

Somite Subdomains, Muscle Cell Origins, and the Four Muscle Regulatory Factor Proteins

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Abstract. We show by immunohistology that distinct expression patterns of the four muscle regulatory factor (MRF) proteins identify subdomains of mouse somites. Myf-5 and MyoD are, at specific stages, each expressed in both myotome and dermatome cells. Myf-5 expression is initially restricted to dorsal cells in all somites, as is MyoD expression in neck somites. In trunk somites, however, MyoD is initially expressed in ventral cells. Myogenin and MRF4 are restricted

to myotome cells, though the MRF4-expressing cells are initially less widely distributed than the myogenin-expressing cells, which are at all stages found throughout the myotome. All somitic myocytes express one or more MRFs. The transiently distinct expression patterns of the four MRF proteins identify dorsal and ventral subdomains of somites, and suggest that skeletal muscle cells in somites originate at multiple sites and via multiple molecular pathways.

ALL of the skeletal muscle cells in the trunk and limbs are formed by myogenic cells that originate in the somites (Christ et al., 1977; Chevallier et al., 1977; Kenny-Mobbs, 1985). Somitic myogenesis does not appear to be a single process, rather multiple lineages of myogenic cells appear to arise in distinct regions of a somite. Myogenic cells which originate in the dorsal-medial region of a somite (nearest the neural tube) form axial muscles, whereas myogenic cells which originate in the ventral-lateral half of a somite migrate and form muscles in the limbs and ventral body wall (Ordahl and Le Douarin, 1992). The neural tube/notochord is required for myogenic cells to form in the dorsal-medial, but not ventral-lateral, region of a somite (Ordahl and Le Douarin, 1992; Rong et al., 1992). The descendants of myogenic cells in the somites are further subdivided into multiple types of embryonic, fetal, and satellite cell myoblasts (reviewed by Cossu and Molinaro, 1987; Miller, 1992; Stockdale, 1992; Miller et al., 1993). The cellular and molecular mechanisms underlying the generation and diversification of myogenic cells are not fully understood, though the muscle-specific transcription factors of the MyoD family likely play a central role.

MyoD, myogenin, Myf-5, and MRF4/Myf-6/herculin comprise the basic helix-loop-helix group of muscle regulatory factors (MRFs)¹ (reviewed by Weintraub et al., 1991; Olson, 1992). The MRFs act in concert with additional ubiquitous and muscle-specific transcription regulators to ac-

tivate transcription of many muscle-specific genes. In situ hybridization has been used to determine the expression patterns of the MRF mRNAs (reviewed by Buckingham, 1992; Sassoon, 1993). The first MRF mRNAs to be detected, Myf-5 in the mouse and *qmfl* and 3 in the quail, are found in the dorsal-medial quadrant of epithelial stage somites (Ott et al., 1991; Pownall and Emerson, 1992). The remaining MRF mRNAs then appear sequentially; e.g., in rodent somites, Myf-5 mRNA is followed by the myogenin, MRF4, and MyoD mRNAs (Ott et al., 1991; Hinterberger et al., 1991).

New questions about MRF function in the somites have been raised by recent work. Different studies have, for example, reached varying conclusions about where myogenic cells originate in the somites (summarized by Kaehn et al., 1988). In addition, studies of mice with deleted MRF genes suggest that: (a) Myf-5 is required to properly form not only the myotomes but also the distal ribs (Braun et al., 1992); (b) MyoD appears dispensable during somitic myogenesis (Rudnicki et al., 1992); (c) lack of myogenin greatly reduces myotube formation in vivo (Hasty et al., 1993; Nabeshima et al., 1993); and (d) either Myf-5 or MyoD is required for myoblast formation (Rudnicki et al., 1993). Cusella-De Angelis et al. (1992) concluded that E9.5 mouse somites contain untranslated myogenin mRNA, and that some somitic muscle cells in vivo and in vitro express myosin heavy chain (MHC), but not the myogenin or MyoD proteins. We found that E8.5 mouse somite cells formed myocytes in culture that contained MHC but did not have detectable amounts of any of the four MRFs (Smith et al., 1993).

Further understanding of how the MRFs function in somitic myogenesis clearly requires knowledge of MRF protein expression patterns in individual somite cells. Because

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1. *Abbreviations used in this paper:* MRF, muscle regulatory factor; MHC, myosin heavy chain.

such analyses were lacking, we have now used specific antibodies (Wright et al., 1991; Mak et al., 1992; Smith et al., 1993) to determine where and when the four MRF proteins are expressed in mouse somites. We find that each MRF protein has a distinct expression pattern in the somites. These unexpectedly complex MRF expression patterns define subdomains in somites and suggest that myogenic cells arise in the somites via multiple cellular origins and molecular pathways.

Materials and Methods

Myosin heavy chain proteins were detected with mAb F59, an IgG1, which reacts with all known striated muscle MHC isoforms in the mouse, but does not react with smooth muscle or cytoskeletal MHCs (Miller et al., 1985, 1989; Miller and Stockdale, 1986a,b; Miller, 1990; Smith and Miller, 1992). Myogenin protein was detected with the mouse anti-myogenin mAb F5D, an IgG1, which was provided by Dr. W. E. Wright (University of Texas Southwestern Medical School, Dallas, TX) (Wright et al., 1991; Cusella-De Angelis et al., 1992). The MyoD, Myf-5, and MRF4 proteins were detected with the same three polyclonal antisera which were used in previous work (Smith et al., 1993) and were provided by Dr. S. F. Konieczny (Purdue University, Lafayette, IN). The anti-MRF sera were prepared by Drs. Konieczny and S. J. Rhodes using glutathione-S-transferase-MyoD, -Myf-5, or -MRF4 fusion proteins as immunogens, and the specific reaction of each antiserum with a single MRF was confirmed by both immunoprecipitation and immunofluorescence analyses (Smith et al., 1993).

Embryos were obtained from timed pregnant females of the outbred CD-1 strain of mice (Charles River Breeding Laboratories, Wilmington, MA). After dissection and fixation (see below), the number of somite pairs was counted and noted for each embryo to be analyzed. Embryos of 8.5-d gestation (E8.5) typically had 7–12 somite pairs, E9 embryos had 16–22 somite pairs, E9.5 embryos had 24–28 somite pairs, and E10.5 embryos had 34–38 somite pairs. Somites are numbered from head to tail, with the most rostral being number 1. To compare MRF expression patterns among different groups of somites, those somites numbered 1–8 are termed “neck”, somites 8–12 are termed “forelimb bud”, somites 11–24 are termed “trunk”, somites 25–29 are termed “hindlimb bud” (Milaire, 1976), and somites numbered ≥ 30 are termed “tail”. Somites numbers 11 and 12 are included with both forelimb bud and trunk somites, because they are adjacent to the forelimb bud and also appear to contribute to the ribs (Theiler, 1989). The number of an individual somite could be determined within one in reconstructions of serial sections. We therefore adopted the convention of Hinterberger et al. (1991), in which a somite known, for example, to be either number 8 or number 9 is referred to as somite 8/9.

For analyses of tissue sections, E8.5–E10.5 embryos were dissected free of external membranes, immediately placed in PBS supplemented with 4% paraformaldehyde, and incubated overnight at 4°C with agitation. Fixed embryos were subjected to two washes of 30 min each in PBS at room temperature, followed by incubation for 2 h at room temperature in PBS containing 2% bovine serum albumin, 2% normal horse serum, 2% normal goat serum, and 0.1% Triton X-100 (blocking solution). Fixed and blocked embryos were infiltrated with sucrose by sequential incubations of 3 h at room temperature in blocking solution modified to contain 12.5% sucrose, and overnight at 4°C in blocking solution modified to contain 25% sucrose. Infiltrated embryos were mounted in Tissue-Tek O.C.T. compound (Miles Laboratories, Elkhart, IN), frozen in liquid nitrogen, and either immediately sectioned or stored at -85°C .

Frozen sections of 10 μm thickness were cut at -26°C , collected on slides, washed in PBS for 5 min at room temperature, incubated for 10 min at room temperature in 90% methanol containing 3% hydrogen peroxide to inhibit endogenous peroxidases, and incubated in blocking solution for 60 min at 37°C. Anti-sera were diluted 1:500 and mAb hybridoma supernatants were diluted 1:5 in blocking solution; and sections were incubated with the diluted antibodies overnight at 4°C. After four washes of 30 min each in PBS containing 0.1% Triton X-100 at room temperature with agitation, sections were incubated with a biotinylated secondary antibody, either goat anti-rabbit or horse anti-mouse IgG as appropriate, for 1 h at room temperature, and then washed as above and incubated 60 min at room temperature with an avidin-horseradish peroxidase complex in PBS prepared according to manufacturer's specifications (Vectastain Elite Kit; Vector Laboratories, Burlingame, CA). Antibody binding was detected by developing the slides for 0.5–2 min at room temperature using diaminobenzidine as substrate. Af-

ter a short wash in water, the slides were mounted under a cover glass in PBS containing 90% glycerol. For double labeling, sections stained for a MRF with the horseradish peroxidase system were further incubated in a 1:5 dilution of anti-MHC mAb F59 hybridoma supernatant, washed as above, incubated for 1 h at room temperature in 0.5 $\mu\text{g}/\text{ml}$ fluorescein-conjugated goat anti-mouse IgG (heavy and light) and mounted under glass coverslips in 75% glycerol in PBS containing 2.5% 1,4-diazabicyclo [2, 2, 2] octane to retard fluorescence bleaching. As a control, primary antibodies were omitted from selected sections on a slide. No nuclear staining was observed in these controls, rather there was a brownish stain that was uniform throughout the section, with the intensity of the background increasing with increased time of incubation with substrate. The epidermis was nonspecifically stained in sections analyzed for myogenin; this staining was also seen when the anti-myogenin mAb was omitted and was thus due to the anti-mouse secondary reagents. Unless noted otherwise, the results reported below were reproducibly obtained with at least four independently prepared embryos.

Results

We used immunohistology to analyze MRF expression patterns in the somites of early postimplantation mouse embryos. In mice, the first, most rostral, somites form on embryonic day 8 (E8), and additional somites continue to form until the most caudal of the approximately 60 pairs of somites appears in the tail on E12–13. Thus, the rostral-caudal position of a somite reflects its age, with rostral somites being developmentally older than caudal somites. A somite forms as an epithelial ball which soon subdivides into dermatome, myotome, and sclerotome. For immunohistochemical assays of MRF proteins, we used the specific antibodies described by Smith et al. (1993). We addressed the following questions: (a) At what stages of somite development and where within a somite is each MRF protein initially detectable? (b) Do the MRFs have similar or distinct patterns of expression within a somite? (c) What is the relationship between myocyte differentiation and expression of individual MRFs?

Initial Stages of MRF Protein Expression in Somites

To determine the stage of somite development at which each MRF was initially expressed, we first determined which somites defined the rostral and caudal limits of expression of each MRF in embryos at different stages of development (not shown). The youngest embryos examined, E8.5, contained seven pairs of epithelial stage somites, and none of these somites contained cells that stained with anti-MRF antibodies. In E9 embryos (13–19 somite pairs), Myf-5, myogenin, and MRF4 were each expressed in the most rostral somites, but had different caudal limits of expression. In 16 somite embryos, for example, cells that expressed Myf-5 and myogenin were found in the 9–10 most rostral somites, whereas cells that expressed MRF4 were found in only the 3–4 most rostral somites. No cells were found to express MyoD in embryos with ≤ 19 somite pairs.

In E9.5 (24–28 somite) embryos, Myf-5, myogenin, and MRF4 had rostral and caudal limits of expression that were similar to those in E9 embryos. For all of the E9–E9.5 embryos examined, the number of unstained caudal somites was approximately six for both Myf-5 (range 5–7, $n = 6$) and myogenin (range 5–11, $n = 11$), and ~ 11 for MRF4 (range 10–13, $n = 5$). Cells that expressed each of these three MRFs were found in all of the more rostral somites, i.e., from somite 1 to the somite defining the caudal limit of expression for that MRF. At E9–9.5, the caudal limits of Myf-5 and myo-

genin expression coincided approximately with those somites in which the epithelium was transforming into dermamyotome and sclerotome.

Unlike younger embryos in which MyoD was not detected, nuclei that stained with the anti-MyoD serum were detected in embryos with ≥ 20 somites. An unexpected finding was that somites that did not contain MyoD⁺ nuclei were, at certain stages, interspersed among those that did. In one 23 somite embryo, for example, somites 1, 2, and 3 contained 3, 7, and 2 MyoD⁺ nuclei respectively; somites 6 and 9 each contained 1 MyoD⁺ nucleus, and somites 11, 12, and 13 contained 1, 2, and 1 MyoD⁺ nuclei respectively. All other somites in this embryo did not have MyoD⁺ nuclei. Similar discontinuous MyoD expression was seen in the two 26 somite embryos that we examined. The number of MyoD⁺ nuclei generally was highest in trunk somites. In one 29 somite embryo, for example, MyoD⁺ nuclei were observed in each of somites numbers 1–15, but the largest number of stained nuclei, 21, was in somite 12, whereas ≤ 8 stained nuclei were in somites 1–10. In E10.5 embryos, the MRFs no longer had the clearly different caudal limits of expression which were seen at E9.5, rather each MRF was expressed in about the same number of somites. In embryos with 35–36 somites, for example, each MRF was found in all but the most caudal 4–5 somites.

In accord with the finding that all except the most caudal 5–7 somites expressed Myf-5 in E9–9.5 embryos, the earliest stage of somite development at which Myf-5⁺ cells were detected was when the sclerotome was first forming. In the somite stained with Myf-5 serum shown in Fig. 1 A, for example, the sclerotome was forming in the ventral–medial corner, but the columnar epithelium still extended most of the way around the somite. Two Myf-5⁺ cells were found in the section shown (Fig. 1 A), and serial sections showed that this entire somite had only three Myf-5⁺ cells. Such a small number of Myf-5⁺ or myogenin⁺ cells (≤ 15) was typical of

the two or three most caudal of the somites with MRF expression.

Additional analyses showed that Myf-5 expression precedes myogenin expression by a brief period. Because Myf-5 and myogenin were expressed in approximately the same number of somites, it appeared that both must accumulate to detectable levels at almost the same developmental stage. In analyses of multiple serially sectioned embryos, however, we failed to find myogenin⁺ cells in somites as immature as those which contained Myf-5⁺ cells. Rather, the caudal-most somites with myogenin⁺ cells had well formed dermamyotomes and sclerotomes (Fig. 1 D). To confirm that Myf-5 was expressed prior to myogenin, we serially sectioned four E9.5 embryos and stained alternate sections with the anti-Myf-5 serum and anti-myogenin mAb (not shown). In each of the four embryos, there was at least one somite with Myf-5–expressing nuclei that was located caudal to the first somite with myogenin–expressing nuclei. Because more caudal expression implies earlier expression, these results suggest that the first MRF protein detectable by immunohistochemistry in somites is Myf-5, which soon is followed by myogenin, then by MRF4, and finally by MyoD.

MRF Proteins and Subdomains of Individual Somites

We next found that, within a single somite, there were several differences among the expression domains of the MRFs. One difference was in the extent of expression along the dorsal–ventral axis of a somite. This difference was apparent at early stages of somite development among the small number of Myf-5⁺ and myogenin⁺ cells in the three or four most caudal of the MRF-expressing somites. In such somites, transverse sections showed that the Myf-5⁺ cells were located in the most dorsal portion of the somite nearest the neural tube (Figs. 1, A–C, and 2 A and B), whereas the myogenin⁺ cells were found in both the dorsal and ventral

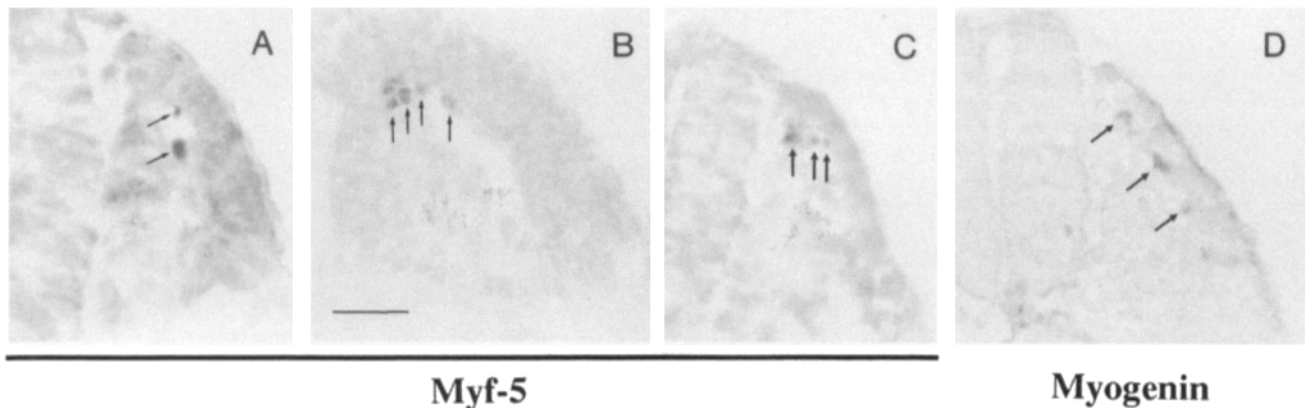


Figure 1. Initial stages of Myf-5 and myogenin expression. Serial transverse sections of E9–9.5 embryos were stained with anti-Myf-5 serum (A–C) or anti-myogenin mAb (D), and the most caudal somites which expressed a particular MRF were identified. (A) In a 13 somite embryo, the most caudal somite with Myf-5⁺ cells (somite number 6/7) was immature, as it had a columnar epithelium that still extended most of the distance around the somite. (B and C). Myf-5 was initially expressed near the neural tube in the dorsal-most portion of the somite, as shown by an oblique section through somite 4/5 of a 13 somite embryo (B) and a transverse section through somite 16/17 of a 24 somite embryo (C) (see also A). (D) In contrast to Myf-5, myogenin was initially expressed in cells located throughout the dorsal-ventral extent of the myotome (cf. Fig. 2). In a different 13 somite embryo, the most caudal somite with myogenin⁺ cells was relatively mature, as it had a columnar epithelium that was limited to the region of the dermamyotome. Thus, Myf-5 was initially expressed at an earlier stage of somite development than myogenin (see text). Dorsal up. Bar, 50 μ m.

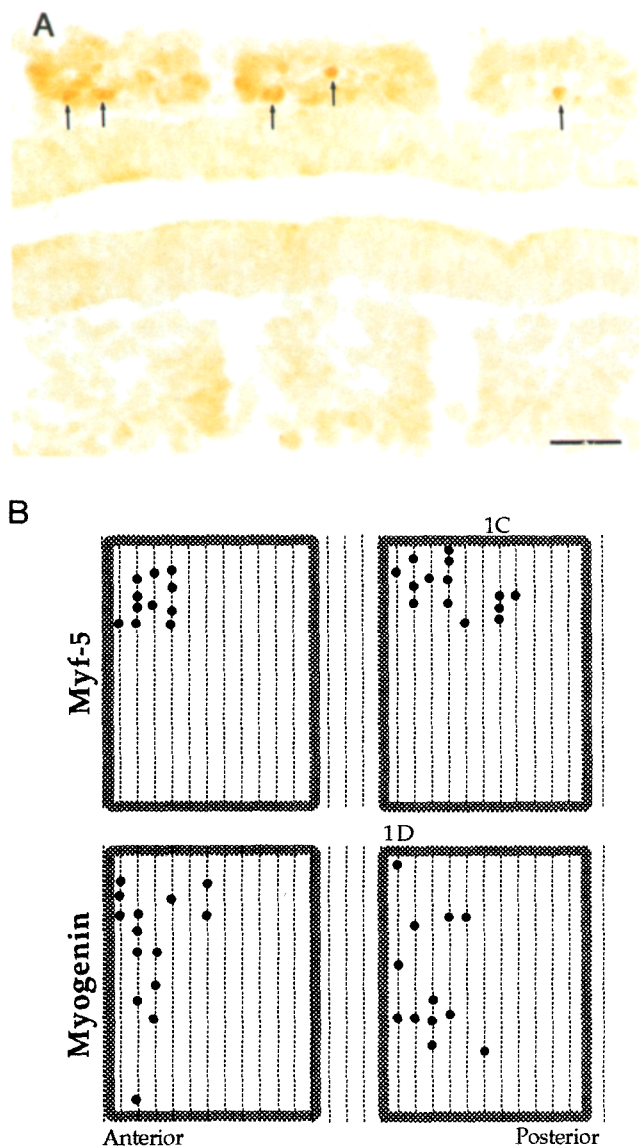


Figure 2. Myf-5 and myogenin are initially expressed in the anterior portion of a somite. Sections of E9 (13 somite) embryos were made and stained with the Myf-5 antiserum (A) or myogenin mAb (B). (A) Frontal sections, with anterior to the left, showed that Myf-5⁺ cells (arrows) were located in anterior portions of somites at this stage. A section through somites numbers 4, 5, and 6 is shown. The slightly oblique plane of section was through the dorsal halves of the somites shown above the neural tube, and through the ventral halves of the contralateral somites shown below the neural tube. The Myf-5⁺ cells (arrows) were found only in the dorsal halves of somites, near the neural tube (cf. Fig. 1, B and C). (B) Myf-5 and myogenin are initially most abundant in the anterior halves of somites; and Myf-5, but not myogenin, is also initially restricted to the dorsal region of a somite. Diagrams are based on reconstructions from serial transverse sections through two 13-somite embryos that were stained for either Myf-5 or myogenin as indicated. Each vertical dotted line represents a single section and each black dot represents the location of a MRF-expressing cell along the dorsal-ventral axis of the somite as determined from photographs or camera lucida drawings. Missing sections were either lost during processing or used as no antibody controls. Somites numbers 4/5 and 5/6 are diagrammed. Photographs of sections labeled 1B and 1D are shown in the corresponding panels of Fig. 1. Bar, 50 μ m.

halves (Figs. 1 D and 2 B). In addition, at this early stage of development, Myf-5⁺ cells were most abundant in the anterior portion of the somite, as shown by a frontal section stained for Myf-5 (Fig. 2 A). Analyses of serial transverse sections, as diagrammed in Fig. 2 B, showed that myogenin⁺ cells were similarly more abundant in the anterior portions of somites at this stage.

The dorsal-ventral differences in MRF expression domains were further analyzed in E9.5 (24–28 somite) and E10.5 (34–37 somite) embryos. In E9.5 embryos, the forelimb bud somites, numbers 8–13, that were analyzed in greatest detail have well formed dermatomes, myotomes, and sclerotomes. In serial transverse sections of E9.5 embryos, these somites had Myf-5⁺ cells that were found in only the dorsal-most 50–60% of the somites (Figs. 3 A and 4). MRF4⁺ cells appeared to occupy a larger region, extending through most of the myotome (Figs. 3 E and 4). Myogenin⁺ cells were more numerous than MRF4⁺ cells and appeared to extend throughout the full dorsal-ventral length of the myotome (Figs. 3 D and 4). Surprisingly, MyoD⁺ cells were differently distributed in neck and trunk somites in 23–26 somite embryos. The MyoD⁺ cells in neck somites were found only in the dorsal half of the somite (not shown), whereas the MyoD⁺ cells in trunk somites numbers 11–13 were found only in the ventral half of the somite (Figs. 3, B and C, and 4). An example of a control assay, in which the primary antibody was left out, is shown in Fig. 3 F. In this example, and in all other such control assays, no myotomal cells were stained above background.

To confirm the distinct dorsal-ventral distributions of Myf-5 and myogenin expression within somites, we serially sectioned E9.5 embryos and stained sections alternately with the anti-Myf-5 serum and the anti-myogenin mAb. As shown in Fig. 5, this staining of alternate sections demonstrated that myogenin-expressing nuclei were found in ventral regions of the myotome that lacked Myf-5-expressing nuclei. The distinct expression patterns were found in both neck somites (e.g., somite 9/10; Fig. 5, A–C) and trunk somites (e.g., somite 11/12, Fig. 5, D–F).

The distinct dorsal-ventral patterns of MRF expression seen in neck and trunk somites of E9.5 embryos were partially recapitulated in hindlimb bud and tail somites of E10.5 embryos (not shown). In the most caudal of the MRF-expressing somites (i.e., tail somites numbers 30–31) of 35–36 somite embryos, Myf-5 was expressed in the dorsal half of the somite and myogenin was expressed throughout the myotome as in E9.5 embryos. Unlike the somites at a similar stage of development in E9.5 embryos, however, the E10.5 tail somites also expressed MRF4 and MyoD, with the MRF4⁺ cells located mostly in the dorsal myotome and the MyoD⁺ cells located mostly in the ventral somite. In E10.5 embryos, these distinct dorsal-ventral MRF patterns were found in only two or three tail somites.

The forelimb somites of E10.5 embryos had different MRF expression patterns than either E10.5 tail somites or E9.5 forelimb somites (Fig. 6). In contrast to its restricted expression in E9.5 forelimb and E10.5 tail somites, MyoD was expressed throughout the myotomes of E10.5 forelimb somites, though staining was more intense in the ventral portion of the somite (Fig. 6 D). MRF4⁺ cells were also found throughout the myotomes of these somites, though the MRF4⁺ cells in the most ventral regions of the trunk somite myotomes

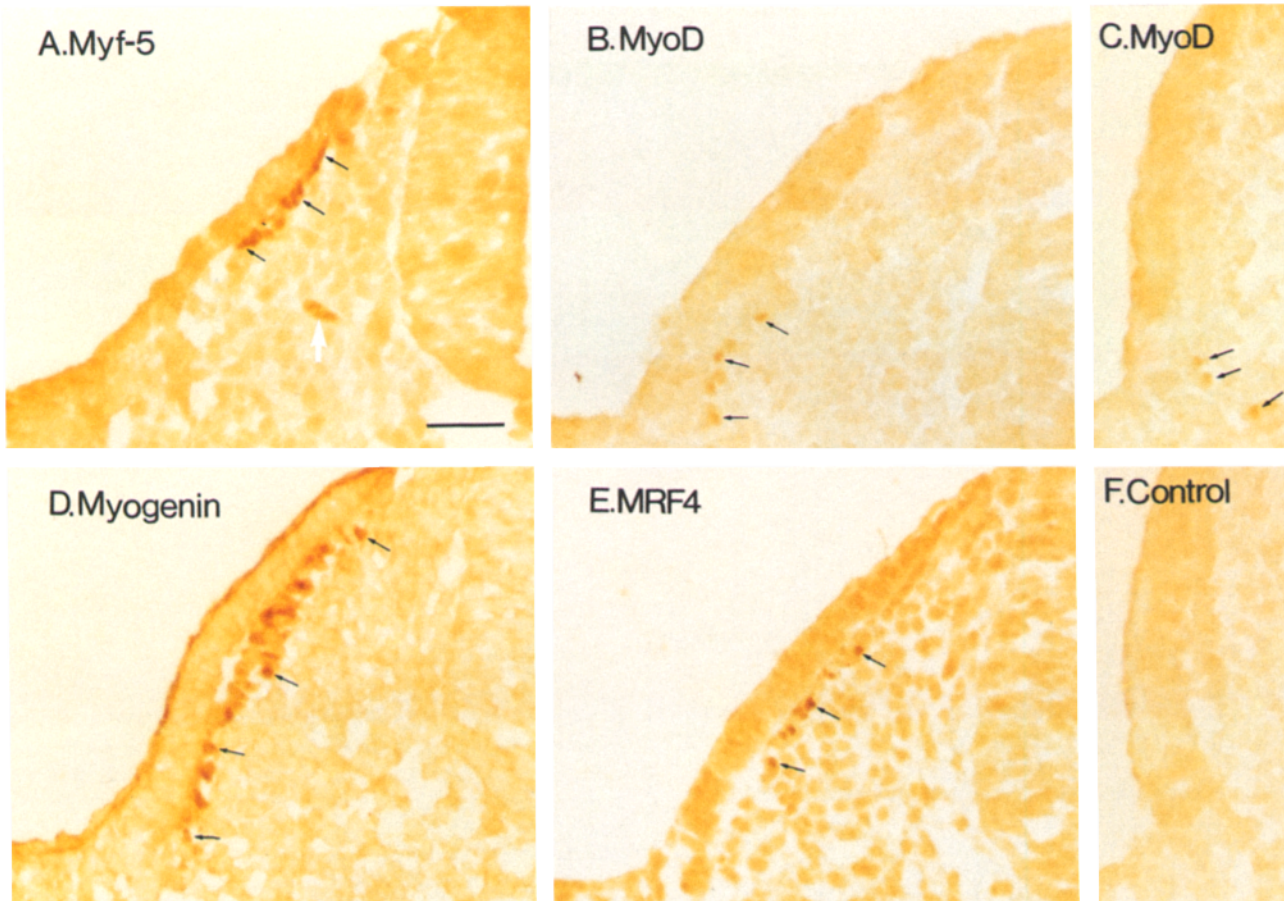


Figure 3. The MRF proteins were expressed in distinct dorsal–ventral subdomains in somites of E9.5 embryos (24–26 somites). Transverse sections of forelimb bud somites (numbers 11 or 12) were stained with the different anti-MRF antibodies as indicated in *A–E*. *F* shows an example of a control assay; the control section was from the same embryo as in *C* and was processed identically except that the anti-MyoD antiserum was omitted. Black arrows point to some of the MRF-expressing nuclei in each section and also indicate the dorsal–ventral extent of cells in the myotome that expressed each MRF. The white arrow in *A* indicates two cells in the presumptive sclerotome that stained with the Myf-5 antiserum. At this developmental stage in these somites, (*A*) Myf-5⁺ cells were only in the dorsal somite; (*B* and *C*) MyoD⁺ cells were only in the ventral somite, though, in contrast to the forelimb bud somites shown here, MyoD⁺ cells were in the dorsal half of neck somites (see text); (*D*) myogenin⁺ cells extended throughout the dorsal–ventral extent of the myotome; and (*E*) MRF4⁺ cells extended farther ventrally than Myf-5⁺ cells but not as far as myogenin⁺ cells. See Fig. 4 for additional data. Bar, 70 μ m.

were fewer in number and much more weakly stained than those in dorsal myotomes (Fig. 6 *C*). In addition, many fewer Myf-5⁺ cells, usually <10 per somite and very weakly stained, were found in E10.5 forelimb somites than in E10.5 tail somites or E9.5 forelimb somites (Fig. 6 *A*). The Myf-5⁺ cells were found throughout the myotomes, though, due to the small number of stained cells, individual sections (e.g., Fig. 6 *A*) showed no stained cells or a more limited distribution. Cells that expressed myogenin and MHC were found throughout the dorsal–ventral extent of the myotomes in E10.5 forelimb somites.

A second striking difference among MRF expression patterns was that Myf-5 and MyoD, but not myogenin or MRF4, were expressed by cells in the dermatome. In sagittal sections, the columnar epithelium of the dermatome has the shape of a shallow bowl, with the nuclei of myotomal cells lying between the lips of the dermatome. In MRF-expressing neck, forelimb bud, and trunk somites of E9–9.5 embryos, Myf-5⁺ cells were found in dorsal myotomes (cf. Figs. 3 *A* and 5 *A*), in dorsal regions of the anterior and posterior lips

of the dermatome (Fig. 7 *A*), and in the dorsal–medial lip of the dermatome, nearest the neural tube (Fig. 5 *F*). In E10.5 embryos, the few Myf-5⁺ cells that remained in these same somites appeared to be restricted to the myotome. Though the few MyoD⁺ cells in E9.5 embryos were only in myotomes, E10.5 embryos had many MyoD⁺ cells in the anterior and posterior lips of the ventral dermatome in the trunk somites (Fig. 7 *D*), and a few in the dorsal dermatome in the neck somites. Myogenin⁺ and MRF4⁺ cells were found only in myotomes at all developmental stages examined (Figs. 7, *B* and *C*).

A final difference among the MRF expression patterns was that a few cells in what appeared to be the sclerotome stained with the anti-Myf-5 serum, but not with antibodies to the other MRFs. In E9.5 embryos, a small number of cells (≤ 5 per somite) that stained with the Myf-5 antiserum were found in the ventral–medial region of the somite, well within the presumptive sclerotome and separated by three or four cell diameters from the layer of cells that form the myotome (Figs. 3 *A* and 4). Such cells were found in six different em-

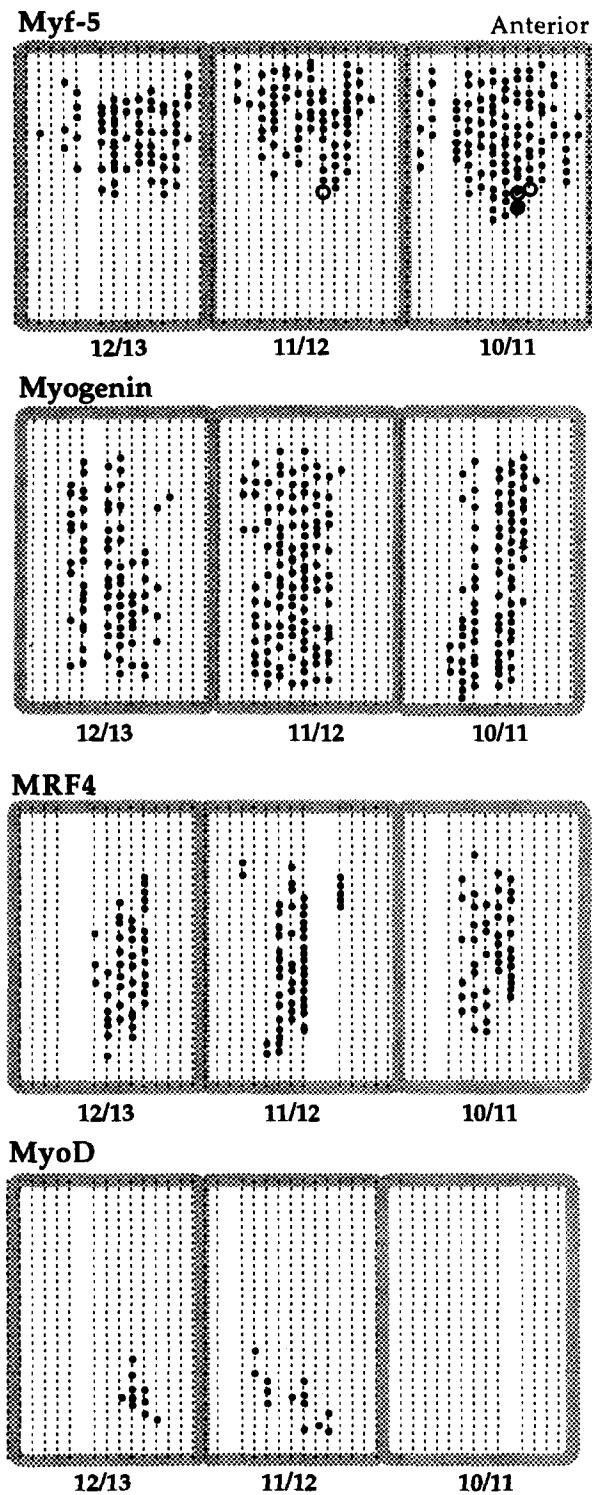


Figure 4. Distributions of MRF-expressing cells within three adjacent somites. Serial transverse sections through the forelimb bud region of E9.5 embryos were prepared, and all sections from a single embryo were stained with one of the anti-MRF antibodies. As in Fig. 2, the diagrams, which were reconstructed from photographs or camera lucida drawings, show the locations of MRF-expressing cells within adjacent somites. Small circles in the Myf-5 somites represent positive cells in the presumptive sclerotome (cf. Fig. 3 A). The numbers below each diagram give the number of the somite which was examined.

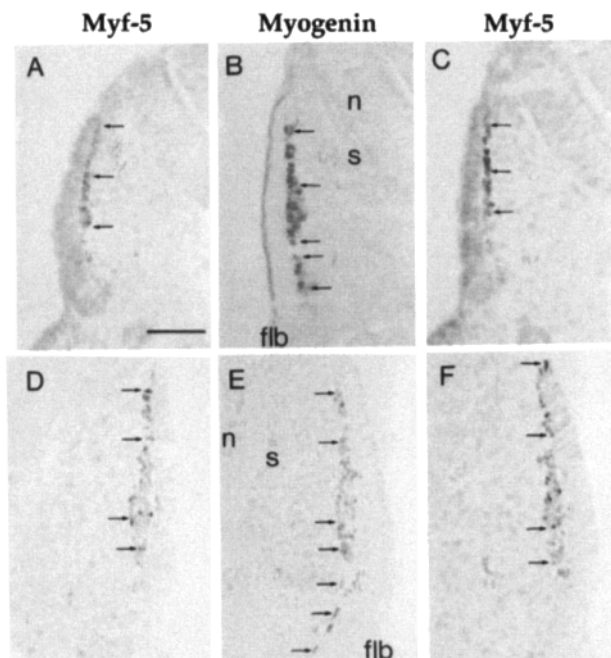


Figure 5. Distinct dorsal-ventral distributions of myogenin and Myf-5 within single somites. Serial transverse sections of E9.5 embryos were made, and those through the left side of neck somite 9/10 (A-C) and the right side of trunk somite 11/12 (D-F) are shown. As indicated, every other section was stained with the anti-Myf-5 serum, and the remaining alternate sections were stained with the anti-myogenin mAb. Arrows indicate some of the cells that express each MRF, as well as the dorsal-ventral extent of the MRF-expressing cells within the myotomes. The myogenin-expressing cells extended farther ventrally than the Myf-5-expressing cells. Also indicated are the neural tube (*n*); presumptive sclerotome (*s*); and forelimb bud (*fib*). Bar, 70 μ m.

bryos that were examined, but were not found in every somite within a particular embryo. In one 26 somite embryo, for example, four pairs of somites in the forelimb bud region were examined in serial transverse sections, and three out of the eight somites were found to contain cells in the sclerotome that stained with the anti-Myf-5 serum. In E10.5 embryos, we did not observe cells in sclerotomes that stained with the anti-Myf-5 serum.

MRFs and Myosin

Previous analyses by immunofluorescence showed that myocytes which formed from early somite cells in vitro could contain MHC in the absence of detectable levels of any of the four MRFs (Cusella-De Angelis et al., 1992; Smith et al., 1993). Because the immunoperoxidase detection system used here was more sensitive than immunofluorescence, we reinvestigated MRF and MHC expression in myocytes formed from somite cells. Cells were cultured from E8.5 somites (Smith et al., 1993) and, after three days of culture, the cells were stained simultaneously with mAb F59 followed by fluorescein-conjugated goat anti-mouse IgG and with an anti-MRF followed by the immunoperoxidase system. Though the peroxidase system did give stronger nuclear signals than fluorescence, the staining patterns, including the

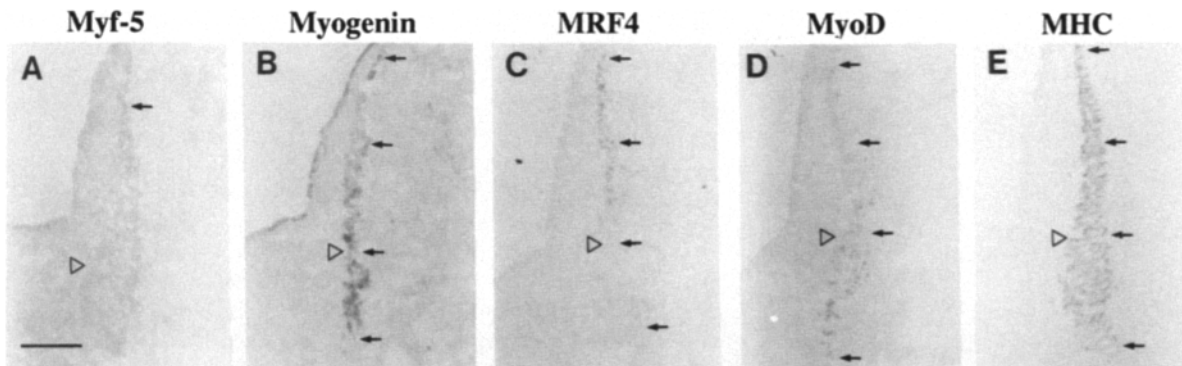


Figure 6. MRF and MHC protein expression in trunk somites of E10.5 embryos. Transverse sections of trunk somites numbers 11 or 12 of E10.5 embryos were stained with the anti-MRF antibodies or anti-MHC mAb as indicated. Arrows indicate regions containing cells that stained with a particular antibody. At this stage, myotomes in trunk somites extend farther ventrally than those in more rostral somites; the open arrowheads indicate where the ventral myotome borders would be in somites numbers 8, 9, or 10. The myogenin and MHC antibodies stained cells strongly throughout the dorsal-ventral extent of the myotome. The MRF4 antibody stained cells strongly in the dorsal myotome, but weakly in the ventral myotome; whereas the MyoD antibody showed the reverse pattern, with more staining in the ventral than dorsal myotome. Bar, 100 μ m.

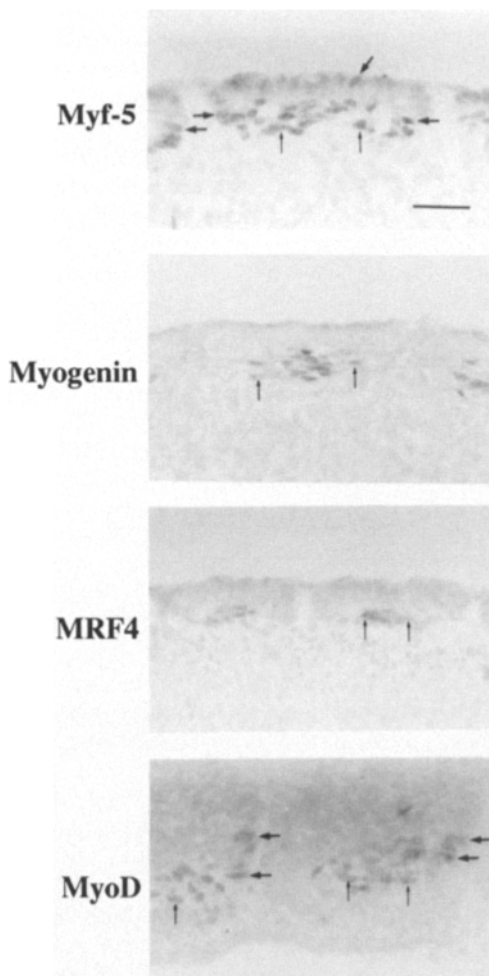


Figure 7. Cells that expressed Myf-5 and MyoD, but not myogenin or MRF4, were found among the columnar epithelial cells of the dermatome. Sections through forelimb bud somites of E9.5 (*Myf-5*, *myogenin*, and *MRF4*) or E10.5 (*MyoD*) embryos were stained with the anti-MRF antibodies as indicated. Large arrows indicate cells in the lips of dorsal dermatomes that were stained with the Myf-5

presence of myocytes that stained with anti-MHC but not anti-MRF, were similar with both methods.

To examine the relationship between MRF expression and myocyte differentiation, we first determined which somites defined the rostral and caudal limits of MHC expression in embryos of different ages. In E9.5 embryos, MHC expression was found in all rostral somites, but was not detected in \sim 11 (range 10–13, $n = 5$) of the most caudal somites; thus, MHC was expressed in more somites than MyoD, about the same number of somites as MRF4, and in fewer somites than Myf-5 or myogenin. MHC staining in E9.5 myocytes was filamentous but not striated; and staining at the ends of myocytes often showed swellings and bifurcations (Fig. 8 B). In E10.5 embryos, MHC was expressed in all but the five or six most caudal somites, which was about the same number of somites as expressed the four MRF proteins at this stage.

In myotomes, each of the four MRFs was expressed both by MHC⁺ myocytes and by cells that did not express MHC. Double labeling showed that cells in the most dorsal regions of myotomes expressed one or more MRFs but not MHC. Such cells were found by E9 for myogenin, Myf-5, and MRF4, and by E10 for MyoD. Examples of cells that expressed MRF4, but not MHC, are shown in Fig. 8. These presumptive myoblasts were located in a hemispherical or triangular region on the dorsal edge of the myotome. MHC⁺ myocytes were found in a band beginning just ventral to the presumptive myoblasts (Fig. 8). Forelimb bud somites in E9.5 embryos contained no more than 30 or 40 myocytes which were found in only the dorsal half of the somite (not

antibody (see also Fig. 5 F) and ventral dermatomes that were stained with the MyoD antibody. Cells in both the anterior and posterior lips of the dermatome stained with Myf-5 and MyoD, though, in the figure shown, only the posterior lip has MyoD⁺ cells. Small arrows in each panel indicate cells in the myotomes that stained with a particular antibody. Sections were through the dorsal half of the somite for Myf-5, myogenin, and MRF4, and through the ventral half of the somite for MyoD. Anterior is to the left. Bar, 40 μ m.

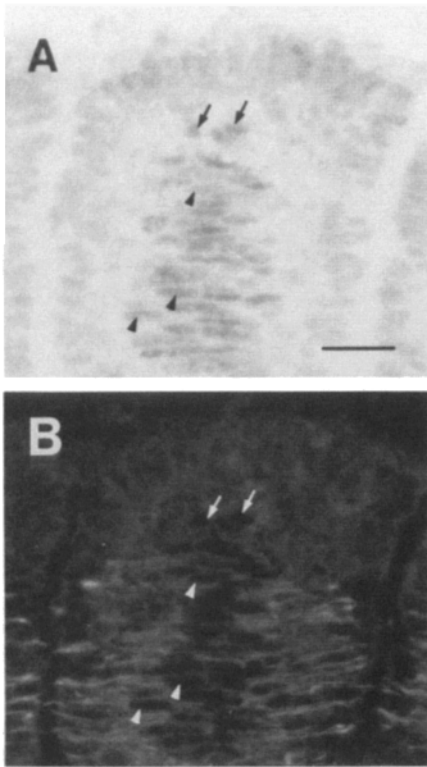


Figure 8. Both myocytes and presumptive myoblasts expressed MRF4. A single sagittal section of trunk somite number 13/14 of an E9.5 embryo was doubly stained with the anti-MRF4 mAb and peroxidase for light microscopy (A) and with the anti-MHC mAb for fluorescence microscopy (B). Arrows indicate presumptive myoblasts in the dorsal region of the myotome that stained with the MRF4, but not the MHC, antibody. Arrowheads indicate myocytes that both expressed MHC and contained MRF4-expressing nuclei. Nuclei in all myocytes appeared to contain MRF4. Bar, 30 μm .

shown), whereas the same somites in E10.5 embryos contained many more myocytes which were found throughout the dorsal-ventral extent of the myotome (Fig. 6 E). Though it was sometimes difficult to assign a particular nucleus to a particular myocyte, it appeared that all myocytes at the forelimb bud region of E9.5 embryos expressed myogenin, and perhaps Myf-5 and MRF4 as well. As an example, myocytes with MRF4-expressing nuclei are shown in Fig. 8. Consistent with MyoD appearing after MHC, the myocytes in E9.5 embryos failed to express MyoD, whereas myocytes in E10.5 embryos did express MyoD. In the most rostral somites at E9.5 (i.e., somites 1-2), some myocytes failed to express Myf-5. Nuclei in myocytes tended to be ovoid, whereas those in presumptive myoblasts tended to be round. In E9.5 embryos, the ventral border of the band of myocytes was close to the ventral limits of Myf-5 and MRF4 expression. By doubling labeling (not shown), myogenin⁺ cells were observed that lay ventral, as well as dorsal, to the myocytes, whereas Myf-5⁺ cells were not found ventral to the myocytes.

Discussion

By immunohistology, we found that the four muscle regulatory factor proteins are expressed in distinct patterns in

mouse somites. Dermatome, myotome, and sclerotome cells, as well as the dorsal and ventral halves of somites, had transiently different staining patterns with each MRF antibody. Because cells in different regions of a somite express the MRFs in different patterns, it seems likely that myogenic cells in the somites have multiple sites of origin and form via multiple molecular pathways.

MRF proteins first accumulate in the anterior half of a somite at about the time the sclerotome begins to form. The Myf-5 protein was found in somites before formation of the dermamyotome and was initially detectable in only dorsal-anterior cells. Because the three or four most recently formed somites may contain Myf-5 mRNA (Ott et al., 1991) but not detectable Myf-5 protein, it appears that a few hours could be required for the Myf-5 protein to accumulate to a detectable level. Accumulation of the myogenin protein, which appears to occur shortly after Myf-5, also was detected first in anterior cells, though myogenin⁺ cells were not additionally restricted to the dorsal half as were Myf-5⁺ cells. These results are consistent with studies showing that the anterior and posterior halves of somites have distinct molecular and functional properties (Stern and Keynes, 1987), and that the muscle-specific expression of desmin, though it begins later than Myf-5, also initiates in dorsal-anterior cells (Kaehn et al., 1988). At later stages, MRF expression patterns in anterior and posterior halves of somite become identical.

To the extent that MRF expression is a marker for committed myogenic cells, the MRF expression patterns in the dermatomes suggest that myogenic cells originate at sites in addition to the dorsal-anterior region of the somite. Though Myf-5 is initially expressed only in the dorsal-anterior somite, it is later expressed by cells in all lips of the dorsal dermatome, suggesting that myogenic cells arise in the posterior, as well as the anterior, half of the dermatome. Also, in specific somites, MyoD is expressed in both the anterior and posterior lips of the ventral dermatome. The ultimate fates of the MyoD- and Myf-5-expressing cells in the E9.5 dermatomes are unknown, though it is known that either MyoD or Myf-5 must be expressed for myoblasts to form (Rudnicki et al., 1993). Dermatome cells likely enter the myotome as myoblasts and form myocytes, consistent with the idea that the dermatome is a continuing source of myogenic cells during somite development (Milaire, 1976; Ordahl, 1993).

The MRFs are expressed in different patterns in dorsal and ventral somites, and the myogenic cells in these regions may thus arise via distinct molecular pathways. In particular, cells in the dorsal dermatome express Myf-5, whereas cells in the ventral dermatome express MyoD, suggesting that Myf-5 and MyoD expression are under different controls in the dorsal and ventral halves of a trunk somite. Because previous work showed that mice with an inactivated *myoD* gene formed muscles normally but had abnormally high Myf-5 levels (Rudnicki et al., 1992), it is possible that Myf-5 expression spreads to the ventral dermatome in the absence of MyoD. Previous work also showed that myogenic cells in the limb bud and visceral arches express the MRF mRNAs in different sequences than cells in the somites (Ott et al., 1991; Hinterberger et al., 1991) and that, in culture, somite cells express a different pattern of MRF proteins than limb bud cells (Smith et al., 1993). Because individual MRFs appear,

in some cases, to activate different subsets of muscle-specific genes (Brennan et al., 1990; Miller, 1990; Yutzey et al., 1990; Block and Miller, 1992; Braun et al., 1992), the different sequences of MRF expression in the somites could lead to distinct muscle cell phenotypes.

Neck and trunk somites differ in structure and MyoD expression. In structure, the myotomes and dermatomes of E10 trunk somites (i.e., beginning with and caudal to somite number 11) extend ventrally farther than those of more rostral somites. This size difference can be clearly seen in the expression patterns of myogenin promoter-*lacZ* and desmin promoter-*lacZ* fusion genes in E10.5 transgenic mice (Cheng et al., 1993; Yee and Rigby, 1993; Li et al., 1993). Somite 11 appears to be the most rostral somite involved in rib formation, so the ventral extensions of the myotomes may contribute to the formation of intercostal muscles. These structural differences may arise due to different patterns of cell death, as seen in the corresponding somites in chickens (Tosney, K. W. 1994. *J. Cell Biochem.* 188[Suppl]:462a). The MyoD protein first accumulates in the dorsal myotome of neck somites, but in the ventral myotome and dermatome of trunk somites. The Pax-3 mRNA also appears to accumulate in the ventral regions of trunk somites (Bober et al. 1994; Williams and Ordahl, 1994; Goulding et al., 1994). Thus, as is the case for myogenin (Cheng et al., 1993; Yee and Rigby, 1993), expression of MyoD, and perhaps the other MRFs, is likely to be controlled by different mechanisms in dorsal and ventral regions of neck and trunk somites.

An unexpected finding was that the anti-Myf-5 serum stained a few cells in the presumptive sclerotome of E9.5 forelimb bud somites. This result raises the possibility that the rib-deficient phenotype seen in mice without Myf-5 (Braun et al., 1992) is due to loss of Myf-5 expression in the sclerotome. Myf-5 mRNA, however, has not been reported in sclerotome cells, though transient expression in a few cells may not be apparent by in situ hybridization. Possible expression of Myf-5 in the sclerotome thus requires confirmation, for example by use of independent anti-Myf-5 sera or additional in situ mRNA analyses.

By E10.5, differences in MRF expression patterns were found in only the two or three most caudal of the MRF-expressing somites. In the more rostral somites at E10.5, each of the MRFs was expressed throughout the myotome, though Myf-5 was expressed by far fewer cells in the more rostral somites than in the more caudal somites. Also, in E10.5 embryos, MRF4, MHC, and MyoD were expressed in about the same numbers of somites as Myf-5 and myogenin, rather than in fewer somites as in E9.5 embryos. Thus, myogenesis appears to occur more rapidly in somites which form later in development.

All somitic myocytes appeared to express myogenin, and likely one or more of the other MRFs. A previous study suggested that at least some myocytes in E9.5–10.5 somites expressed MHC, but not the myogenin or MyoD proteins (Cusella-De Angelis et al., 1992). In E9.5 embryos, we found myocytes that failed to express MyoD, a result that was expected because MyoD is expressed after MHC. In contrast to Cusella-De Angelis et al. (1992), however, we detected the myogenin protein more than one day earlier in development and the myocytes that we observed in E9–9.5 embryos did express myogenin. Because both groups used the same anti-myogenin mAb, the different results were likely due to differ-

ent fixation conditions or the higher sensitivity of the peroxidase system compared to fluorescence. In addition, with the exception of a subgroup of myocytes in the most rostral somites that failed to express Myf-5, the myocytes in E9.5 embryos appeared to express MRF4 and Myf-5 in addition to myogenin. Thus, though it is possible that we missed critical somites or developmental stages, we did not find evidence of myocytes in vivo that differentiated in the absence of MRF expression. The origin of the MRF⁻/MHC⁺ myocytes in somite cell cultures (Cusella-De Angelis et al., 1992; Smith et al., 1993; this work), therefore, remains unclear, though it is likely that they are descendants of cells that previously expressed MRFs. Our results also showed, as in cultures (Smith et al., 1993), that each MRF is expressed in both myocytes and myoblasts.

Though immunohistology uncovered aspects of MRF protein expression that were not anticipated from in situ hybridization studies, the expression patterns of the MRF mRNAs are consistent with the overall expression patterns of the MRF proteins. In rodents, Myf-5 mRNA is the first detectable MRF mRNA and is followed in sequence by the myogenin, MRF4, and MyoD mRNAs (reviewed by Buckingham, 1992). The MRF proteins appeared in the same sequence, and each MRF protein was first detectable within a few hours of the first appearance of its mRNA. The first MRF mRNAs to be expressed are found in the dorsal-medial region of epithelial stage somites (Ott et al., 1991; Pownall and Emerson, 1992). Similarly, the Myf-5 protein was first detectable in the dorsal half of somites before full separation of the sclerotome and dermatomyotome. Also, in the neck somites, the Myf-5 mRNA and protein both become less abundant by E10.5. Additional aspects of a MRF protein expression that were revealed by the single cell resolution of immunohistology included initial expression in the anterior half of a somite and the different expression patterns in dorsal vs. ventral somites, neck vs. trunk somites, and myotome vs. dermatome and sclerotome.

Some limitations of the assay must be kept in mind when interpreting MRF antibody staining patterns. First, because MRF function is modulated by phosphorylation and interactions with additional proteins (reviewed by Olson, 1992; Weintraub, 1993), the presence of a MRF protein in a cell does not necessarily indicate that it is active as a transcription factor. Second, because detection limits of antibodies may differ and immunohistology is not quantitative, our assays could have missed low amounts of MRFs or failed to detect additional variations in MRF levels which have functional significance. Third, due to the lack of a reliable double fluorescence system, it is not clear if all cells in a particular region express the same combination of MRFs, or if cells with different MRF expression patterns are intermingled. Finally, an antibody may react with non-MRF proteins. The antibodies used here react with only a single MRF in precipitation and immunocytology assays, and each fails to react with those additional bHLH proteins (e.g., E12, Id) against which it has been tested (Wright et al., 1991; Smith et al., 1993). Each antibody also reacts with only one MRF in mice, because each gave a distinct pattern of staining in the somites and each stained somites only when the corresponding MRF mRNA is expressed. Reaction of an antibody with additional proteins thus seems unlikely in E8.5–E10.5 embryos.

MRF protein expression patterns may distinguish the multiple myogenic cell lineages and signaling systems that occur in somites. As determined by half somite transplantations, distinct myogenic cell lineages arise in the dorsal (medial) and ventral (lateral) regions of chicken somites (Ordahl and LeDouarin, 1992). We find that dorsal and ventral somite cells, and thus perhaps the two lineages of myogenic cells, express distinct patterns of the MRFs. Also, a signal(s) from the neural tube/notochord is required for differentiation of the dorsal, but not ventral, myogenic cells (Christ et al., 1992; Rong et al., 1992) and local signals allow survival of ventral dermomyotome in trunk, but not limb bud, somites (Tosney, K. W. 1994. *J. Cell Biochem.* 18D[Suppl.]:462a). MRF expression in the dorsal-medial and ventral-lateral regions of all somites and in the extended ventral regions of trunk somites may be activated by different combinations of intercellular and intracellular mechanisms. This possibility is supported by the finding that distinct regulatory elements govern *myogenin* gene transcription in dorsal and ventral trunk somites (Cheng et al., 1993; Yee and Rigby, 1993). Distinct regulatory elements also control *myf-5* gene expression in somite and head muscle cells (Papoutian et al., 1993).

Myogenic cells may migrate to limb buds from the lateral surface of the ventral dermatome (Milaire, 1976) and apparently do not express MRFs during migration (Buckingham, 1992; Ordahl, 1993; Sassoon, 1993; Cheng et al., 1992; Yee and Rigby, 1993). Thus, because cells in the ventral myotome express a distinct MRF pattern and are located on the medial side of the dermatome, they could be considered a third type of myogenic cell, different from both the dorsal myotomal cells and the ventral cells that migrate into the limb buds. The unexpectedly complex expression patterns of the MRF proteins thus provide molecular markers for subdomains of mouse somites and suggest that myogenic cells in the somites arise at multiple sites and via multiple molecular pathways and signals.

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