sections. For the Myf-5/LacZ lines, the most intense transgene expression was in the visceral arches and their craniofacial muscle derivatives, beginning at day 8.75 post coitum (p.c.). This correlates with endogenous Myf-5 expression in visceral arches. However, while Myf-5 is also expressed in somites starting at day 8 p.c., transgene expression in the trunk is not observed until day 12p.c. Thus, the Myf-5/Lacz construct responds to early *Myf-5* activators in the visceral arches but not in the somites, suggesting that myogenic determination in the nonsmoitic head mesoderm may be under separate control from that of the somitic trunk mesoderm. MRF4/LacZ lines displayed an entirely different pattern from Myf-5. Transgene expression appeared in muscles starting at day 16.5 p.c. and became increasingly

prominent at later times. However, an early wave of myotomal expression that is characteristic of the endogenous MRF4 was not recapitulated by the transgene.

INTRODUCTION

Skeletal muscle is one of many derivatives of mesoderm in vertebrates. Muscles of the head originate from prechordal and paraxial mesodermal cells (Noden, 1991; Couly et al., 1992), most of which populate the visceral arches and then migrate into the developing head. Muscles of the trunk and limbs arise from the somites, which are segmental blocks of paraxial mesoderm that form in pairs on either side of the neural tube (Lyons and Buckingham, 1992; Ordahl and Le Douarin, 1992).

An important contribution to the current view of skeletal myogenesis came from the cloning of *MyoD* (Davis et al., 1987; Tapscott et al., 1988) and its three close relatives, *myogenin* (Wright et al., 1989; Edmondson and Olson, 1989), *Myf-5* (Braun et al., 1989), and *MRF4/herculin/Myf-6* (Rhodes and Konieczny, 1989; Miner and Wold, 1990; Braun et al., 1990). These genes encode transcription factors of the basic-helix-loop-helix (B-HLH) family (Murre et al., 1989) that bind in vitro to consensus "E-box" recognition sites. These sites are prominent and functionally significant in many genes expressed specifically in differentiated muscle (Murre et al, 1989; Weintraub et al, 1991). Forced expression of any of the *MyoD* family genes in a variety of nonmuscle cultured cells can convert them to a skeletal muscle phenotype, suggesting that these regulators play a significant role in determination and differentiation of skeletal muscle (Olson, 1990; Weintraub et al., 1991). The ability of ectopic MyoD and/or Myf-5 to activate skeletal muscle-specific genes in developing *Xenopus* embryos (Hopwood and Gurdon, 1990; Hopwood et al., 1991) and the hearts of transgenic mice (Miner et al., 1992) supports this view.

Analysis of the expression of these myogenic regulatory genes in several cultured skeletal muscle cell lines has revealed that proliferating myoblasts, which are determined to form muscle, express MyoD (MM14 [Mueller and Wold, 1989]), Myf-5 (L6, BC3H1 [Braun et al., 1989; Mueller and Wold, 1989]), or both (C2C12 [Braun et al, 1989; Miner and Wold, 1990]), while their differentiated conterparts express myogenin always

(Emerson, 1990) and MRF4 sometimes (L6J1-C, C2C12 [Rhodes and Konieczny, 1989; Miner and Wold, 1990]). However, it is not at all clear how these few established cell lines are related to muscle and its progenitors in the animal. In situ hybridization experiments on mouse embryo sections have shown that there is a complex pattern of sequential accumulation and disappearance of MyoD family RNAs in developing muscle, and that the pattern varies among different skeletal muscle groups (for review see Buckingham, 1992). Myf-5, the earliest marker of muscle, is first detected at 8 days p.c. in anterior somites, just before the myotome can be recognized as distinct from sclerotome and dermotome by cytological criteria (Ott et al., 1991). Similar expression is sequentially observed in more posterior somites as these structures form in their characteristic rostral-caudal sequence. Myf-5 RNA is subsequently detected at day 9.25 p.c. in the hyoid arch and is followed at day 10 p.c. in the mandibular arch and at day 10.5 p.c. in the forelimb bud. Myogenin and MyoD are expressed in all muscle masses following *Myf-5* activation. In contrast, MRF4 is never detected in visceral arches or limb buds by in situ hybridization. However, a wave of transient MRF4 RNA expression is observed in myotomes between days 9 and 11.5 p.c. (Bober et al., 1991; Hinterberger et al., 1991). Late in development, Myf-5 expression declines and becomes undetectable by day 14 p.c. and remains that way (Ott et al., 1991), while MRF4 reappears at day 16 p.c. in all fetal skeletal muscles and becomes the predominant *MyoD* family regulatory gene expressed in the adult (Rhodes and Konieczny, 1989; Miner and Wold, 1990; Bober et al., 1991; Hinterberger et al., 1991).

The basis for the complex and stringently controlled pattern of differential expression of the MyoD family of regulators is not well understood. Studies of genomic regulatory elements have mainly concentrated on the *myogenin* and *MyoD* genes. Remarkably, only 200 base pairs of proximal 5' flanking sequence from the *myogenin* gene were sufficient to direct myocyte specific expression (Salminen et al., 1991). The emerging picture for human *MyoD* is quite different. Its 5' flank has been tested for

regulatory activity in both cell culture and in transgenic mice. Experiments in cultured cells led to the identification of a region positioned between 18 and 22 kb upstream of the coding region which enhanced transcription from the proximal *MyoD* promoter in 23A2 myoblasts but also, surprisingly, in their nonmyogenic parental cell line C3H 10T1/2, where endogenous MyoD is not normally expressed. In spite of the apparently inappropriate expression in nonmyogenic cultured cells, this enhancer element drove expression in a muscle-restricted pattern in transgenic mice (Goldhamer et al., 1992).

In contrast to *MyoD* and *myogenin*, little is presently known about the regulation of *MRF4* and *Myf-5*. Myf-5 transcripts appear before any of the other MyoD family RNAs (Ott et al, 1991). MRF4 is most notably expressed in late fetal and postnatal muscle where it quantitatively predominates over the other MyoD family transcripts (Miner and Wold, 1990), suggesting a role for *MRF4* in maintenance of differentiated muscle. Furthermore, the close physical linkage of *Myf-5* and *MRF4*, presumably the result of an ancient gene duplication, presents an interesting problem in the evolution, organization and utilization of regulatory elements. The two genes share a stringent specificity for expression in skeletal muscle, but show highly disparate developmental regulation. In preliminary experiments we found expression of reporter genes carrying *Myf-5* and *MRF4* flanking sequences in cultured cell lines to be minimal. To overcome this assay limitation and to gain access to the full developmental diversity of MRF4 and Myf-5 expressing cells in the animal, we have produced transgenic. These experiments have allowed us to identify sequence elements from *Myf-5* and *MRF4* that specify expression in their distinct, spatially and temporally restricted patterns.

MATERIALS AND METHODS

Construction of the transgenes

5.5 kb of the 5' flanking region of the mouse *Myf-5* gene were isolated as a *Bam*HI to *Sac*I fragment from the original mouse *Myf-5/herculin* phage described previously (Miner and Wold, 1990). This fragment contains the *Myf-5* proximal promoter and putative transcription start site (Ott et al., 1991). A bacterial *ß-galactosidase* gene containing an introduced consensus Kozak translation initiation sequence was placed downstream of this in Bluescript II KS+ (Stratagene) to make MYF5Z.

The original *Myf-5/herculin* phage contained only about 350 base pairs of *herculin* (*MRF4*) 5' flanking DNA. Genomic Southern blots (J. M., unpublished) had indicated the existence of another *Bam*HI site approximately 10 kb upstream of the previously identified site (Miner and Wold, 1990), or 6.5 kb upstream of the MRF4 coding region. We cloned this 10 kb *Bam*HI fragment by constructing a phage lambda library from ~9-11 kb *Bam*HI fragments of mouse genomic DNA (electroeluted from an agarose gel) using the vector lambda gem-12 (Promega). The library was probed with the *MRF4* transcribed region, and the insert of a positive phage was subcloned into Bluescript II KS+. Restriction analysis showed that this phage contained the MRF4 coding region as well as 6.5 kb of 5' flanking DNA, as expected. To be sure of including the *MRF4* transcription start site in the *lac*Z construction, we used the ~6.5 kb *Bam*HI to *Sal*I fragment as a foundation and then ligated 52 base pairs of additional contiguous sequence, synthesized as two complementary oligonucleotides by the Caltech Microchemical Facility. This sequence was added because preliminary primer extension assays (J. M., unpublished) had indicated that it should contain the major transcription start site. Therefore, the 3' end of the mouse *MRF4* sequences used here is nucleotide 64 of the reported *herculin* genomic sequence (Miner and Wold, 1990).

Preparation of DNA and production of transgenic mice

MYF5Z and MRF4Z plasmids were purified by cesium chloride density gradient ultracentrifugation. To liberate the constructs from vector sequences, MYF5Z was cut with *Kpn*I, *Bam*HI, and *Sca*I, and MRF4Z was cut with *Bam*HI and *Xho*I. To isolate the fragments for microinjection, the restriction digests were loaded onto 10-40% preformed sucrose gradients and ultracentrifuged in a SW41 rotor at 26,000 RPM for 24 hours (Maniatis et al., 1982). 300 µL fractions were collected and analyzed by agarose gel electrophoresis. DNA was ethanol-precipitated out of the appropriate fractions and dissolved in 10 mM Tris (pH=7.5), 0.1 mM EDTA (pH=8). Transgenic mice were produced by pronuclear microinjection of single cell mouse embryos from a (C57BL/6XDBA/2)F1X(C57BL/6XDBA/2)F1 cross as described (Hogan et al., 1986; Miner et al., 1992).

Analysis of transgenic mice

Transgenic founder mice were identified by Southern blot or polymerase chain reaction analysis of tail DNA. Male founders and male offspring of female founders were mated in most cases with C57BL/6 X DBA/2 hybrid females, though sometimes the parental inbred strains were used. For postnatal analyses, pups of various ages were sacrificed, skinned, fixed in 4% formaldehyde in phosphate buffered saline (PBS), and stained in PBS containing 35 mM potassium ferricyanide, 35 mM potassium ferrocyanide, 1.5 mM magnesium sulfate, and 1 mg/ml X-gal (US Biochemicals Corp.) overnight at 37°C. For prenatal analyses, timed pregnant females were sacrificed at the desired day of gestation, and embryos were dissected out of the uterine horns into PBS. They were fixed and stained as above, though for older embryos the staining solution was supplemented to contain 0.2% sodium deoxycholate and 0.1% Nonidet P-40 to enhance X-gal penetration.

In situ Hybridization

In situ hybridization was performed on 5 to 7-µm paraffin sections. The procedures used for section treatment, hybridization, and washings are described by Lyons et al. (1990). Hybridizations were carried out at 50°C for ~16hr in 50% deionized formamide, 0.3M

NaCl, 20mM Tris-HCl (pH 7.4), 5mM EDTA, 10mM sodium phosphate (pH 8), 10% dextran sulfate, 1X Denhardt's solution, 50µg/ml of yeast RNA, with 50-75,000 cpm/µl of cRNA labeled with ^{35}S-labeled UTP (>1000 Ci/mmole, Amersham). Washing was at 65°C in 50% formamide, 2XSSC, and 10mM DTT. Slides were then treated with RNase A (20µg/ml) (Boehringer Mannheim) for 30 min at 37°C. After washes, slides were processed for standard autoradiography with Kodak NTB-2 nuclear track emulsion and exposed for 7 days. Analysis was carried out with both light- and dark-field optics on a Zeiss Axiophot microscope.

For the Myf-5 probe, a 310-bp *Bal*I-*Apa*I fragment of the first exon of the mouse *Myf-5* gene was used as described by Ott et al. (1991). For the MRF4 probe, the 680-bp 3' *Pst*I fragment of the mouse gene was used as described by Bober et al. (1991). For the lacZ probe, the 3kb *Bss*HII fragment of the MRF4Z construct, which also contains the SV40 polyadenylation signal sequence, was used.

RESULTS

Production of transgenic mice

5.5 kb of *Myf-5* and 6.5 kb of *MRF4* 5' flanking regions were each linked to the bacterial *ß-galactosidase* gene. These constructs (Fig. 1) are called MYF5Z and MRF4Z, respectively. Multiple transgenic lines were produced by pronuclear microinjection. All four DNA-positive lines produced with the MYF5Z construct expressed the transgene; two of the four lines, MYF5Z-21 and 29, showed the most intense lacZ staining and hence were more comprehensively characterized. The two other lines, MYF5Z-9 and MYF5Z-46, showed an overlapping but reduced expression pattern. Seven MRF4Z transgenic lines were produced: three (MRF4Z-28, 45 and 49) expressed the transgene in a muscle-specific manner, three did not express the reporter gene at all, and a single line, called MRF4Z-4, exhibited ectopic expression in several diverse tissues characteristic of strong, site-of-insertion postition effects (Allen et al; Gossler et al., 1989).

MYF5Z trangenic mice

To survey developmental expression patterns of the transgene in detail, we used histochemical staining for ß-galactosidase beginning at day 8.75 p.c. (E8.75) and continuing through birth. This assay is highly sensitive, and parallel controls showed all staining, except in the gut of postnatal pups, to be specified by the activity of the transgene. The E8.75 (15 somites) embryos examined from MYF5Z-21 and 29, two of the best-expressing lines, contained lacZ-positive cells in the hyoid and mandibular arches but not in somites (Fig. 2A). Since prior in situ hybridizations had shown that Myf-5 transcripts begin to accumulate in somites at E8.0 and in visceral arches at E9.25 (Ott et al., 1991), it appears that this construct contains sequences sufficient to specify the early visceral arch expression, but insufficient to drive early somitic expression. As development proceeded, both the number of cells expressing lacZ and the intensity of expression increased. At E10.5, the hyoid arch exhibited very intense expression, and lacZ-positive cells were

visible in the developing ocular muscles (Fig. 2B). At E12, myotomal staining became detectable, then increased in intensity at E12.5 (Fig. 2C). Also at E12.5, staining was visible in the developing muscles of the head which are derived from the visceral arches and in the muscle masses of the proximal forelimb. When myotomal expression of the transgene becomes detectable, it already encompasses the full length of the embryo (Fig 2C,D), so the anterior-posterior sequence of endogenous *Myf-5* activation that corresponds to early myotome segregation is not recapitulated by the transgene. Transgene expression was most prominent at E13.5 with the appearance of lacZ-positive cells in the intercostal muscles and developing abdominal muscles (Fig. 2D). Beginning at day 14, when endogenous Myf-5 RNA levels begin to decrease (Ott et al., 1991), muscle-specific lacZ expression declined and became undetectable shortly after birth (data not shown). The expression patterns described were present in at least two different transgenic lines (Fig. 2D,E), confirming that the transgene expression in these tissues resulted from regulatory elements present in the MYF5Z construct, not from site-of-integration position effects. This lacZ pattern reflects a substantial subset of the endogenous Myf-5 expression pattern as described previously by in situ hybridization (Ott et al., 1991).

To compare endogenous and transgene expression directly, and to verify the sensitivity of the whole mount embryo X-gal staining assay, E11.5 and E12.5 embryos from MYF5Z-21 and MYF5Z-29 transgenic lines were sectioned, and in situ hybridization experiments with Myf-5 and lacZ probes were performed on adjacent sections. The lacZ expression pattern observed in these experiments was identical to the whole-embryo X-gal staining pattern. In most cases Myf-5 and lacZ RNA distribution was similar, with lacZ representing a subset of the endogenous Myf-5 expression. At E11.5 (Fig. 3A,B), head muscles were positive with both Myf-5 and lacZ probes, while somitic expression was observed only with the Myf-5 probe. At E12.5 (Fig. 3 C,D), trunk muscles as well as the proximal forelimb muscles expressed the transgene.

In individual MYF5Z transgenic lines, lacZ was expressed ectopically in a few non-skeletal muscle tissues (Fig. 2B-D, 3B,D), as observed by both whole-mount staining of embryos with X-gal and in situ hybridization experiments. Since these expression patterns were each characteristic of one line only, and not shared by any of the other three transgenic lines (Fig. 2D,E), we suggest that the observed ectopic expression is probably due to enhancer elements trapped near the insertion site of the construct (Lacy et al., 1983; Kothary et al., 1988; Al-Shawi et al., 1990).

MRF4Z transgenic mice

Three MRF4Z lines expressed the lacZ transgene in multiple muscle groups beginning at E16 (data not shown). Expression of the transgene increased throughout the perinatal period (Fig. 4) in parallel with the observed pattern of endogenous MRF4 expression (Bober et al., 1991; Hinterberger et al., 1991). However, MRF4Z transgenic mice did not express the transgene transiently in embryonic myotomes as the endogenous *MRF4* gene is expressed.

Some muscles expressed the MRF4Z transgene more intensely than others. For example, muscles of the limbs showed faint lacZ staining (Fig. 4A), whereas the spinodeltoideus muscle (Fig. 4A), levator auris longus muscle (Fig. 4B), and intervetebral muscles (Fig. 4C) expressed lacZ particularly strongly. To compare the spatial distribution of the endogenous MRF4 and lacZ transcripts, abdominal muscles were dissected and in situ hybridization reactions were performed with MRF4 and lacZ probes on adjacent sections (Fig. 5). Both MRF4 and lacZ were positive in all muscle fibers, but showed no activity in the nearby connective tissue.

Because three of four independent transgenic MRF4Z lines exhibited the same general patterns of lacZ expression, and because this pattern parallels that of endogenous MRF4, we attribute this expression to regulatory sequences present in the transgene. However, one MRF4Z line, MRF4Z-4, expressed lacZ in a different and unexpected fashion (Fig. 6). With the exception of the spinotrapezius muscle, which expressed

intensely starting at E16, MRF4Z-4 transgene expression did not correlate with the endogenous MRF4 pattern (Bober et al., 1991; Hinterberger et al., 1991) or with the other MRF4Z lines. Instead, this line expressed lacZ in a variety of other tissues at different developmental time periods including vibrissae (Fig. 6A), hand pads, olfactory bulbs, knee and elbow joints (Fig. 6A), and mid-brain. This diversity suggests a dominant position effect coupled, perhaps, with damage to one or more copies of the transgene.

DISCUSSION

The MyoD family of regulators have been inferred to play a significant role in skeletal myogenesis. Three lines of evidence support this idea. First, all four MyoD family members can recruit diverse cultured nonmuscle cells to a skeletal muscle phenotype (Olson, 1990; Weintraub et al., 1991), and both MyoD and Myf-5 have been shown to be capable of activating skeletal muscle genes ectopically in developing embryos (Hopwood and Gurdon, 1990; Hopwood et al., 1991; Miner et al., 1992). This argues for a direct or indirect role for the MyoD family in the myogenic determination process. Second, molecular studies suggest a direct interaction between MyoD family regulators and transcriptional enhancers of many terminally differentiated muscle-specific genes (Olson, 1990; Weintraub et al., 1991). This is consistent with a direct and ongoing role in execution and maintenance of terminal muscle differentiation. Finally, the MyoD family of transcription factors (most notably Myf-5) are expressed early in premuscle cells (Sassoon et al., 1989; Ott et al., 1991; Bober et al., 1991; Hinterberger et al., 1991), which reinforces a role for these regulators in myogenic determination prior to overt differentiation of muscle. However, little is known about the molecular and cellular processes that govern the segregation of the myogenic lineage from other mesodermal derivatives in the developing mouse embryo. If the activities of these MyoD family regulatory proteins are partly or wholly responsible for establishing the skeletal muscle phenotype, then learning how expression of these regulators is initiated, maintained, and terminated during embryogenesis is crucial for understanding how myogenic cell fate is specified and executed. Apart from the postulated importance of MyoD family products in regulating myogenesis, their complex and diverse patterns of expression during development make them very useful markers for distinct subdivisions of myogenic populations.

Here we have identified DNA sequences from the *Myf-5/MRF4* locus that direct expression of the bacterial *ß-galactosidase* gene in patterns reflecting that of endogenous

Myf-5 and MRF4. The MYF5Z transgenic mice, which carried 5.5kb of upstream Myf-5 sequences, expressed the *lacZ* reporter gene in a large subset of the cells which express endogenous Myf-5 RNA. Myf-5, the earliest known marker for myogenic precursors, is initially expressed in a group of cells in somites and visceral arches which later become muscle cells of the trunk and head, respectively (Ott et al, 1991). Our results suggest that visceral arch expression of Myf-5 is under separate control from early Myf-5 expression in the somite, since we have shown that Myf-5 visceral arch regulation is recapitulated in our construct, but early somitic expression is not. Although confirmation will require isolation of sequences that do direct early somitic expression, the clear implication is that there are distinct regulatory pathways by which Myf-5 expression, and perhaps muscle determination, is initiated in somitic trunk versus unsegmented head mesoderm. In a similar vein, expression of Myf-5 observed in our experiments subdivides somitic expression by time (early versus late) and by the subset of somitic Myf-5-positive cells that also show lacZ expression by in situ hybridization. In addition, the failure of this MYF5Z construct to be expressed when transfected into C2C12 cells (A.P., unpublished), which do express some endogenous Myf-5 (Miner and Wold, 1990), is consistent with the organization of *Myf-5* control regions into multiple segregated regulatory elements and it is likely that only a subset of these are included in this construct.

The MRF4Z transgenic mice expressed the transgene in muscle cells of embryos starting at E16.5, and the intensity of lacZ staining increased through the first week of postnatal development. This general expression pattern reflects that of endogenous MRF4 as determined by in situ hybridization and Northern blot experiments (Bober et al., 1991; Hinterberger et al., 1991). However, endogenous MRF4 also displays a separate, transient expression pattern in myotome between E9-11.5 (Bober et al., 1991; Hinterberger et al., 1991), but we did not detect any somitic expression of the transgene in MRF4Z lines. Interestingly, this construct, when transfected into C2C12 cells, which express some endogenous MRF4 upon differentiation (Miner and Wold, 1990), is active only in the

presence of high concentrations of cotransfected MyoD family regulatory factors (A.P., unpublished). Thus, for both *MRF4* and *Myf-5*, sequences that normally regulate early myotomal as well as C2C12 transcription were apparently not included in the constructs. Of course there are additional mechanisms of regulation that may contribute to differences between transgene expression and endogenous RNA levels, including specific methylation of sequences within the transgene, or post-transciptional mechanisms such as RNA stability. It is also possible that all regulatory regions are present in our constructs but are dependent on the linked state of *Myf-5* and *MRF4*.

In these experiments some ectopic ß-galactosidase was observed in individual lines and was not reproduced in other transgenic lines with the same construct. This raises the general issue, crucial in experiments of this design, of proper attribution of transgene expression patterns. Two criteria were applied to assign expression to the elements from *MRF4* and *Myf-5*. First, for any pattern attributed to the construct, transgene expression was observed in independent transgenic lines carrying the same construct, arguing that the signal was due to the construct itself and not to position effects at the site of insertion. The second criterion was to compare directly the transgene expression pattern with that of the endogenous gene by in situ hybridization. In this study, all elements of transgene expression that were shared by multiple lines of transgenic mice from the same construct also correlated with RNA expression from their respective endogenous genes, supporting the idea that the elements used in these experiments represent true regulatory regions of *Myf-5* and *MRF4*. in situ hybridization experiments were especially informative in the MRF4Z pups where penetrance of histochemical reagents into more mature muscle became a technical limitation. The line-specific ectopic transgene expression patterns were most likely due to adventitious enhancer trapping, in which the construct integrates near one or more endogenous, active genes, and the site of integration exerts some regulatory influence over the transgene (Lacy et al., 1983; Kothary et al., 1988; Al-Shawi et al., 1990). Three of 11 lines displayed position effect phenomena at some stage of development, with two

other lines, MYF5Z-21 and MYF5Z-29, showing some ectopic expression together with the expected pattern. In the most extreme case, MRF4Z-4, the typical MRF4 pattern was almost entirely suppressed and a wide array of ectopic sites were substituted. Curiously expression in just one major muscle, the spinotrapezius (Fig. 6), was very prominent, and correlated with the temporal regulation of the endogenous MRF4.

Of the isolated regulatory elements, perhaps the most interesting is the *Myf-5* 5' flank, which responds to early signals in the developing head; it will be interesting to more narrowly define the sequences responsible. Since craniofacial muscles are derived from unsegmented paraxial mesoderm (Couly et al., 1992) and not from the somitic mesoderm that gives rise to trunk and limb muscles, a regulatory element that responds to early myogenic signals in the head but not in the somites may ultimately permit molecular-level description of how these two myogenic lineages are differentially specified. Also, while cell culture experiments have shown that forced expression of any of the four MyoD family regulators can initiate a myogenic pathway, the issue of whether individual regulators are functionally different in vivo remains open and will require both gain-of-function and loss-of-function manipulations. The cis-regulatory segments defined here should serve as effective tools for testing the in vivo consequences of specifically altering the expression pattern of myogenic regulators by, for example, expressing *MRF4* in visceral arches and their derivatives.

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Fig. 1. Structure of the mouse *MRF4/Myf-5* locus and the respective transgenes. The stippled areas approximate transcribed regions, but for *Myf-5* only the location of the first exon is known. *LacZ* segments are not drawn to scale. See Materials and Methods for construction details. B, *Bam*HI; R, *Eco*RI; K, *Kpn*I.

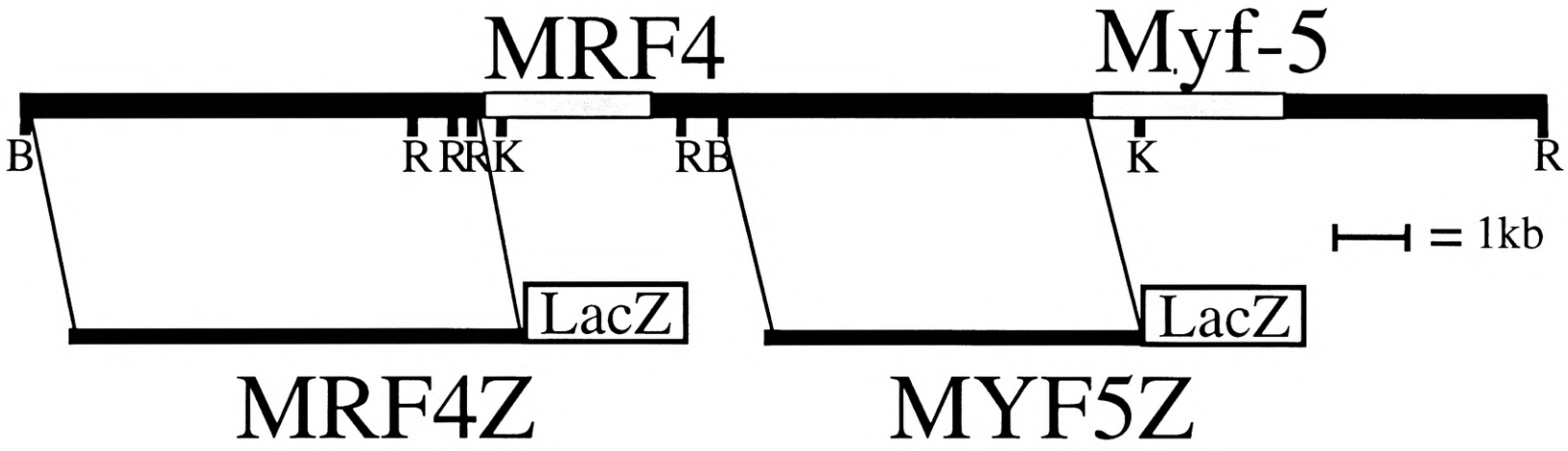
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Fig. 2. Whole mount histochemical staining of MYF5Z embryos at various developmental stages. (A) A 15 somite (E8.75) MYF5Z-21 embryo. The hyoid arch and the mandibular arch are positive for lacZ activity. (B) E10.5 MYF5Z-21 embryo. The hyoid arch is the most intensely stained structure and staining is also visible in the mandibular arch and in the ocular muscles. Ectopic staining specific to MYF5Z-21 embryos is observed in the nervous system as well as in the epithelium of the forelimb-bud. (C) E12.5 MYF5Z-21 embryo. Staining is evident in muscular derivatives of the visceral arches, in the ocular muscles, in developing proximal forelimb muscles, and in the segmented myotomes. Additional ectopic expression is observed in the forebrain. (D) E13.5 MYF5Z-21 embryo. Staining is similar to that observed in (C), with additional muscle-specific staining in intercostal, proximal hindlimb, and dorsal neck and head muscles. Ectopic lacZ expression is observed in the ribs. (E) E13.5 MYF5Z-29 embryo. Muscle specific staining is identical to that observed in (D), indicating that this staining is due to regulatory sequences in the transgene and not those found at the site of integration. f, forebrain, h, hyoid arch, icm, intercostal muscles, m, mandibular arch, om, ocular muscles, r, rib, arrowhead, myotome.

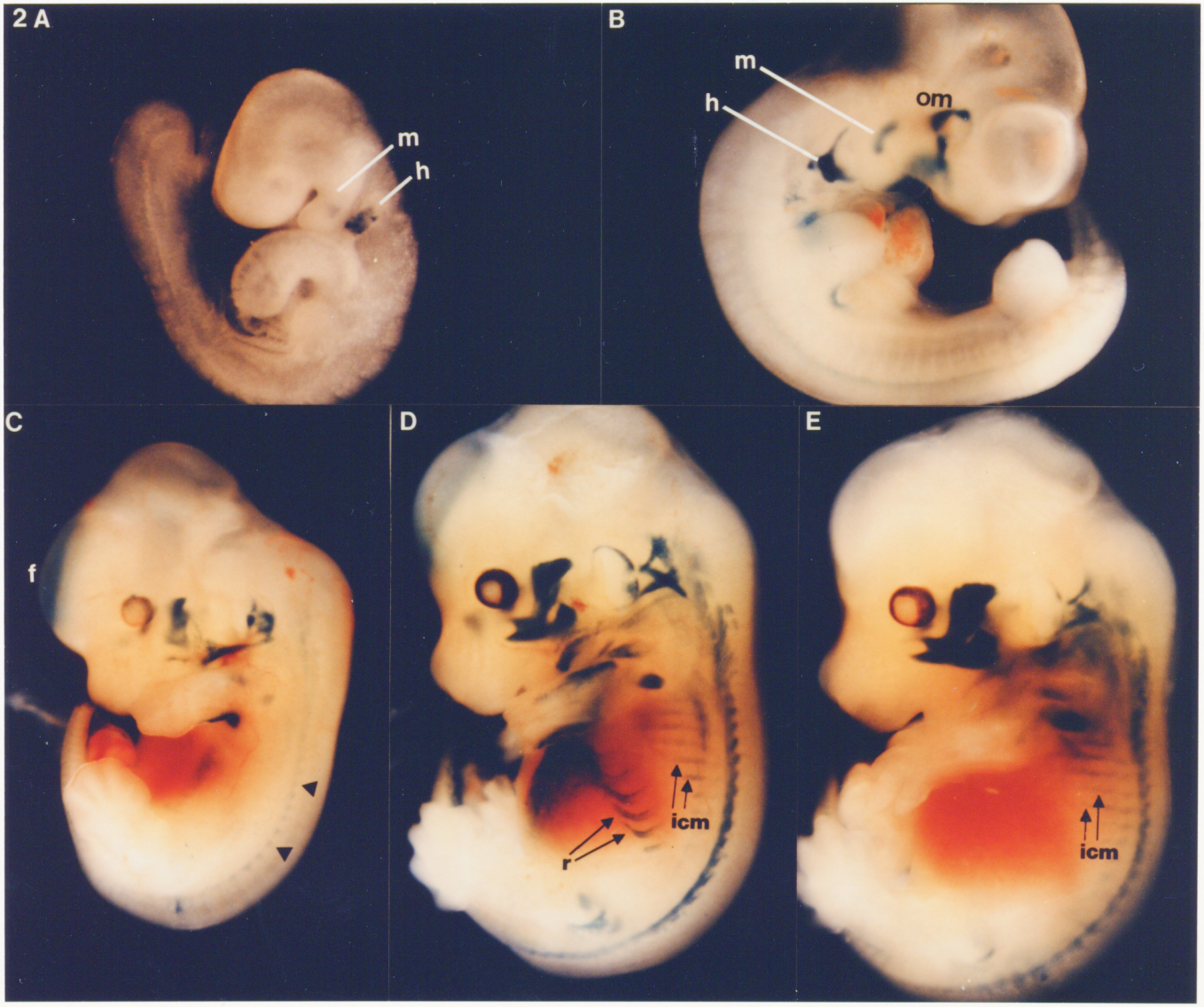
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Fig. 3. Expression of Myf-5 and lacZ in MYF5Z transgenic embryos as detected by in situ hybridization. (A,B) Parasagittal sections of E11.5 MYF5Z-21 embryo hybridized to Myf-5 (A) and lacZ (B) probes. Both Myf-5 and lacZ are expressed in jaw (jm) and shoulder (sm) muscles, while only Myf-5 shows significant hybridization in intercostal muscles (icm), and tail somites (s) at this stage. While lacZ is expressed in ocular muscles (om), these muscles are not present in the more lateral section (A) probed with Myf-5. Notice that the pigment layer of the retina refracts under dark field illumination. Some ectopic expression of lacZ is observed in the neural tube (nt), dorsal ganglia (arrowheads), and surrounding the heart. (C,D) Frontal sections of an E12.5 MYF5Z-29 embryo hybridized to Myf-5 (C) and lacZ (D) probes. Both Myf-5 and lacZ are expressed in the back (bm) and limb muscles (lm), with limb muscles showing more intense signal with the Myf-5 probe than with the lacZ probe. The transgene also shows some faint ectopic expression in the heart (h) and neural tube (nt) which was not observed in the whole embryo X-gal assay. li, liver. Scale bar = 500 microns.

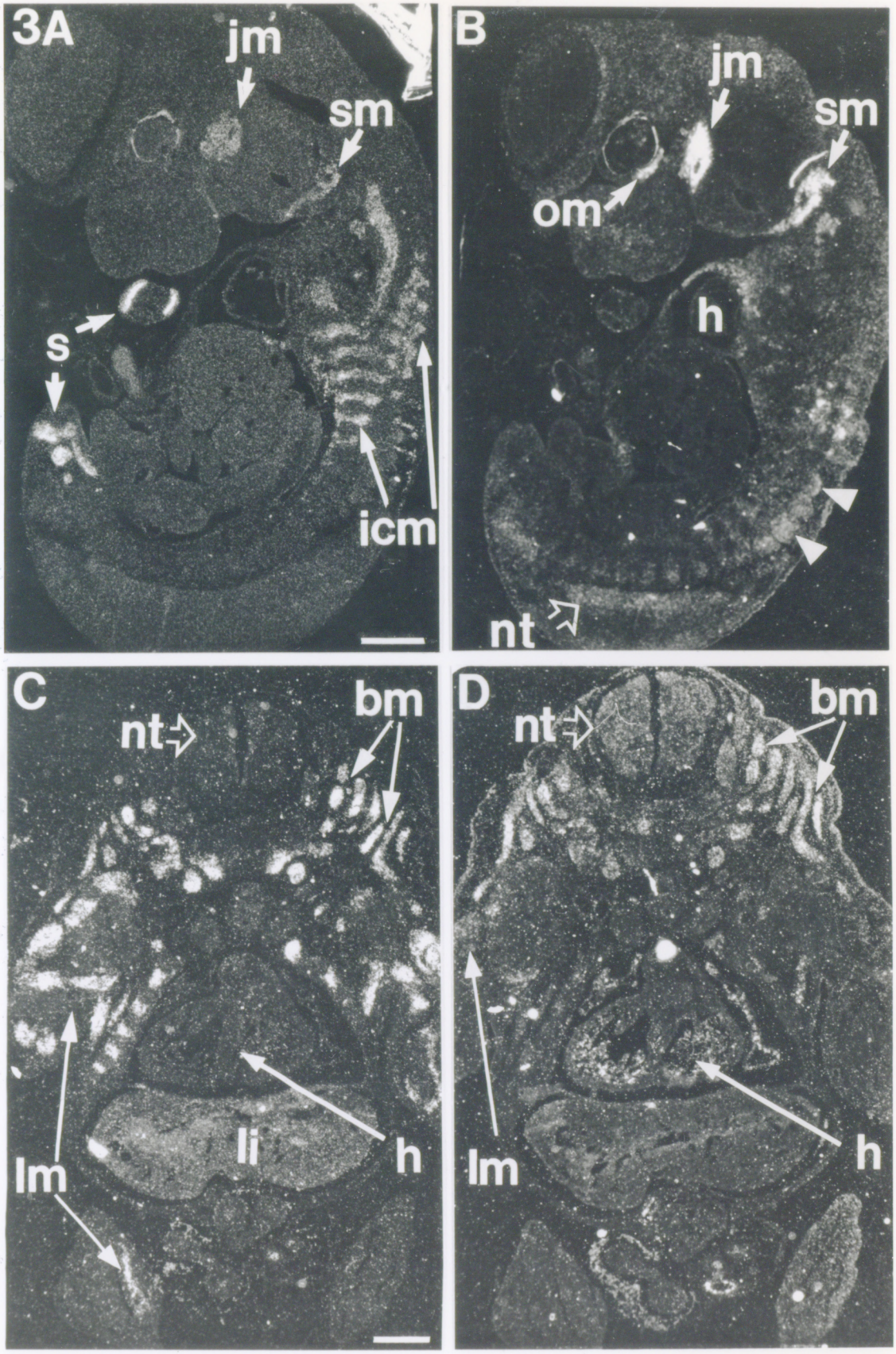
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Fig. 4. Whole mount X-gal histochemical staining of MRF4Z postnatal mice. (A) A lateral view of the trunk of a two day-old MRF4Z-28 mouse pup. Most muscles of the trunk contain fibers that are positive for lacZ activity, including latissimus dorsi, pectoralis, and the abdominals. The spinodeltoideus muscle (s) is consistently the most intensely stained in MRF4Z transgenics; the limbs exhibit considerably less lacZ activity. (B) An enlarged dorsal view of the same pup showing staining in muscles of the head and the neck. (C) A dorsal view of the trunk of a seven day-old MRF4Z-45 pup. Most muscles are lacZ positive, staining is striking in the paired intervertebral muscles (arrowheads) along the anterior-posterior axis. The top is anterior in all three cases.

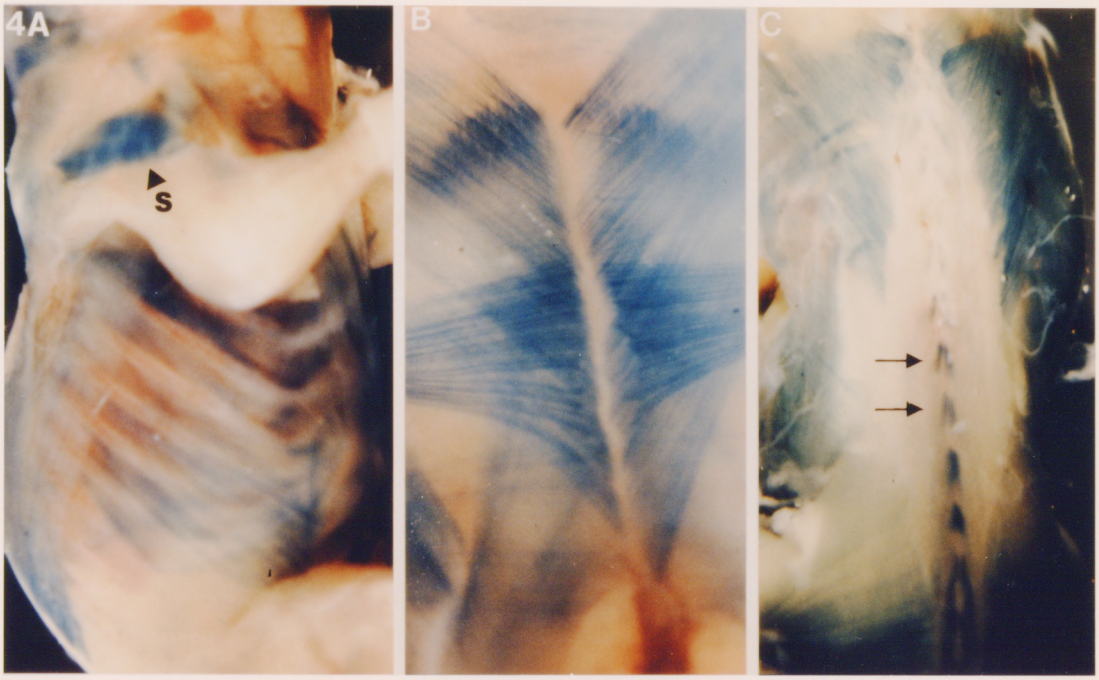
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Fig. 5. MRF4 and lacZ transgene expression in abdominal muscle sections of a 6.5 day old MRF4Z-45 trangenic pup as detected by in situ hybridization. (A) A phase-contrast micrograph of a section showing abdominal muscle fibers (m) associated with some adjacent connective tissue (ct). (B) Dark field micrograph of A hybridized with the lacZ probe. (C) Dark field micrograph of an adjacent section hybridized with the MRF4 probe. All muscle fibers are positive with both probes, while the connective tissue associated with the muscle is negative. Scale bar = 100 microns.

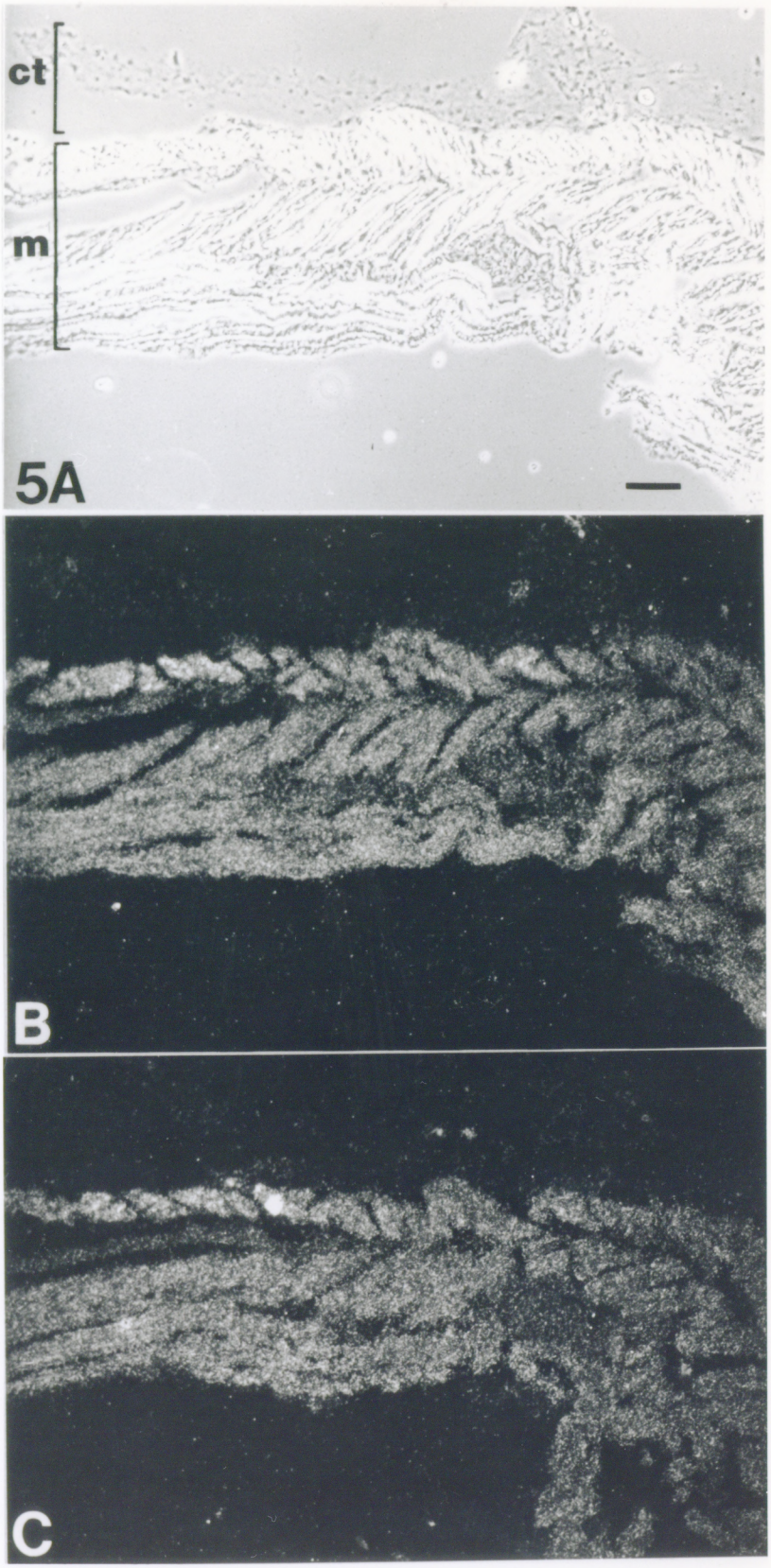
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Fig. 6. Whole mount histochemical staining of the MRF4Z-4 transgenic mice demonstrates presumed insertion site-dependent expression specific to this transgenic line. (A) E15.5 embryo. Staining is evident in the vibrissae (whisker pads), around the opening of the mouth, and in connective tissue of the elbow and the knee. The only muscle staining observed in this line is in the spinotrapezius muscle of the back. (B) A dorsal view of the trunk of a two day-old pup showing intense staining of the spinotrapezius muscle of the back. Staining in elbow and knee connective tissue persists.


6A
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Chapter 5

Differential Expression of Muscle-Specific Genes in Head and Trunk of Mouse

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Differential Expression of Muscle-Specific Genes in Head and Trunk of Mouse

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ABSTRACT

Vertebrate skeletal muscle is derived from dorsal mesoderm. While trunk muscles are of somitic origin, many cranial muscles arise from cells of the rostral non-segmented paraxial mesoderm that migrate to visceral arches and later form muscles of the neck and face. In an initial comparative investigation, we measured expression of a panel of muscle-specific regulatory and differentiation genes in developing and adult head and trunk lineages. The MyoD family of myogenic regulatory factors (MRFs) play a crucial role in the formation and differentiation of all known skeletal muscle. In the trunk, prior studies have shown that expression of the first muscle differentiation genes quickly follows expression of Myf-5, the earliest marker for developing skeletal muscle in all lineages studied. In contrast, we find here that in the head lineage, expression of muscle-specific differentiation genes is significantly delayed relative to Myf-5. All other MRFs and downstream genes first appear about three days after Myf-5 expression is initiated. These results suggest that Myf-5 activity is under sustained negative regulation in early cranial muscle precursors. In the adult head and limb muscles, no significant differences in MRF expression was detected; however, differential expression of specific myosin and actin isoforms was observed. One marked difference between head and trunk lineages is that MRF4 is not detectably expressed in head muscle precursors of the embryo. As an in vivo assay for its individual function, MRF4 was expressed in transgenic animals in early muscle precursors under the direction of Myf-5 head-specific cis-regulatory elements. This shift in MRF identity did not detectably perturb the normal cranial myogenic program.

INTRODUCTION

During embryogenesis of vertebrates, multiple lineages of myoblasts originate from the dorsal mesoderm, differentiate, and ultimately fuse to form multinucleated myotubes (reviewed by Wachtler and Christ, 1992). All skeletal muscles of the trunk are derived from somites, the metameric units of paraxial mesoderm along the rostrocaudal axis of the trunk that characterize vertebrate embryos. The myotome, a compartment within the somite, is the first differentiated skeletal muscle mass to form in the embryo. A second myogenic lineage arises from the ventro-lateral region of limb-level somites and migrates into the limb buds where they later differentiate and fuse (reviewed by Ordahl and Le Douarin, 1992; Stockdale, 1992). In contrast, muscles of the head are mainly derived from prechordal plate and anterior (non-segmented) paraxial mesoderm (reviewed by Couly et al., 1992; Noden, 1991). A majority of these muscle precursors migrate ventrally to occupy the visceral arches before they migrate again to their final position and differentiate.

The cloning and characterization of muscle-specific genes, both regulators and downstream differentiation markers, has been crucial to the study of myogenesis. The Myogenic Regulatory Factors (MRFs) belong to the basic helix-loop-helix (b-HLH) class of transcription factors, and include Myf-5, MyoD, myogenin and MRF4/herculin/Myf-6 (reviewed by Weintraub, 1993). The MRFs' roles in initiating and executing muscle differentiation in tissue culture is well characterized. Furthermore, they are required for muscle formation in vivo (Hasty et al., 1993; Nabeshima et al., 1993; Rudnicki et al., 1993). Consistent with their roles in myogenesis, MRF expression in vivo is largely restricted to skeletal muscle precursors and mature myofibers. Within muscle precursors, the MRFs show distinct and dynamic expression patterns in different muscle lineages: Myf-5 is the first of the MRFs to appear in the early myotomes, limb buds, and visceral arches

of mouse embryos; embryonic expression of MRF4, on the other hand, is restricted to myotomes only (reviewed by Lyons and Buckingham, 1992). Furthermore, the earliest MRF expression in the myotome is initiated dorsally, in close proximity to midline structures such as the neural tube and notochord which are important for induction of the myotome. In contrast, the visceral arch and limb muscle precursors begin expressing the MRFs and initiate myogenesis in a different cellular environment, after these cells have migrated laterally and ventrally towards their terminal destination. Recently, a close examination of the localization of the MRF proteins at the cellular level within the myotome, together with the analysis of germline deletions of Myf-5 and MRF4 have pointed towards an even more complicated scenario: different combinations of MRFs are expressed in, and are required for, distinct subdomains of the expanding myotome (Braun et al., 1994; Patapoutian et al., 1995; Smith et al., 1994). Similar situations could be present in the developing limb buds and visceral arches.

Many muscle-specific differentiation genes contain binding sites for MRFs within their regulatory regions, and they are expressed in the developing skeletal muscle following the appearance of MRFs. Expression of the differentiation genes, both at the RNA and protein levels, is well characterized in the myotome and limb buds of mouse embryos (reviewed by Lyons and Buckingham, 1992). Some of these, such as the skeletal and cardiac alpha actin isoforms are detectable within half a day of Myf-5 expression in both myotomes and limb buds, while others commence expression at later times during differentiation. The expression pattern of the differentiation genes has not been previously characterized in the developing head muscle. In this study, using RT-PCR and immunofluorescence, we found that the earliest detectable muscle-specific differentiation gene expression begins in cranial muscle three embryonic days after Myf-5 is first detected: the lag in trunk somites is a half day, at most. This suggests the presence of sustained negative regulation in head muscle precursors during this time point that keeps Myf-5 inactive for an extended period

of time. MRF4 is not expressed in the early head or limb bud lineages; and unlike the situation in myotomes, cells of the head lineage goes through extensive migration after becoming Myf-5 positive, but before differentiating. To test its possible in vivo role, MRF4 was ectopically expressed in early cranial muscle precursors under Myf-5 regulatory sequences and find no overt change in the normal cranial myogenic pathway.

RESULTS

Muscle-specific gene expression in head muscle precursors.

To rapidly survey the expression of a large panel of muscle-specific regulatory and differentiation genes in the developing head muscle precursors, we used quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). E7.5 whole mouse embryos; the head and trunk dissections of E8.5 to E16.5 embryos; and three adult muscle groups, rectus femoris (thigh muscle), masseter (head muscle) and temporalis (head muscle) were the sources of total RNA used. Transcript levels from one gene to another cannot be compared using RT-PCR; however, relative levels of transcripts from a given gene can be compared among different RNA samples if assay linearity is established. Thus, linearity of the RT-PCR over a substantial range of substrate RNA and PCR cycle number are crucial issues and were demonstrated in two sets of control experiments: First, cycle number titrations were performed with individual sets of primers to ensure that the product was in linear range (data not shown); second, titrations were carried out with varying amounts of initial input RNA to verify linearity with respect to starting material. One such control is shown in Fig. 1, and demonstrates linearity of GAPDH product with respect to the amount of input RNA over a 100-fold range.

As in the somites, different MRFs are expressed in the head in a characteristic temporal fashion during embryogenesis. RT-PCR results showed no detectable MRF expression in E7.5 embryos at 26 cycles (Fig. 2). Under the same conditions of amplification, Myf-5 was expressed at E8 in the trunk (Patapoutian et al., 1995); myogenin, at E8.5; MRF4, at E9.5; MyoD, at E10.5 (Fig. 2). In the head, Myf-5 was present at E8.5 which agrees with the onset of Myf-5 regulatory region activity in the visceral arches as visualized by a Myf-5-lacZ reporter construct (Patapoutian et al., 1993). None of the other MRFs were

detectable in the head before E11.5 (Fig. 2). MRF4 did not begin to accumulate in the head muscle until E16.5 (Fig. 2).

We next surveyed the expression of skeletal muscle differentiation genes in head and trunk at different embryonic stages. This panel consisted of FGF-6, M-Cadherin, α skeletal actin, α cardiac actin, different isoforms of myosin light chain (MLC1F, MLC3F, and MLC1A), different isoforms of myosin heavy chain (MHC-embryonic, MHC-perinatal, MHC-adult, and MHC-β), the muscle specific form of neural cell adhesion molecule (NCAM-msd), muscle creatine kinase (MCK), gamma and epsilon subunits of acetylcholine receptors (ACHR-ε and ACHR-γ) and MEF-2C. Expression of these genes in the trunk have been described previously (Lyons and Buckingham, 1992), and the trunk RNA samples served here as controls. No muscle differentiation genes from this panel were detected in head samples before E11.5 (Fig. 2 and data not shown), although the expression of Myf-5 reported above served as a positive control for the presence and detectability of genes expressed specifically in the head myogenic lineage. Similarly, the absence of myogenin and α skeletal actin in the head samples showed that the dissection of head from trunk (where these genes are active) was successful.

To verify the RT-PCR results regarding the lack of early muscle-specific differentiation gene expression in the head lineage, we probed E10.5 and E11.5 sections of visceral arch and myotomal domains with antibodies to α actinin (Fig. 3) and myosin heavy chain (data not shown). While both differentiation markers were clearly expressed in myotomes at both timepoints, the visceral arches showed expression only at E11.5 and not at E10.5, verifying the RNA-level analysis.

Muscle-specific gene expression in adult head and trunk muscles.

To further investigate at later developmental times the distinctions between head and trunk lineages, we compared gene expression of two head muscle groups (masseter and temporalis) to a thigh muscle (rectus femoris) which is often used as the prototype adult muscle and serves here as a major representative of the appendicular lineage. Masseter and temporalis are both derived from 1st arch mesoderm in the avian system. MRF gene expression, as well as MEF-2C and FGF-6 appeared equivalent in the adult samples tested (Fig. 4A). Also, while many of the muscle-specific differentiation genes described above appeared comparable, a few were differentially expressed (Fig. 4B and data not shown). Instead of the MHC-adult isoform, adult masseter mainly expressed MHC-perinatal; both adult and perinatal isoforms were expressed at low levels in temporalis. α cardiac actin was almost completely undetectable in the head muscles (Fig. 4B), but was expressed at relatively higher levels in rectus femoris. Thus, while known regulatory genes appear to be expressed at similar levels in head and limb muscles, RNA levels for several presumed downstream transcriptional targets show significant differences.

Ectopic expression of MRF4 in early head muscle precursors:

Among the main differences in MRF expression pattern between myotomes and visceral arches is the absence of MRF4 expression in head muscle precursors. To test for possible in vivo functional implications of this difference, we used cis-regulatory sequences from Myf-5 regulatory sequences, described previously (Patapoutian et al., 1993), to drive the MRF4 gene in visceral arches. The Myf-5~MRF4 construct was coinjected with the previously described MYF5Z (Patapoutian et al., 1993), which contains the same regulatory sequences driving the bacterial β-galactosidase gene. We obtained two transgenic lines, one expressing both constructs (TgMRF4-1), and the other containing the Myf-5~MRF4 construct only (TgMRF4-2). Whole mount embryo staining of TgMRF4-1 with X-gal showed lacZ staining in visceral arch muscle precursors beginning at E8.5, and the overall staining pattern was as previously reported (data not shown; Patapoutian et al.,

1993). RT-PCR analysis demonstrated the presence of exogenous MRF4 in head muscle precursors in both transgenic lines (the reverse primer is from SV40 3' untranslated region) (Fig. 5, and data not shown). However, expression of other MRFs and skeletal differentiation markers appeared normal in the transgenic animals at different developmental timepoints (Fig. 5, and data not shown), indicating that combined Myf-5 (endogenous) and MRF4 (transgene) activity is not sufficient to drive premature myocyte differentiation or to alter the character of differentiated head muscle at later times.

DISCUSSION

Two fundamental findings have made myogenesis an attractive system to study vertebrate cell type determination and differentiation: the ability to culture myoblasts and induce them to differentiate (Konigsberg, 1963), and the cloning of the MyoD gene and its relatives that have the potential to transform diverse permissive cell types into a myogenic phenotype in cell culture (Davis et al., 1987). Transfection assays have shown that all four MRFs have similar capabilities of activating the myogenic pathway. Results from cell culture studies led to a working model of myogenic regulation, in which MRFs (mainly Myf-5 and MyoD) are expressed in dividing myoblasts, but their activity is negatively regulated until the onset of differentiation which is signaled by expression of myogenin (reviewed by Lassar et al., 1994). Multiple mechanisms have been shown to negatively modulate MRF activity in biochemical assays and in cell culture systems: For example, Id family members, which are HLH proteins lacking a functional basic region are present in myoblasts and bind directly to the MRFs or their partners and repress their activity. Downregulation of Id-type molecules is observed under differentiation conditions, and this is thought to unleash the MRFs to carry out the myogenic differentiation process (Benezra et al., 1990; Neuhold and Wold, 1993). Twist, another b-HLH molecule, can also act as a negative regulator of MRF activity by directly binding to the MRFs (Hebrok et al., 1994; Yun et al., 1995). C-Jun, a leucine zipper class DNA binding factor, can also bind to the MRFs in vitro and depress muscle differentiation in transfected cells in culture (Bengal et al., 1992). A second class of possible negative regulators of MRF action are kinases. Phosphorylation of the basic domain of the MRFs by protein kinase A and C inhibits DNA binding in vitro and can repress myogenesis in transfection assays (Li et al., 1992; Hardy et al., 1993). More recently, a family of cyclins, presumably acting as components of cyclin dependent kinases (CDKs) associated with cell cycle progression, have been implicated as negative regulators of MRF function (reviewed by Marx, 1995).

In addition to the formidable array of prospective negative modulators of MRF action, there is also evidence that one or more positive acting b-HLH co-regulators of the E-family are required for proper activity of MRFs (Weintraub et al., 1991). Although it remains an interesting possibility that MRFs, in some environments, may form homomeric complexes, their function in muscle differentiation is likely to require dimerization to positive E-type partners. The implication is that the absence of positive regulators in specific embryonic lineages could limit MRF action. Therefore, although the regulation of MRF activity has been thoroughly studied, a crucial issue is whether the picture of MRF modulation initially obtained from cell culture and biochemistry also applies to myogenic specification and differentiation in the developing mouse embryo.

Possible negative regulation of Myf-5 in early head muscle precursors

This study presents a survey of expression of various muscle regulators and differentiation genes in the head muscle lineage. We find that Myf-5 is expressed in visceral arches for three days before any other muscle-specific transcripts are detectable. The most straightforward explanation for this finding is that Myf-5 function is negatively regulated in head muscle precursors while these cells are in the visceral arches. However, the mechanisms of Myf-5 regulation are not clear. In vivo analysis of the expression pattern of MRFs, Id-1, and m-twist during myotomal development in the somite has shown that, to a large extent, a mutually exclusive expression pattern between the positive and negative HLH proteins (Wang et al., 1992; Wolf et al., 1991; Yun et al., 1995). Furthermore, the expression of some muscle-specific differentiation markers is detected only half a day after the initial appearance of Myf-5 in the myotome and limb-bud; the rapid kinetics and mutually exclusivity of MRF and negative HLH/bHLH factors argue against the extended use of global negative regulation of MRF activity in these lineages. Indeed, it is only the domain at the dorsomedial lip of the newly forming myotome that seems to express both m-

twist and Myf-5 (Yun et al., 1995). In contrast, we observe in the head lineage a pattern of muscle-specific gene expression that may call for sustained negative regulation of Myf-5 in early visceral arches. It will now be of interest to monitor at high resolution the expression of potential negative regulators, such as Ids 1-4, m-Twist, and G-1 cyclins in the early head muscle precursors. The global negative regulation of Myf-5 in visceral arches nominates the head muscle lineage as the only embryonic example reminiscent of the myogenic differentiation process in cell culture, where dividing myoblasts express MRFs for an extended period subject to apparent negative regulation until the time of induced differentiation. A second explanation for extended Myf-5 expression without differentiation would be the lack of E-family positive-acting bHLH expression in the head lineage at early times. The unique modulation of Myf-5 activity in the head lineage might be due to the separation of induced commitment to myogenesis in visceral arches from the sites of muscle differentiation that occurs later, after the complete migration of head muscle cells to the presumptive face.

Differential expression of muscle-specific genes in adult head and trunk muscles

The MRFs are transcription factors that can bind to regulatory sequences of muscle-specific genes and activate their expression. The MRFs are required for the expression of these differentiation genes in vivo (Hasty et al., 1993; Nabeshima et al., 1993; Rudnicki et al., 1993). However, as observed previously from embryonic muscle-specific gene expression patterns, there is not a simple correlation between the timing of different MRF expression and the expression of putative downstream genes (reviewed by Lyons and Buckingham, 1992). For example, although muscle creatine kinase (MCK) regulatory sequences are routinely accepted and used as a direct reporter of MyoD/myogenin activity in cell culture transfection assays, MCK is only expressed three days after MyoD and five days after myogenin in the developing mouse trunk. Similarly, comparison of gene expression

between head and limb adult muscle groups described here, shows that although MRFs and MEF-2C are expressed in a similar temporal fashion, major differences in the expression of some muscle-specific differentiation genes exists between the two muscle groups: MHC-perinatal, as its name implies, is an isoform of myosin heavy chain previously shown to be expressed in the trunk and limb muscles during perinatal stages of development; however, here we show that it is expressed prominently in adult head masseter muscle, while the normally expressed "adult" MHC isoform is virtually absent (Fig. 4). There are many possible explanations for the differential expression of skeletal muscle-specific genes during various developmental times or within distinct muscle groups: Different affinities of cis-regulatory elements for individual MRFs and MEF-2s; methylation status and chromatin accessibility of individual regulatory elements (Donogue and Sanes, 1994); or the differential presence of co-regulators (positive or negative) preferential for some muscle differentiation genes. It is clear from this work, however, that there exist a set of distinctions that correlate directly with head lineage vs. limb, and others that define different muscles within the head.

Ectopic expression of MRF4 does not effect the cranial myogenic program

A major difference in gene expression between early head and trunk muscle precursors is the expression of MRF4 in myotomes compared to its complete absence from the visceral arches. In an attempt to detect a specific role for MRF4 expression in embryonic myocytes or their precursors, we ectopically expressed it in early head muscle precursors of transgenic mice by the previously described Myf-5 regulatory sequences. Exogenous MRF4 was properly expressed at the expected time in the heads of these transgenic mice; however, we did not observe any molecular or physiological changes in these mice. Although a formal possibility exists that functional MRF4 protein is not present, it is unlikely since the RNA was present at high levels, and the same genomic MRF4 cassette has previously proven functional in cultured cell experiments (Miner and Wold, 1990).

The lack of phenotype observed at the earlier timepoints (E8.5 to E10.5) could be mainly due to the proposed sustained negative regulation of Myf-5, which might act on MRF4 as well.

MRF4 knockouts have been useful to elucidate the specific function of this gene during early embryogenesis and adult life. Among other phenotypes, these mice show a severe downregulation of the embryonic isoform of myosin heavy chain (MHC-emb) which is expressed in myotomes concurrently with MRF4 (Braun and Arnold, 1995; Patapoutian et al., 1995; Zhang et al., 1995). However, as we show here, MHC-emb is expressed in wildtype head muscles at E11.5, at least four days before MRF4 expression is detected there, suggesting that MRF4 is not necessary for embryonic head lineage expression of MHC-emb, even though it appears important for this gene in trunk and limb. The transgenic mice expressing ectopic MRF4 in head muscle precursors also showed normal levels of MHC-emb transcripts, and initial proper activation. Therefore, although the phenotype of MRF4 null mice suggests that MRF4 is necessary for the expression of MHC-emb in some sub-lineages, the expression of MHC-emb in head lineage prior to MRF4 expression is consistent with the emerging view that no particular muscle-specific gene seems to be completely dependent on MRF4.

EXPERIMENTAL PROCEDURES

RNA isolation and RT-PCR

RNA was prepared by the method of Chomczynski and Sacchi (1987) from the trunk and head regions of embryos from different developmental timepoints and adult musculature. Quantitative RT-PCR was carried out according to the method of Robinson and Simon (1991). Both reverse transcription and PCR were performed in the same tube in a single buffer with specific primers. AMV-RT (Promega) was used instead of MMLV-RT, and in some cases Taq antibody (Clontech) was also added to block Taq polymerase activity at lower temperatures. 20-28 cycles were used for different primer sets (Table I). An initial titration was carried out to assure that amplifications at high cycle numbers were still in linear range. All primer sets were designed to span at least one intron to distinguish RNA from DNA contamination, and the sizes of the products were between 200 and 500 bases.

Frozen sections and antibody staining

Embryos were fixed in 4% paraformaldehyde at 4^{o}C overnight. Embryos younger than e11 were sunk into 15% sucrose & 7.5% gelatin in PBS solution and then frozen in OCT (Tissue-Tek). Older embryos were immediately frozen in OCT. Sections of 10-20μm thickness were obtained using a cryostat, and were blocked for 20 minutes in 10% goat serum and 3% BSA in PBS before applying the primary antibody for 1-3 hours at room temperature. The secondary antibody solution was applied for 1 hour. Antibodies against myosin heavy chain, (MF20; 1:10 dilution; mouse IgG2b; Developmental Studies Hybridoma Bank), striated muscle specific α actinin (1:400 dilution; mouse IgG1; Sigma), were used. Secondary antibodies were conjugated to Fluorescein and specific to mouse IgG isotypes and used as 1:100 dilution in 3% BSA in PBS (Southern Biotechnology Associates).

Production and analysis of transgenic mice

The 5.5 kb of the 5' flanking region of the mouse Myf-5 gene as described previously (Patapoutian et al., 1993) was used to drive the MRF4 (herculin) gene attached to an SV40 3' untranslated region (Miner and Wold, 1990). This construct, MYF5~MRF4, was then cut to isolate it from vector sequences, run on an agarose gel and purified by Qiaex Gel Extraction Kit (Qiagen). DNA was eluted in 10 mM Tris (pH=7.5), 0.1 mM EDTA (pH=8). This construct mixed with MYF5Z (Patapoutian et al., 1993) was used for pronuclear microinjection of single cell mouse embryos from a (C57BL/6XDBA/2)F1 X (C57BL/6XDBA/2)F1 cross as described (Hogan et al., 1986) and two independent transgenic founder lines were obtained. One of these lines contained only the MYF5~MRF4 construct (TgMRF4-1), while the other contained both constructs (TgMRF4-2). Transgenic mice and embryos were identified by polymerase chain reaction (PCR) analysis of tail or placenta DNA. 1μg of genomic DNA was used in 29 cycles of amplification with forward: CCAGAAGGCCACCGAGCAGGTTAG (from Myf-5 promoter) and reverse: CCCTGGATACAAAGGAGAGCCCTC (from MRF4 exon 1) primers. Male transgenic animals were mated in most cases with C57BL/6 X DBA/2 hybrid females to propagate the line or to obtain transgenic embryos.

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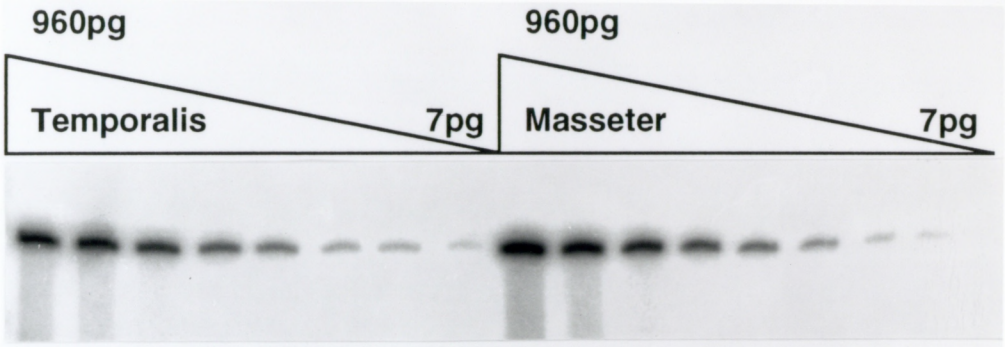
Table I: Primers used in RT-PCR.

Gene	Forward Primer	Reverse Primer	Cycle #
MRF4-endogenous	CTACATTGAGCGTCTACAGGACC	CTGAAGACTGCTGGAGGCTG	27
MRF4-exogenous	CAGTGGCCAAGTGTTTCGGATCATTC	GCAAGTAAAACCTCTACAAATGTGGTATGGC	27
Myf-5	TGAATGTAACAGCCCTGTCTGGTC	CGTGATAGATAAGTCTGGAGCTGG	26
MyoD[a]	AGGCTCTGCTGCGCGACC	TGCAGTCGATCTCTCAAAGCACC	26
myogenin[a]	GAGCGCGATCTCCGCTACAGAGG	CTGGCTTGTGGCAGCCCAGG	28
FGF6	GTGCTCTCTTCATTGCCATGAACAG	CCCCGTGAGCCTTCATCC	28
MEF2C[b]	CATGCCGCCATCTGCCCTCAG	CCCTTTCGTCCGGCGAAGGTC	23
MEF2D[c]	CAAGCTGTTCCAGTATGCCAG	AAGGGATGATGTCACCAGGG	26
M-cadherin	CAGGTTCACCATCCTTGAAGGT	TGGGTCGTAGTCTTTGGAGTAGC	26
ACHR-γ	CAGCGCAATGGATTAGTGCAGG	GTCAGGCACTTGGTTGTAGTGGG	27
NCAM (MSD)	TCCTCCACAGGCTCCTGCTAAC	CGCTCTGTACTTGACCAGATAGTG	26
MLC1F	AAAGACGTGAAGAAGCCCGCTG	ATAACCTCCCTGGTCCTTGTTG	23
MLC3F	CTTCAGTGCTGACCAGATTGCC	ATAACCTCCCTGGTCCTTGTTG	21
α skeletal actin	TTATCGGTATGGAGTCTGCGGG	CACAGCACGATTGTCGATTGTGG	22
α cardiac actin	AACCCACCAAAGCTGTGCCAG	CTTCAGTGAGCAGGGTTGGG	20
MCK	TTCGGCAACACCCACAACAAGTTC	ACATAGTTGGGGTCCAGGTCGTC	22
MHC-embryonic	GCAAAGACCCGTGACTTCACCTCTAG	GCATGTGGAAAAGTGATACGTGG	23
MHC-perinatal	GAAGACCGCAAGAATGTGCTCC	CCTCCTGTGCTTTCCTTCAGCC	22
MHC-adult	AACATCGAAGCCGTCAAGGGTC	GTGATTTCTCCTGTCACCTCTC	21
GAPDH	GTGGCAAAGTGGAGATTGTTGCC	GATGATGACCCGTTTGGCTCC	22

[a] Hannon et al. 1992; [b]Martin et al. 1993, [c]Martin et al. 1994.

Figure 1. Linear dependence of the output signal on input RNA during the amplification of control GAPDH. Seven RNA samples of two-fold serial dilutions from two separate head muscle groups were subjected to the RT-PCR protocol with primers to GAPDH at 24 cycles. The products were quantitated using a Molecular Dynamics phosphorimager and plotted as signal versus amount of input RNA in log scale.


960pg
Temporalis
7pg
960pg
Masseter
7pg

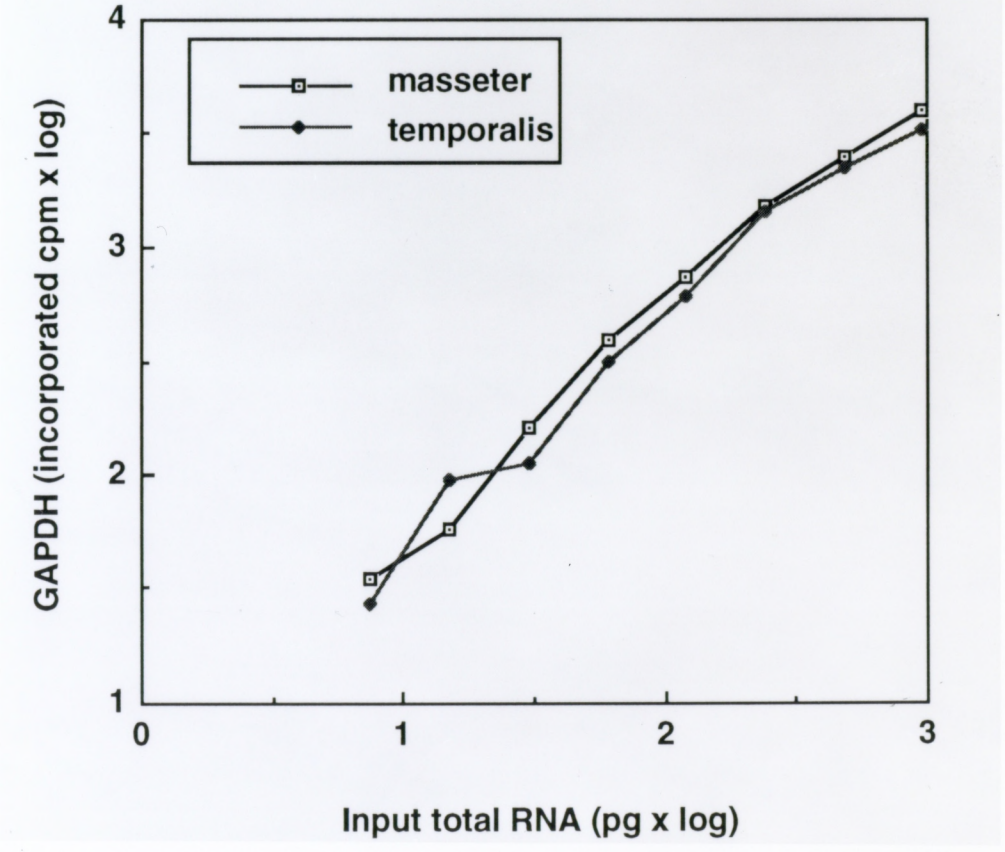

masseter
temporalis
GAPDH (incorporated cpm x log)
Input total RNA (pg x log)
4
3
2
1
0
1
2
3

Figure 2. Myf-5 is the only muscle-specific gene to be expressed in head muscle precursors before E11.5. Total RNA was made from head and trunk dissections from various stage embryos and subjected to the RT-PCR protocol with various primers to muscle-specific genes at different cycle numbers (see table I). (*) E7.5 represents RNA from the whole embryo.

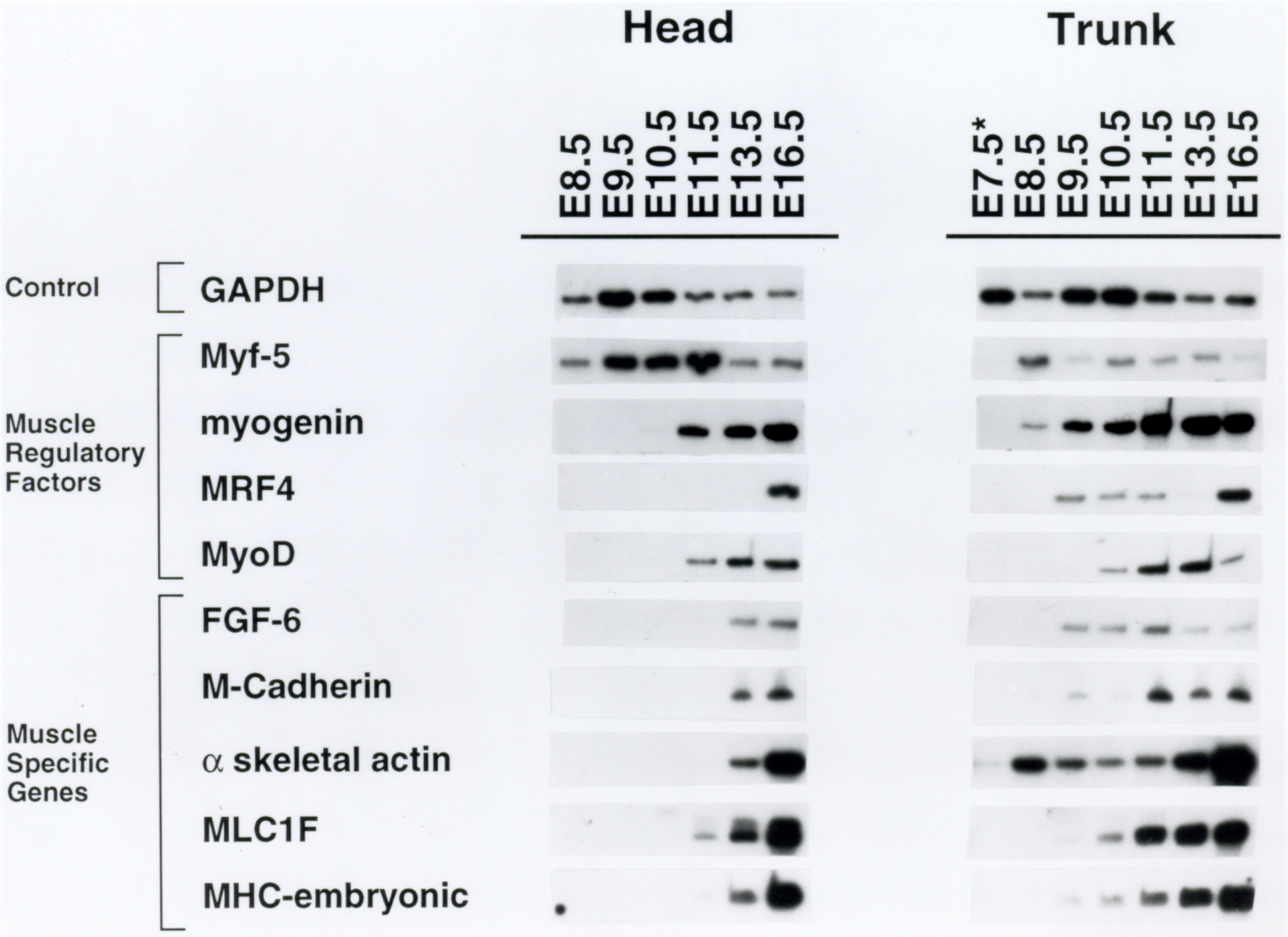

Head
Trunk
E8.5
E9.5
E10.5
E11.5
E13.5
E16.5
E7.5*
E8.5
E9.5
E10.5
E11.5
E13.5
E16.5
Control
GAPDH
Muscle Regulatory Factors
Myf-5
myogenin
MRF4
MyoD
Muscle Specific Genes
FGF-6
M-Cadherin
α skeletal actin
MLC1F
MHC-embryonic

Figure 3. Skeletal muscle-specific α actinin is not expressed in visceral arches prior to E11.5. Transverse sections of E10.5 (A, B) and E11.5 (C, D) wildtype embryos were probed with an antibody against α actinin. Expression in visceral arches was observed at E11.5 (C) but not at E10.5 (A). α actinin was expressed in myotomes of thoracic region at both timepoints (B, D).

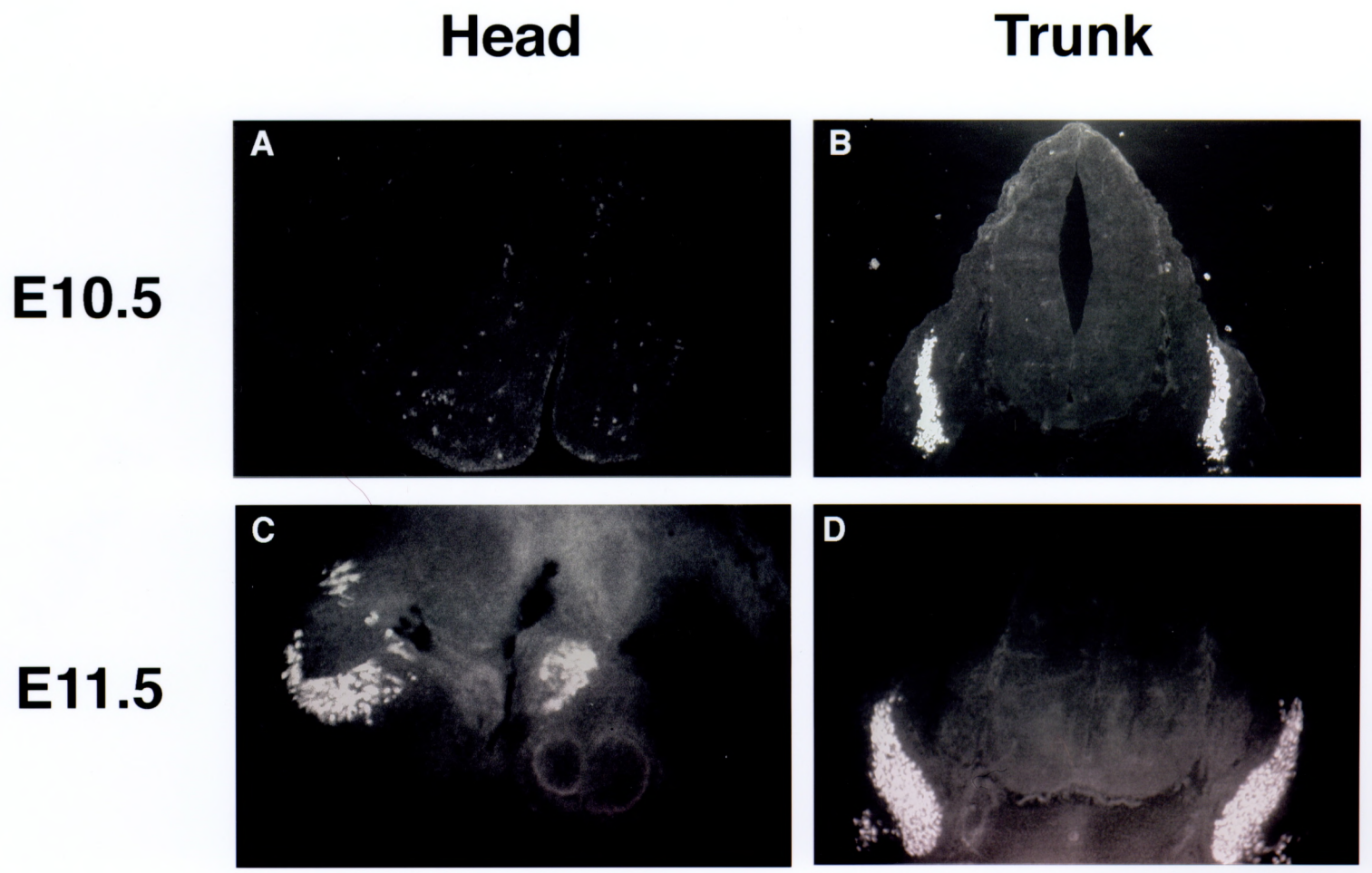

Head
Trunk
E10.5
E11.5
A
B
C
D

Figure 4. Expression of muscle-specific gene expression in adult head musculature. RT-PCR from rectus femoris (thigh muscle), masseter (head muscle), and temporalis (head muscle) of adult mice was performed with primers to GAPDH control, muscle-specific regulators (A) and downstream differentiation markers (B). Most of the regulators are expressed comparably among the three samples. MHC isoforms and α cardiac actin are differentially expressed.


Rectus Femoris
Masseter
Temporalis
GAPDH
Myf-5
Myogenin
MRF4
MyoD
MEF-2C
FGF-6

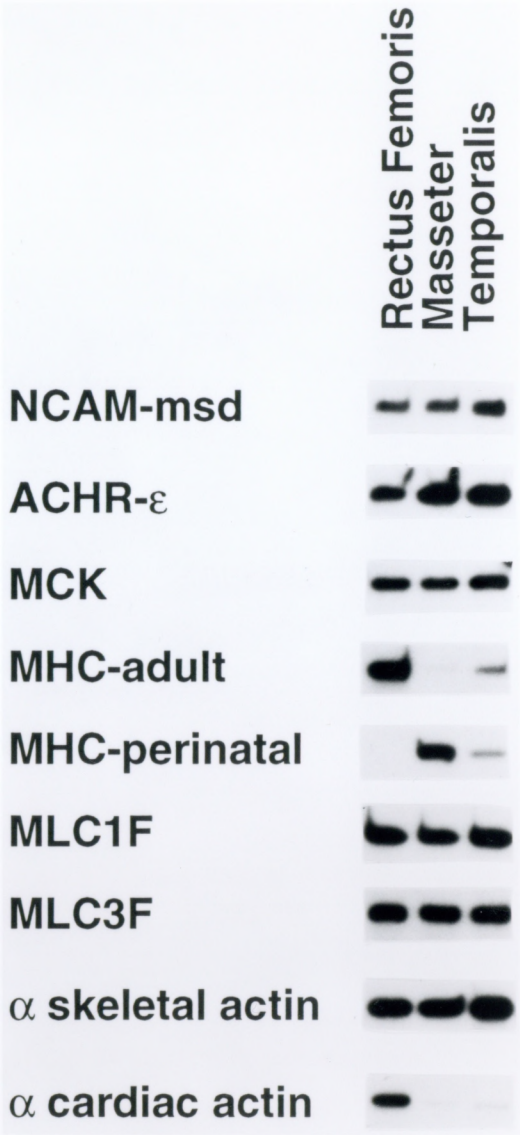
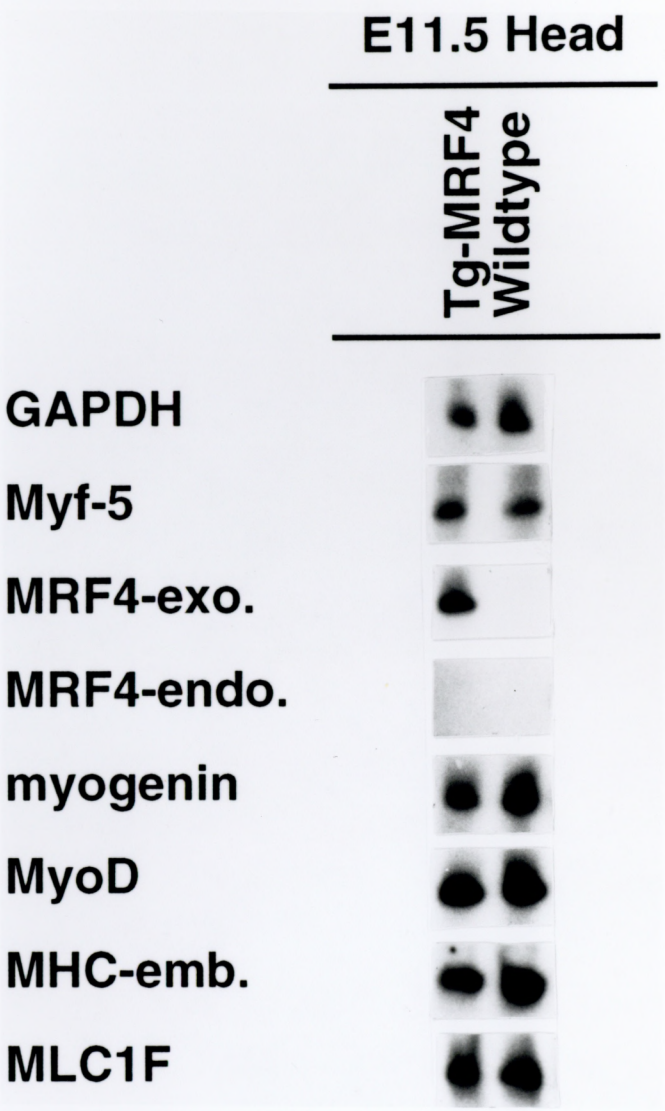

Rectus Femoris
Masseter
Temporalis
NCAM-msd
ACHR-ε
MCK
MHC-adult
MHC-perinatal
MLC1F
MLC3F
α skeletal actin
α cardiac actin

Figure 5. Expression of muscle-specific gene expression in transgenic mice containing the Myf5~MRF4 construct (TgMRF4-1). RNA from E11.5 Head of transgenic and wildtype littermates were used for this RT-PCR assay. Endogenous MRF4 was not expressed at this time, however, exogenous MRF4 was only detectable in the transgenic sample. No other major difference in gene expression is detectable at this time. Abbreviations: exo., exogenous; endo., endogenous; emb., embryonic; MLC1F, myosin light chain 1 fast.


E11.5 Head
Tg-MRF4
Wildtype
GAPDH
Myf-5
MRF4-exo.
MRF4-endo.
myogenin
MyoD
MHC-emb.
MLC1F