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A unique pattern of expression of the four muscle regulatory factor proteins distinguishes somitic from embryonic, fetal and newborn mouse myogenic cells

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SUMMARY

A unique pattern of expression of the four muscle regulatory factor (MRF) proteins was found to distinguish early somitic from embryonic, fetal and newborn limb myogenic cells in vitro. Expression of the myosin heavy chain (MHC), MyoD, myogenin, Myf-5, and MRF4 proteins was examined by immunocytochemistry in cultures of four distinct types of mouse myogenic cells: somitic (E8.5), embryonic (E11.5), fetal (E16.5) and newborn limb. In embryonic, fetal and newborn cultures, the MRF proteins were expressed in generally similar patterns: MyoD was the first MRF expressed; MyoD and myogenin were expressed by more cells than Myf-5 or MRF4; and each of the four MRFs was found both in cells that expressed MHC and in cells that did not express MHC. In cultures of somitic cells, in contrast, Myf-5 was expressed first and by more cells than MyoD or myogenin; MRF4 was not detected; and the MRFs were never found to be coexpressed with MHC in the same cell. Thus, some somitic cells had the unexpected ability to maintain MHC expression in the absence of detectable MRF protein expression. The different myogenic programs of embryonic, fetal and newborn myogenic cells are not, therefore, a simple result of qualitatively different MRF expression patterns, whereas myogenesis by somitic cells does include a unique pattern of MRF expression.

Key words: myogenesis, myoblasts, muscle regulatory factors, somites, mouse

INTRODUCTION

One of the hallmarks of skeletal myogenesis is the formation of diverse types of myoblasts and skeletal muscle fibers. Distinct types of myoblasts - termed somitic, embryonic, fetal and satellite cells - appear sequentially at different stages of development, migrate to muscle-forming regions of the embryo, and fuse to form multinucleated muscle fibers. The different types of myoblasts can be distinguished based on their different culture requirements, abilities to fuse, and the morphologies and biochemical phenotypes of the myotubes that they form (reviewed by Cossu and Molinaro, 1987; Miller and Stockdale, 1987; Stockdale, 1990; Miller, 1991, 1992). These distinct types of myoblasts participate in the formation of the diverse types of muscle fibers (e.g. fast and slow) which are found in vertebrates. In the mouse, the first differentiated muscle cells appear in the somites on about embryonic day 9 (E9). Subsequently, primary (beginning at approx. E13) and secondary (beginning at approx. E16) generations of muscle fibers form in the limbs and trunk (Kelly, 1983). Each step of myogenesis can be controlled by intrinsic or extrinsic factors such as the stage of development and location in the embryo at which a myogenic cell arises, thyroid hormone or growth factor levels, extracellular matrix components, and interactions with innervating neurons. It is not yet clear how different types of myogenic cells are generated, though members of the MyoD family of muscle-specific transcription factors are likely candidates for regulators of myogenic cell diversification.

The basic helix-loop-helix family of muscle regulatory factors (MRFs) has four known members - MyoD, myogenin, Myf-5, and MRF4/Myf-6/herculin (reviewed by Weintraub et al., 1991). Expression of any one of the four proteins will convert many types of non-myogenic cells into cells capable of myoblast fusion and muscle-specific gene expression. The multiplicity of MRFs suggests that each might regulate a distinct subset of genes, and that differential MRF expression might thus underlie myogenic cell diversity. Supporting this possibility are the findings that (i) the MyoD, myogenin, Myf-5, and MRF4 mRNAs are expressed in distinct patterns in developing and adult rodent

muscles (Bober et al., 1991; Hinterberger et al., 1991; Hannon et al., 1992); (ii) myogenic cell line phenotypes can be altered by changing which MRFs are expressed (Brennan et al., 1990; Miller, 1990; Block and Miller, 1992); and (iii) the promoters of several muscle-specific genes are differentially activated by individual MRFs in co-transfection assays (Yutzey et al., 1990; Chakraborty et al., 1991; Fujisawa-Sehara et al., 1992).

Recently, Cusella-De Angelis et al. (1992) found that myotubes formed from embryonic and fetal limb myoblasts express the MyoD and myogenin proteins, whereas a population of myosin-expressing cells formed by somitic myoblasts fail to express MyoD and myogenin. Though Myf-5 and MRF4 expression patterns were not examined, these results raised the intriguing possibilities that somitic myoblasts might be able to differentiate in the absence of MRF proteins and that different myogenic programs might be controlled by differential expression of the MRFs. To further examine the possibility that MRF expression patterns might control myogenic cell diversification, we have used immunocytochemistry to determine the patterns of expression of all four MRF proteins in somitic, embryonic, fetal and newborn mouse myogenic cells in vitro. Our results suggest that differential MRF expression is one, though likely not the only, mechanism for generating myogenic cell diversity.

MATERIALS AND METHODS

Dissection, culture and nomenclature

Embryos from outbred CD-1 mice (Charles River) were staged according to Thieler (1989). To obtain somitic myoblasts, whole somite chains with attached neural tube were dissected from embryonic day 8.5 (E8.5) embryos that had 5-12 somite pairs. Embryonic myoblasts were from whole limb buds of E11.5 embryos; and fetal and newborn myoblasts were from limb muscles, separated from skin and bone, of E16.5 and postnatal day one (P1) mice, respectively. Tissues were dissociated by trituration (E8.5 and E11.5) or by two 15 minute incubations at 37°C in 0.05% trypsin, 0.5 mM EDTA in PBS (E16.5 and P1). Cells were cultured at a density of $40\text{-}60\times10^3$ cells per cm^2 on dishes coated with gelatin or with 10-20 μg/ml of ECL substrate (Upstate Biotechnology). All cultures were grown in DMEM supplemented with 15% fetal calf serum, 4% chicken embryo extract, 1 mM sodium pyruvate, 10 mM Hepes (pH 7.4), 2 mM L-glutamine and penicillin. Medium was used at 0.2-0.3 ml/cm^2 and cultures were incubated in a humidified atmosphere of 95% air, 5% CO_2 at 37°C.

Myoblasts are myogenic, mononucleated, mitotic cells that do not express skeletal muscle myosin heavy chains (MHCs); myocytes are mononucleated cells that express MHC(s); and myotubes are multinucleated, non-mitotic cells that express MHC(s). Myoblasts from E8.5 somites, and from E11.5, E16.5, and P1 limbs are termed somitic, embryonic, fetal and newborn respectively to indicate time and place of origin and to reflect their distinct myogenic programs (Cossu and Molinaro, 1987; Cusella-De Angelis et al., 1992; Smith and Miller, 1992). Cultures are designated by age of donor and duration of incubation. For example, an E11.5+3 culture was prepared with myoblasts from E11.5 donors and incubated for 3 days.

Generation of antisera, in vitro translation and immunoprecipitation

The entire protein coding regions of the rat MRF4 (Rhodes and Konieczny, 1989), mouse MyoD (Davis et al., 1987), rat myogenin (Wright et al., 1989) and human Myf-5 (Braun, et al., 1989) cDNAs were ligated in-frame into the glutathione S-transferase (GST) expression vectors pGEX-1, pGEX-2T, or pGEX-3X (Smith and Johnson, 1988). GST fusion proteins were isolated from IPTG-treated *E. coli* using glutathione-coated agarose beads (Smith and Johnson, 1988) and separated by SDS-PAGE in a 10% gel. After the gel was fixed and stained, the appropriate GST-MRF fusion proteins were excised from the gels and homogenized in PBS. Each GST-MRF protein (approx. 250 μg) was mixed with an equal volume of Freund's complete adjuvant and injected into New Zealand white rabbits. Subsequent injections were performed with equal mixtures of GST-MRF protein and Freund's incomplete adjuvant. After 3-4 boosts, serum was collected and stored at −20°C in the presence of 0.02% sodium azide.

Capped RNA was synthesized in vitro with T3 and T7 RNA polymerases from 1 μg of linearized pBluescript KS+ plasmid containing the rat MRF4, mouse MyoD, rat myogenin, or human Myf-5 cDNAs (Lin and Konieczny, 1992). After phenol extraction and ethanol precipitation, 10% of the RNA was translated in vitro using rabbit reticulocyte lysates containing [^{35}S]methionine per manufacturer's instructions (Promega). Synthesis of the ^{35}S-labeled MRF proteins was confirmed by SDS-PAGE and autoradiography. Immunoprecipitation of each MRF protein was performed by incubating the in vitro translated protein with 300 μl RIPA buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Nonidet-P40) containing 2 μl of each antiserum or control preimmune serum in separate reactions. The mixture was rocked gently at 4°C for 2 hours after which 100 μl of formalin-fixed *Staphylococcus aureus* cells were added for an additional 2 hours. Pellets were recovered by centrifugation and rinsed three times in 0.5 ml RIPA buffer and twice in 10 mM Tris-HCl, pH 7.0, 200 mM LiCl, 0.1% 2-mercaptoethanol. Washed pellets were resuspended in SDS-PAGE sample buffer, boiled, and analyzed by SDS-PAGE in a 12% gel. The gels were fixed, treated with En^{3}Hance (DuPont), dried, and exposed to Kodak XAR film for autoradiography (Lin and Konieczny, 1992).

Antibody specificity

Myosin heavy chain was detected with mAb F59 which reacts with all known striated muscle MHC isoforms in the mouse (Miller et al., 1985, 1989; Miller and Stockdale, 1986a,b; Miller, 1990). Individual MHC isoforms were analyzed by double immunofluorescence with mAb F59 and either mAb BF-45, specific for embryonic MHC, or mAb R11D10, specific for slow MHC (Smith and Miller, 1992). Myogenin protein was detected, in some cases, with the mouse anti-myogenin mAb 5FD (Wright et al., 1991). The specificities of the polyclonal antisera generated against GST-MRF fusion proteins were confirmed by immunoprecipitation (Fig. 1), and by immunofluorescence analysis of mouse cell lines that expressed different patterns of the four MRF mRNAs (Table 1). When compared, the anti-myogenin mAb and the anti-myogenin serum gave similar results. The myogenic BC3H-1 (Schubert et al., 1974) and C_2C_{12} (Blau et al., 1985) lines and the non-myogenic C3H10T1/2 (Reznikoff et al., 1973) line were from the American Type Culture Collection. The BF1 and BR4 cell lines were derived as described in Table 1 and Block and Miller (1992).

Immunocytochemistry

Cultures were fixed with 2% paraformaldehyde in PBS for 15 minutes at room temperature, permeabilized with 0.5% Triton X-100 in PBS for 20 minutes, and blocked in 5% horse serum, 2% fetal calf serum in PBS for one hour at 37°C. The fixed cultures were incubated overnight at 4°C in the first antibody, either a polyclonal anti-MRF serum diluted 1:500 or anti-myogenin mAb 5FD

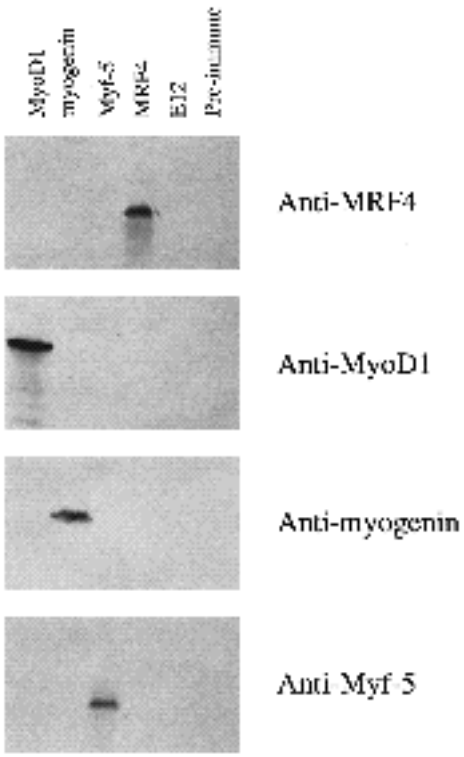

Fig. 1. Specificity of anti-MRF antisera demonstrated by immunoprecipitation. ^{35}S-labeled MRF4, MyoD, myogenin, and Myf-5 proteins were synthesized in vitro, incubated with antisera generated against glutathione S-transferase-MRF fusion proteins, immunoprecipitated with fixed *S. aureus* cells, and the immunoprecipitated proteins were analyzed by SDS-PAGE and autoradiography as described in Materials and Methods. Each antiserum immunoprecipitated only that MRF protein used to generate the serum (*e.g.* anti-MRF4 precipitated only MRF4); no cross-reactions with other MRFs or with the bHLH protein E12 were seen. Each antiserum also failed to immunoprecipitate one or more additional bHLH proteins, including Id, Myc, and E47 (not shown). Preimmune serum, taken before immunization from the same rabbit in which the antiserum was generated, did not precipitate the MRF later used for immunization.

(Wright et al., 1991) hybridoma supernatant diluted 1:5 in 2% horse serum, 2% fetal calf serum, 0.1% Triton X-100 in PBS. Cultures were gently agitated in four washes of PBS of 30 minutes each, and incubated for 1 hour at room temperature with secondary antibody diluted to 0.5 μg/ml in PBS, 2% horse serum, 2% fetal calf serum, 0.1% Triton X-100. Secondary antibodies were either fluorescein-conjugated goat anti-rabbit IgG (H+L) or fluorescein-conjugated goat anti-mouse IgG (H+L; Vector Laboratories). For each MRF and MHC, data were obtained by examining a total of 6000 cells in six E11.5 cultures, 2000 cells in four E16.5 cultures, and 1500 cells in three P1 cultures. Total cell densities were determined also (not shown); total cell numbers (myogenic plus non-myogenic) in each type of culture doubled every 18-36 hours until confluence was reached. For somitic cell cultures, all cells in an area of 0.8 cm^2 (>1000 cells) were examined in each of four experiments.

For those cells analyzed by double immunofluorescence, cultures were further incubated in the second antibody to be tested (1:5 dilution of either mAb F59 or mAb 5FD supernatants); washed as above; incubated for 1 hour at room temperature in 0.5 μg/ml Texas red-conjugated goat anti-mouse IgG (H+L); and mounted under glass coverslips in 75% glycerol in PBS containing 2.5% 1,4-diazabicyclo [2, 2, 2] octane to retard fluorescence bleaching. Nuclei were stained by including 1 μg of bisbenzamide (Hoechst 33258) per ml with the secondary antibody. Three types of nuclei were counted in each culture: (i) those that expressed the MRF under study but were not in MHC-expressing cells, (ii) those that expressed the MRF and were in MHC-expressing cells, and (iii) those that did not express the MRF but were in MHC-expressing cells.

RNA analyses

Total RNA samples were prepared (Chomczynski and Sacchi; 1987) and analyzed by electrophoresis in 1% agarose-formaldehyde gels with 100 μg/ml ethidium bromide included in the sample buffer. The separated RNAs were transferred and u.v. cross-linked to a nylon membrane (Micron Separations Inc., Westboro, MA), and the amount of transferred RNA was observed by ethidium bromide fluorescence of the rRNA bands (Block et al., 1991). MyoD, myogenin, Myf-5, and MRF4 cDNA probes were prepared by random primer labeling using [^{32}P]dCTP and the Klenow fragment of DNA polymerase I (Feinberg and Vogelstein, 1983). Templates for labeling reactions were the gel purified *Eco*RI inserts from pEMSVC11s for mouse MyoD (Davis et al., 1987), pEMSV-mouse myogenin (Wright et al., 1989), pEMSV-human Myf5 (Braun et al., 1989), or pEMSV-rat MRF4 (Rhodes and Konieczny, 1989). Probes were labeled to the same specific activity and hybridized with transferred RNAs in 50% formamide, 5× SSC, 1× PE (PE=50 mM Tris, pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, 0.2% ficoll, 0.2% polyvinylpyrolidone, 5 mM EDTA, 0.2% bovine serum albumin), 100 μg/ml salmon sperm DNA at 42°C for 16 hours. Membranes were washed with 0.1× SSC, 0.1% SDS at 65°C for MyoD and myogenin, or 0.5× SSC, 0.1% SDS at 60°C for Myf-5 and MRF4.

Reverse transcription-PCR was used to detect mouse Myf-5 and MRF4 mRNAs. MRF4 mRNA was assayed using a forward primer, TTAGAAGTGGCAGAGGGCTC, and reverse primer AGGTGCGCAGGAAATCCGCA, which amplified nucleotides 73-547 of the MRF4 coding sequence (Rhodes and Konieczny, 1989; Miner and Wold, 1990) and produced a 475 bp fragment with diagnostic *Bst*XI and *Pst*I sites (Block and Miller, 1992). Myf-5 mRNA was assayed using a forward primer, CCAAGGTGGAGATCCTCAGG, and a reverse primer, TCATAAAGTGGCAAGACAGT, which amplified nucleotides 356-900 of the Myf-5 coding sequence (Buonanno et al., 1992) and produced a 545 bp fragment that was cleaved by *Sma*I into diagnostic fragments of 175 and 370 bp. Both primer pairs spanned introns, so that amplification from contaminating genomic DNA would have generated DNAs more than 250 bp longer than those amplified from reverse transcribed mRNAs. Samples (1.0 μg) of total RNA were reverse transcribed and subjected to 30 cycles (1 minute at 94°C, 0.5 minute at 60°C, 1 minute at 72°C for MRF4; and 1 minute at 94°C, 0.5 minute at 50°C, 1.5 minute at 72°C for Myf-5) of hot start amplification (Perkin-Elmer RT-PCR kit). Amplified cDNAs were treated with *Bst*XI, *Pst*I, or *Sma*I, and analyzed by Southern blotting with ^{32}P-labeled MRF4 or Myf-5 probes.

RESULTS

To find out if differential expression of the four muscle regulatory factors (MRFs) might underlie myogenic cell diversification, we determined if the diverse types of mouse myogenic cells expressed the MRFs in distinct patterns in vitro. At least four types of mouse myoblasts have been shown by in vitro assays to arise during mouse development (Cossu and Molinaro, 1987; Smith and Miller, 1992;

Cusella-De Angelis et al., 1992). These different types of myoblasts are termed somitic, embryonic, fetal and satellite cell to reflect location and time of appearance during development, as well as the different myogenic programs that they express. Accordingly, we determined the patterns of expression of the four MRF proteins in cultures of mouse cells obtained from E8.5 somites (somitic myoblasts), E11.5 forelimbs (embryonic myoblasts), E16.5 limb muscles (fetal myoblasts), and P1 limb muscles (newborn or satellite cell myoblasts). MRF and myosin heavy chain expression patterns were compared to examine the relationship between individual MRFs and terminal differentiation.

MRF expression in cultures of embryonic, fetal and newborn muscle cells

Antisera that reacted specifically with each of the four MRFs were prepared using glutathione-S-transferase-MRF fusion proteins as immunogens and characterized by immuno-precipitation and immunofluorescence. The 5FD mAb, which is specific for myogenin, was used in some cases (Wright et al., 1991). Each of the anti-MRF sera immunoprecipitated only that single MRF against which the serum was generated (Fig. 1). The antibodies also were specific for individual MRFs when used for immunofluorescence, as shown by analyses of cell lines that expressed different combinations of the MRF mRNAs (Table 1). The antibodies thus provided specific reagents to analyze MRF expression in the diverse types of mouse myogenic cells.

To examine MRF expression patterns, cultures of embryonic, fetal and newborn muscle cells were established and assayed by immunofluorescence for expression of MHC and the MRF proteins at one, two, three, and five days in vitro. Double immunofluorescence analyses also were used in some cases as noted. As in previous work, cultures of E11.5, E16.5 and P1 myogenic cells differed in the rate of differentiation and the morphologies of the MHC-expressing cells that formed. For example, the MHC-expressing cells formed from embryonic myoblasts were mostly mononucleated myocytes, whereas fetal and newborn myoblasts formed mostly multinucleated myotubes (Cossu and Molinaro, 1987; Cusella-De Angelis et al., 1992; Smith and Miller, 1992). Differentiation, measured by MHC expression, was more rapid in embryonic cell cultures than in fetal and newborn cell cultures (Fig. 2). Though embryonic, fetal and newborn myogenic cells expressed different myogenic programs, MRF protein expression patterns were nonetheless very similar in cultures of the three types of myogenic cells (Fig. 2). Aspects of MRF and MHC expression that were shared among the embryonic, fetal and newborn cell cultures included:

(1) Expression of the MyoD protein preceded that of the myogenin and MHC proteins by about one day. Double immunofluorescence analysis of fetal cell cultures confirmed that MyoD expression preceded myogenin, because $MyoD^+$/$myogenin^-$ cells were abundant in early stages of culture, whereas in later stages of culture almost all cells that expressed MyoD also expressed myogenin (not shown). In later stages of culture, myogenin and MyoD were expressed in approximately equal numbers of nuclei.

(2) The Myf-5 protein was expressed in each type of culture, but fewer nuclei eventually expressed Myf-5 than

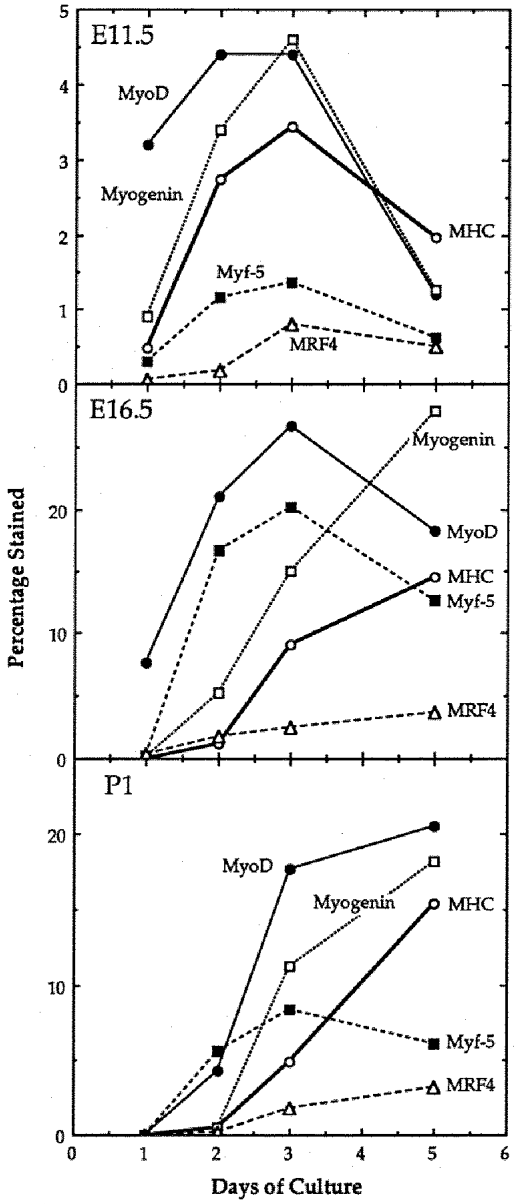

Fig. 2. Patterns of expression of the MyoD, myogenin, Myf-5, and MRF4 proteins in cultures of embryonic, fetal and newborn limb muscle cells. Cultures of embryonic (E11.5), fetal (E16.5), and newborn (P1) myogenic cells were established and analyzed by immunofluorescence for expression of MyoD, myogenin, Myf-5, MRF4, and MHC. To determine the percentage of nuclei that showed positive staining with a particular anti-MRF or were in cells that were stained with the anti-MHC mAb F59, observations were made of a total of 6000 cells in six E11.5 cultures, 2000 cells in four E16.5 cultures, and 1500 cells in three P1 cultures. As expected, based on the mixed myogenic and non-myogenic cell populations of the donor tissues, nuclei within myogenic MHC-expressing cells reached a maximum of approx. 3% of all nuclei in E11.5 + 3 cultures and approx. 15% of all nuclei in E16.5 + 5 and P1 + 5 cultures. The number of myosin-expressing cells per dish was about the same in E11.5 + 5 compared to E11.5 + 3 cultures, but the percentage of myosin-expressing cells was lower in the older cultures due to an approx. 2-fold increase in non-myogenic cells. Similar patterns of MRF expression were seen in the three types of culture.

Table 1. Expression of MRF mRNAs and reactivity with anti-MRF antibodies by mouse cell lines

Cell line* and culture stage	Expression of mRNA†				Reactivity with antibody to†			
	MyoD	Myogenin	Myf-5	MRF4	MyoD	Myogenin	Myf-5	MRF4
C_2C_{12} Myotubes	+	+	+	−	+	+	+	−
BC3H-1 Myocytes	−	+	+	−	−	+	+	−
BF1 Myoblasts	−	−	+	−	−	−	+	−
Myotubes	−	+	+	−	−	+	+	−
BR4Myoblasts	−	−	±	+	−	−	n.d.	+
Myotubes	+	+	+	+	+	+	n.d.	+
C3H10T1/2 cells	−	−	−	−	−	−	−	−

*All cell lines tested, except C3H10T1/2, were myogenic. The BF1 cell line was derived by expression of transfected human Myf-5 in BC3H-1 cells and the BR4 cell line was derived by expression of transfected rat MRF4 in BC3H-1 cells (Block and Miller, 1992).

†Cultures were examined by northern blotting for MRF mRNA expression or by immunofluorescence with antisera prepared against the indicated MRF for MRF protein expression. Cultures were analyzed at either the myoblast or differentiated myocyte/myotube stage as indicated. The autoradiographic signals of MRF mRNAs and staining by antibodies of MRF proteins in nuclei were classified as positive (+), very weak (±), or negative (−). n.d., not determined.

MyoD or myogenin. Though Myf-5 and myogenin were expressed to about the same extent in early stages of fetal and newborn cultures, MyoD and myogenin were found in approx. 1.5-3 times as many nuclei as Myf-5 in later stages of all cultures. In fetal and newborn cultures, expression of Myf-5, like that of MyoD and myogenin, preceded MHC expression, whereas in embryonic cultures, Myf-5 expression appeared to lag behind MHC expression.

(3) The MRF4 protein was expressed in a very small percentage of nuclei in early stages of culture, but this percentage increased as culture duration was lengthened. At all culture stages examined, MRF4 was expressed in a smaller percentage of nuclei than the other MRFs or MHC.

(4) All MHC-expressing cells in the limb cell cultures expressed at least one MRF. In 3 day old fetal and newborn cultures, for example, double immunofluorescence showed that every myocyte and myotube examined expressed both MyoD and myogenin, and a subset of the myocytes and myotubes also expressed Myf-5 and MRF4 (Fig. 3). In every multinucleated myotube that was observed, each nucleus within a single myotube contained the same MRFs.

(5) Expression of the MRFs was not limited to MHC-expressing cells. MyoD, myogenin, Myf-5, and MRF4 were each found in mononucleated cells that did not express MHC, as well as in myocytes and myotubes that did express MHC (Fig. 3).

To confirm the immunocytochemical assays, we used northern blotting and RT-PCR analyses to analyze MRF mRNA expression (not shown). Total RNA was prepared from embryonic, fetal and newborn cultures after one, two, three, and five days of incubation. Northern blots using 5-10 μg samples of total RNA showed that the MyoD and myogenin mRNAs were easily detectable, whereas the Myf-5 and MRF4 mRNAs were not detected under these conditions using the rat MRF4 and human Myf-5 cDNA probes. The Myf-5 and MRF4 mRNAs were, however, detected with more sensitive RT-PCR assays (see Materials and Methods). The combined northern and PCR assays showed that the MRF mRNAs were expressed in patterns similar to those of the MRF proteins: MyoD mRNA levels peaked very early, usually on day one; myogenin mRNA expression peaked about a day later than MyoD; and the Myf-5 and MRF4 mRNAs appeared at the times when the corresponding proteins were detected (not shown). Thus, the mRNA and immunocytochemical analyses suggested that embryonic, fetal and newborn myogenic cells expressed similar patterns of the MRFs in vitro.

MRF and MHC protein expression in somitic cell cultures

The patterns of MRF and MHC protein expression in somite cell cultures were markedly different from those in embryonic, fetal and newborn cultures. Cells were obtained from E8.5 somites (with neural tube and notochord), cultured, and examined by immunofluorescence for expression of MHC and the four MRFs. As expected (compare Cusella-De Angelis et al., 1992; Smith and Miller, 1992), the MHC-expressing cells that formed in the somite cell cultures were mononucleated myocytes. Analyses with MHC isoform-specific mAbs demonstrated that the embryonic and slow MHC isoforms were coexpressed in the myocytes formed in somitic cell cultures (not shown). The number of cells that expressed MHC increased as the duration of culture was lengthened, showing that the myocytes were formed in culture and were not simply survivors of the isolation procedure. MHC-expressing myocytes at all culture durations amounted to a small percentage (<5%) of the total cells. Initial experiments also showed that the somite cell cultures contained cells that expressed detectable amounts of MyoD, myogenin, and Myf-5, but not MRF4. Double immunofluorescence analyses were then used to determine if MHC and the MRFs were expressed in the same or in different cells.

The double immunofluorescence assays confirmed that the MHC, MyoD, myogenin, and Myf-5, but not MRF4, proteins were expressed in somite cell cultures, though MHC and the MRF proteins were never found to be coexpressed in the same cell (Table 2 and Fig. 4). Double immunofluorescence analyses with the anti-MHC mAb F59 and the different anti-MRF antibodies were used to analyze one and three day old E8.5 somite cell cultures. No MRF4 protein was detected in these cultures, whereas the MyoD, myogenin, and Myf-5 proteins were found to be expressed

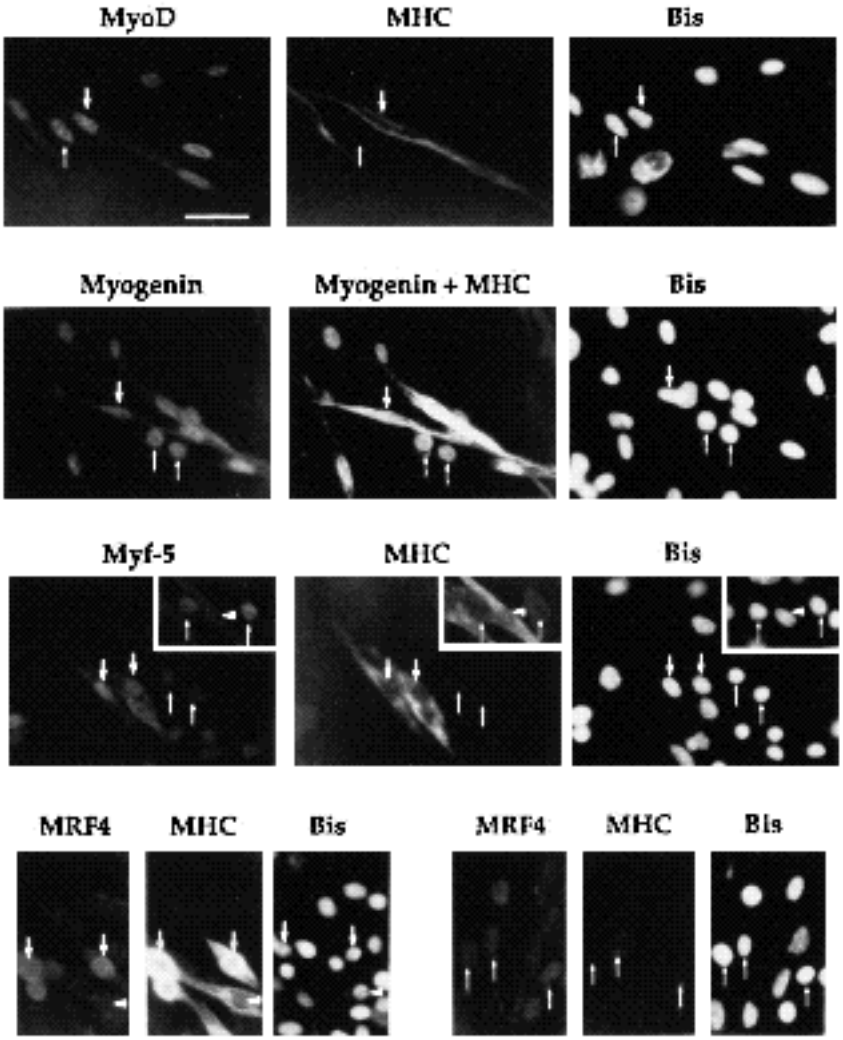

Fig. 3. In fetal cell cultures, each MRF was found both in cells that expressed MHC and in cells that did not express MHC. The pattern of MRF and MHC expression by individual cells in E16.5 + 3 cultures was analyzed by triple fluorescence. Each field of cells was examined, as indicated, for (i) expression of one of the MRFs (myogenin expression was assayed with mouse mAb 5FD in this instance; MyoD, Myf-5, and MRF4 were assayed with specific rabbit antisera; secondary antibodies were fluorescein-conjugated); (ii) expression of MHC with mAb F59 (Texas red); and (iii) location of nuclei by staining with bisbenzimide (blue fluorescence, bis). In the myogenin analysis, signals for both myogenin and MHC were seen in the Texas red channel due to the use of two mouse IgG mAbs. Three types of expression patterns are noted. Upward pointing arrows indicate cells that expressed the MRF examined but did not express MHC. Downward pointing arrows indicate cells that coexpressed the MRF and MHC. Arrowheads, in the Myf-5 insets and MRF4 photographs, indicate cells that expressed MHC but did not express Myf-5 or MRF4. Note also that each field contains cells that failed to express both MHC and the MRFs. Bar, 20 μm.

in the nuclei of a small percentage (<5%) of the cells. Remarkably, however, MHC and MRFs were never found to be simultaneously expressed in the same cell. Several hundred MHC- or MRF-expressing cells were examined in somite cultures by double immunofluorescence. Though the MHC and MRF proteins were easily detectable, no clear examples were found of cells that simultaneously expressed both MHC and a MRF (Table 2). This result was the opposite of that noted above for embryonic, fetal and newborn cultures where every MHC-expressing cell was found to coexpress at least one MRF.

The observed lack of coexpression of Myf-5 and MHC in somitic cells is illustrated in Fig. 4. This finding did not appear to be due to a limitation of the method, because our observations of embryonic, fetal and newborn cell cultures showed that the double immunofluorescence assay was capable of detecting simultaneous expression of MRFs and MHC within a single myocyte (Fig. 3). Also in contrast to the embryonic, fetal and newborn cultures where MyoD was the first and most abundantly expressed MRF, the Myf-5 protein appeared to be the first MRF expressed in somitic cell cultures and it was expressed by a larger number of cells than was myogenin or MyoD (Table 2 and not shown).

Table 2. Expression of muscle regulatory factors and myosin heavy chain in cultures of E8.5 somite cells

MRF assayed	Culture duration (days)	Number of cells observed that expressed*		
		MRF only	MRF plus MHC	MHC only
MyoD	1	0	0	46
	3	26	0	89
Myogenin	1	1	0	43
	3	32	0	96
Myf-5	1	43	0	37
	3	138	0	110
MRF4	1	0	0	10
	3	0	0	116

*E8.5 somite plus neural tube cells were cultured for one or three days, and double immunofluorescence analysis with a specific anti-MRF and the anti-MHC mAb F59 was used to determine the number of three types of cells: (i) cells termed "MRF only" expressed the indicated myogenic regulatory factor (MRF) but did not express myosin heavy chain (MHC); (ii) cells termed "MRF plus MHC" coexpressed the MRF and MHC (no such cells were observed); and (iii) cells termed "MHC only" expressed MHC but did not express the MRF. Numbers represent the sum of each type of cell observed in four independent experiments. For each experiment, all cells in an area of 0.8 cm^2 (>1000 cells) were examined.

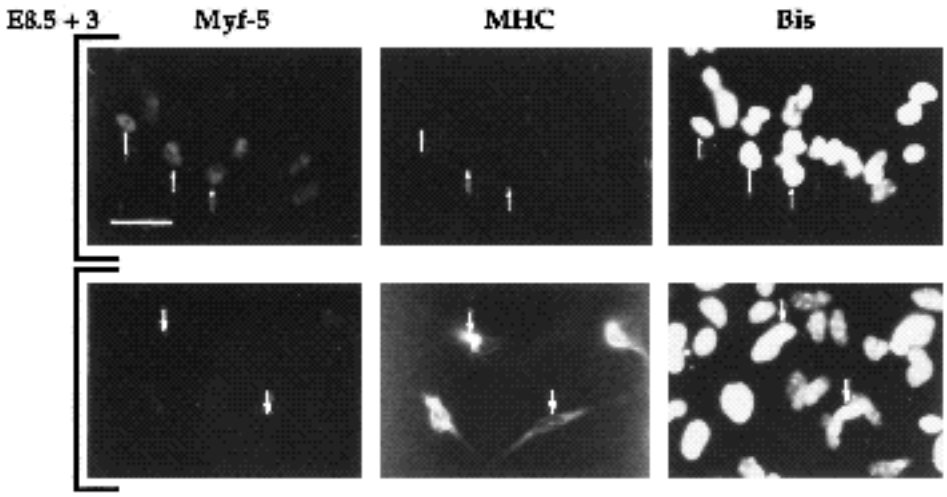

Fig. 4. Myf-5 and myosin heavy chain were never observed to be simultaneously expressed in the same cells in cultures of E8.5 somite cells. 3 day old cultures of E8.5 somite plus neural tube cells were examined by triple fluorescence using anti-Myf-5 serum to identify nuclei that expressed Myf-5, mAb F59 to identify cells that expressed myosin heavy chain (MHC), and bisbenzimide to stain all nuclei in the field (bis). Upward pointing arrows indicate cells that expressed Myf-5 but did not express MHC. Downward pointing arrows indicate the contrasting type of cells that expressed MHC but did not express Myf-5. Each field also contains cells in which neither Myf-5 nor MHC were expressed. Myf-5 and MHC were never found to be coexpressed in the same cell (compare Table 2). Bar, 20 μm.

DISCUSSION

Myogenic cells from early somites expressed MHC and MRFs in vitro in a unique pattern which was distinct from that of myogenic cells from embryonic, fetal and newborn limbs. In somitic cell cultures, Myf-5 was expressed first and by more cells than MyoD or myogenin; MRF4 was not detected; and the MRFs were never found to be coexpressed with MHC in the same cell. Some somitic cells thus had the surprising ability to maintain MHC expression in the absence of detectable MRF expression. In limb cell cultures, in contrast, MyoD was expressed first; MyoD and myogenin were expressed by more cells than Myf-5 or MRF4; and at least one of the MRFs was expressed by all myocytes and myotubes. The diverse myogenic programs of embryonic, fetal and newborn cells do not appear, therefore, to be linked to different patterns of MRF expression, whereas somitic cells display a distinct pattern of MRF expression.

Our results extend previous work on myogenic cell diversity in the mouse. Earlier work showed that somitic, embryonic, fetal and newborn (satellite cell) mouse myoblasts are four distinct types of myogenic cells which have different responses to drugs and growth factors, and form myotubes with different morphologies and MHC isoform expression patterns (Cossu and Molinaro, 1987; Cusella-De Angelis et al., 1992; Smith and Miller, 1992). Cusella-De Angelis et al. (1992) also found that somite cells in culture expressed MHC in the absence of myogenin or MyoD, results that were confirmed here and extended by our additional observations of Myf-5 and MRF4 expression patterns. The unique MRF expression pattern of somitic cells is a further characteristic that distinguishes them from embryonic, fetal and newborn myogenic cells.

The origins of the MHC-expressing and MRF-expressing populations of somite-derived cells remain to be determined. One possibility is that the MRF^+/MHC^- cells may convert into MRF^-/MHC^+ myocytes. If such conversion occurs, then MRF expression might be required for initiation, but not for maintenance, of the somitic cell myogenic program. To be consistent with the absence of MRF and MHC coexpression among the several hundred MHC^+ somitic cells that we observed, any such a conversion must be accompanied by, at most, a relatively short period of MRF and MHC coexpression. MRF proteins appear to have very short half-lives, so MRF levels probably change rapidly upon changes in transcription rates (Edmondson et al., 1991).

Alternatively, the MRF^+/MHC^- and MRF^-/MHC^+ somite cells might be independent. If so, then one group of somite-derived cells would have the unexpected ability to express MHC in the absence of detectable MRF expression, whereas a separate group would express one or more MRFs but fail to progress to MHC expression. Two lineages of somitic myogenic cells have been identified by transplant assays (Ordahl and LeDouarin, 1992), and both lineages would have been included in our cultures. In addition, MRF-independent muscle cell formation occurs in nematodes that lack the single *Caenorhabditis elegans* homolog of MyoD (Chen et al., 1992); and the slow MHC gene, which is a MHC isoform expressed by somitic cells in culture (Smith and Miller, 1992), can potentially be transcribed in a MRF-independent manner (Thompson et al., 1991).

Embryonic, fetal and newborn limb cells expressed the MRFs in similar patterns. In general, MyoD was the first MRF to be expressed; MyoD and myogenin were expressed in more cells than Myf-5; and MRF4 expression was low but increased as culture duration was lengthened. MRF protein and mRNA expression patterns were similar, suggesting that transcription and translation are closely coupled. Minor differences in timing and extent of Myf-5 expression relative to MyoD were noted among different types of cultures, but the significance of these differences is unknown. In contrast to the somitic cell cultures, the myocytes and myotubes in the limb cell cultures expressed MyoD and myogenin; and some additionally expressed Myf-5 and MRF4. Thus, both initiation and maintenance of the embryonic, fetal and newborn myogenic programs are likely to require expression of one or more MRFs.

In embryonic, fetal and newborn cell cultures, each of

the four MRFs was found in mononucleated cells that had no detectable MHC, as well as in myocytes and myotubes. Thus, MRF protein expression need not be immediately followed by MHC accumulation, and, conversely, MHC expression is not a necessary prerequisite for any of the MRFs to be expressed. Similar results are found with myogenic cell lines (e.g. Miller, 1990; Block and Miller, 1992), and are likely to be due to MRF proteins existing in nuclei as forms that are incapable of activating muscle-specific gene transcription (Vaidya et al., 1989; Weintraub et al., 1991; Martin et al., 1992). MRF expression is antagonistic to cell division (Crescenzi et al., 1990; Sorrentino et al., 1990), but it remains to be determined whether the MRF^+/MHC^- cells in culture are post-mitotic cells in the early stages of terminal differentiation or remain true myoblasts capable of division. It is also not known if the same sequence of MRFs is expressed by each cell of a particular type.

A comparison of expression of the MRF proteins in vitro and the MRF mRNAs in vivo reveals both similarities and differences (this work; Bober et al., 1991; Hinterberger et al., 1991). Somites in vivo first express Myf-5 mRNA, followed sequentially by myogenin, MRF4 and MyoD mRNAs. In somitic cell cultures, Myf-5 protein was similarly expressed first, and MyoD and myogenin were expressed subsequently, but MRF4 protein was not detected. Embryonic limbs in vivo also express Myf-5 mRNA first, followed half a day later by MyoD and myogenin mRNAs, and a day later by MRF4 mRNA. In limb cell cultures, the MRF4 protein similarly appeared last, but MyoD was usually the first MRF protein expressed, followed by Myf-5 and myogenin. Such disparities might be due to untranslated mRNAs (Cusella-De Angelis et al., 1992), alterations of MRF expression by culture, or coexistence of diverse types of myoblasts in a developing muscle which would complicate interpretation of in situ hybridizations.

Several lines of evidence now support the proposition that different myogenic programs can be produced by differential expression of the MRFs. In the BC3H-1 myogenic cell line, for example, overexpression of MyoD or MRF4 activates several additional muscle functions that are not in the myogenic program of the parent cells (Brennan et al., 1990; Miller, 1990; Block and Miller, 1992). In cotransfection assays, different MRFs also have different abilities to activate promoters (Yutzey et al., 1990; Chakraborty et al., 1991; Fujisawa-Sehara et al., 1992); and ectopic MRF expression, for example MyoD in mouse heart and XMyoD or XMyf-5 in frog embryos, activates only a subset of skeletal muscle genes (Hopwood and Gurdon, 1990; Hopwood et al., 1991; Miner et al., 1992). Finally, the mRNAs of the four MRFs are expressed in different patterns during rodent somite and limb development (Bober et al., 1991; Hinterberger et al., 1991), and somitic cells in culture express the MRF proteins in a unique pattern.

On the other hand, differential expression of the MRFs is unlikely to be the sole mechanism of myogenic cell diversification. In particular, embryonic, fetal and newborn mouse myoblasts express the MRFs in very similar patterns, though these cells have distinct properties as myoblasts and express diverse myogenic programs upon differentiation. What mechanisms besides simple differential expression of the MRFs might regulate myogenic cell diversification? Perhaps the seemingly similar patterns of MRF protein expression mask differences in MRF function. The immunocytochemical assays were not quantitative, so different types of myogenic cells might have expressed very different quantities of each MRF. Also, the function of individual MRFs can be regulated by dimerization with E2A gene products, binding to c-Jun, levels of Id-related proteins, phosphorylation, translation rate, and turnover rate (Lassar et al., 1991; Sun et al., 1992; Bengal et al., 1992; Li et al., 1992; reviewed by Weintraub et al. 1991). Alternatively, myogenic cell diversity may be established and maintained by regulators of muscle-specific genes other than the MRFs; for instance, *myd*, MEF-2, M-CAT binding factor, or other as yet unidentified factors (Pinney et al., 1988, Gossett et al., 1989; Mar and Ordahl, 1990). Differential expression of the four bHLH muscle regulatory factors is thus likely to be one, though not the only, molecular mechanism for generating myogenic cell diversity.

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