Myogenic Programs of Mouse Muscle Cell Lines: Expression of Myosin Heavy Chain Isoforms, MyoD1, and Myogenin

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Abstract. Different mouse muscle cell lines were found to express distinct patterns of myosin heavy chain (MHC) isoforms, MyoD1, and myogenin, but there appeared to be no correlation between the pattern of MHC expression and the patterns of MyoD1 and myogenin expression. Myogenic cell lines were generated from unconverted C3H10T1/2 cells by 5-azacytidine treatment (Aza cell lines) and by stable transfection with MyoD1 (TD cell lines) or myogenin (TG cell lines). Myogenic differentiation of the newly generated cell lines was compared to that of the C_2C_{12} and BC3H-1 cell lines. Immunoblot analysis showed that differentiated cells of each line expressed the embryonic and slow skeletal/β-cardiac MHC isoforms though slow MHC was expressed at a much lower, barely detectable level in BC3H-1 cells. Differentiated cells of each line except BC3H-1 also expressed an additional MHC(s) that was probably the perinatal MHC isoform. Myogenin mRNA was expressed by every cell line, and, with the exception of BC3H-1 (cf., Davis, R. L., H. Weintraub, and A. B. Lassar. 1987. *Cell.* 51:987–1000), MyoD1 mRNA was expressed by every cell line. To determine if MyoD1 expression would alter the differentiation of BC3H-1 cells, cell lines (termed BD) were generated by transfecting BC3H-1 cells with MyoD1 under control of the β-actin promoter. The MyoD1 protein expressed in BD cells was correctly localized in the nucleus, and, unlike the parental BC3H-1 cell line that formed differentiated MHC-expressing cells, which were predominately mononucleated, BD cell lines formed long, multinucleated myotubes (cf., Brennan, T. J., D. G. Edmondson, and E. N. Olson. 1990. *J. Cell. Biol.* 110:929–938). Despite the differences in morphology and MyoD1 expression, BD myotubes and the parent BC3H-1 cells expressed the same pattern of sarcomeric MHCs.

VERTEBRATE muscle contains a heterogeneous population of fast and slow skeletal muscle fibers that have different biochemical phenotypes and contraction rates. The contraction rate of a striated muscle cell is largely determined by the ATPase activity of the myosin heavy chain (MHC)[1] isoform or isoforms expressed in the cell (Schwartz et al., 1981; Reiser et al., 1985; Sweeney et al., 1988). MHC isoform expression is, therefore, both the molecular determinant of and a marker for skeletal muscle fiber diversification during development (Bandman, 1985; Whalen, 1985; Stockdale and Miller, 1987). In the rat and mouse, multiple striated muscle MHC isoforms have been identified, and the expression of each of these MHCs is regulated so that each isoform is expressed only in certain striated muscle cells and at certain stages of development (Whalen et al., 1981; Izumo et al., 1985; Emerson and Bernstein, 1987; Naruzawa et al., 1987; Weydert et al., 1987; Bär and Pette, 1988; Vivarelli et al., 1988; Schiaffino et al., 1988, 1989; Harris et al., 1989).

Observations in vivo and in vitro suggest that MHC isoform expression is regulated by multiple cellular processes that can occur in either myoblasts or myotubes during the multi-stage process of muscle fiber formation. Though MHC isoform expression is regulated by extrinsic factors such as thyroid hormone levels and the activity pattern of the innervating motor neuron (Jolesz and Sréter, 1981; Pette and Vrbová, 1985; Izumo et al., 1986), factors intrinsic to myogenic cells also influence the MHC phenotype of muscle fibers (Kelly, 1983; Miller and Stockdale, 1987; Cossu and Molinaro, 1987). For example, sequential transitions or modulations of MHC isoform expression occur within avian and mammalian myotubes in culture in the absence of innervation (Cerny and Bandman, 1986; Silberstein et al., 1986; Weydert et al., 1987; Miller and Stockdale, 1989). Additionally, multiple types of avian and mammalian myoblasts have been shown to appear sequentially at different stages of muscle development, and multiple types of avian myoblasts have been found that are intrinsically committed to form distinct types of fast and slow MHC-expressing myotubes (White et al., 1975; Rutz and Hauschka, 1982; Miller and Stockdale, 1986*a*, 1986*b*; Cossu and Molinaro, 1987; Schafer et al.,

1. *Abbreviations used in this paper*: MHC, myosin heavy chain; P2, postnatal day 2.

1987; Vivarelli et al., 1988; Hoh et al., 1989; Miller and Stockdale, 1989).

Recently, the molecular analysis of myogenesis has advanced with the identification of several myogenic regulatory factors including MyoD1, *myd*, myogenin, Myf-5, MRF4, and the *ski* oncogene that can convert some types of nonmyogenic cells into myoblasts (Lassar et al., 1986; Davis et al., 1987; Pinney et al., 1988; Braun et al., 1989*a*; Colmenares and Stavnezer, 1989; Edmondson and Olson, 1989; Rhodes and Konieczny, 1989; Wright et al., 1989; Miner and Wold, 1990). MyoD1, myogenin, Myf-5, and MRF4 form a family of homologous, DNA-binding proteins that appear to regulate expression of each other and of other muscle-specific proteins (Braun et al., 1989*a*; Lin et al., 1989; Thayer et al., 1989). During mouse embryogenesis, myogenin and MyoD1 mRNAs are expressed in different patterns in the developing somites and limbs (Sassoon et al., 1989). These findings raised the possibility that different patterns of MHC isoform expression might result from different patterns of expression of the individual MyoD1-like proteins. To begin to test this possibility, the expression of MHC isoforms, MyoD1, and myogenin has been analyzed in the differentiated cells formed by previously described and newly generated mouse myogenic cell lines.

Materials and Methods

Cell Lines and Culture

Table I lists the myogenic cell lines used in this study. C3H10T1/2 clone 8 (Reznikoff et al., 1973), BC3H-1 (Schubert et al., 1974), and C_2C_{12} (Yaffe and Saxel, 1977; Blau et al., 1985) cells were obtained from the American Type Culture Collection (Rockville, MD). The Aza2 myogenic cell line was generated in this laboratory by expansion of a myogenic clone resulting from 5-azacytidine conversion of 10T1/2 cells (Taylor and Jones, 1979; Konieczny and Emerson, 1985). Myogenic cell lines TD33, TD38, and TD45 were obtained from independent experiments in which ~10^5 10T1/2 cells were co-transfected with 0.2 μg pSV2neo and 10 μg pEMC1ls using cationic liposome-mediated transfection (Lipofectin reagent, Bethesda Research Laboratories, Gaithersburg, MD). MyoD1 is expressed from a Moloney sarcoma virus LTR promoter on pEMC1ls (Davis et al., 1987). Myogenic colonies resistant to 500 μg/ml of active G-418 were selected, recloned twice, and expanded. The TG1 cell line was obtained by co-transfection of 10T1/2 cells with 0.2 μg pSV2neo and 10 μg pEMSV-mouse myogenin (pEMSV-Myo8; Edmondson and Olson, 1989). ~10^3 G-418-resistant colonies were obtained per 1 μg pSV2neo. In control experiments, no myogenic colonies were found among several hundred G-418-resistant colonies resulting from transfection of 10T1/2 cells with pSV2neo alone. After several unsuccessful attempts to express MyoD1 in BC3H-1 cells by co-transfection with pEMC1ls and pSV2neo, the BD1, BD2, and BD10 lines were obtained in independent experiments by transfecting BC3H-1 cells with 0.2 μg of pEM340 (a gift of Dr. C. P. Emerson, Department of Biology, University of Virginia), a plasmid which contains MyoD1 cDNA expressed under control of the β-actin promoter and the *neo*^{r} gene for selection.

To eliminate effects due to different culture conditions, all cell lines were grown in the same manner (Miller, 1984). Dividing myoblasts that did not express striated muscle MHCs were maintained at <75% confluence in a medium consisting of BME supplemented with 15% FCS (Gibco Laboratories, Grand Island, NY), 1 mM glutamine, and 100 U/ml penicillin (growth medium). To induce myogenic differentiation, nearly confluent cultures were switched to a low mitogen medium consisting of DME supplemented with 10% horse serum (Hazleton Systems, Inc., Aberdeen, MD), 1% FCS, glutamine, and penicillin (differentiation medium). Media were used at 0.2 ml/cm^2; dishes were gelatin-coated; and cultures were incubated in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. FCS was selected for ability to support clonal growth of primary adult human myoblasts (Lev et al., 1987) and horse serum was selected for ability to support muscle colony formation from embryonic day 19 mouse muscle cells (Hauschka et al., 1979).

Monoclonal Antibodies

The preparation and properties of mAbs S46, S58, F47, and F59 have been described (Crow and Stockdale, 1984; Miller et al., 1985; Stockdale and Miller, 1987; Evans et al., 1988; Miller and Stockdale, 1989; Miller et al., 1989). An additional mAb, mAb S22, was prepared similarly using chicken slow MHCs as immunogens. The reactivities of these mAbs with mouse MHC isoforms were determined and are shown in Table II. R1ID10, a mAb that reacts specifically and with high affinity with human β-cardiac MHC (Khaw et al., 1984), was also found to be specific for the slow skeletal/β-cardiac MHC of the mouse (Table II). Three additional slow MHC-specific mAbs, NOQ7.5.4D, NA7, and NA8, were also used (Narusawa et al., 1987; Harris et al., 1989; Bandman et al., 1990). All experiments used either 1:10 dilutions of hybridoma supernatants (mAbs S22, S46, S58, F47, F59, and NOQ7.5.4D), 500 ng/ml of purified antibody (mAb R1ID10); or 1:500 dilutions of ascites fluid (mAbs NA7 and NA8). An adult mouse MHC isoform termed IIX or IID has been described (Bär and Pette, 1988; Schiaffino et al., 1989); mAbs S58, S46, S22, and R1ID10 did not react with this MHC because the electrophoretic mobility of the slow MHC that reacted with these mAbs was more rapid than that reported for IIX/D MHC (Schiaffino et al., 1989). Both mAbs F47 and F59 reacted with the IIX/D MHC (not shown). The mouse embryonic MHC isoform expressed in E14 limbs and

Table I. Mouse Muscle Cell Lines

Cell line	Origin	Differentiated cell type	Differentiation index*
Aza2	5-azacytidine-converted 10T1/2	Mononucleated myocytes and multinucleated myotubes	10–30%
BC3H-1‡	Brain tumor	Mononucleated myocytes‖	20–50%
BD1, BD2, and BD10	MyoD1-transfected BC3H-1	Multinucleated myotubes¶	20–50%
C_2C_{12}§	Adult leg	Multinucleated myotubes¶	30–50%
TD33, TD38, and TD45	MyoD1-transfected 10T1/2	Multinucleated myotubes¶	10–30%
TG1	Myogenin-transfected 10T1/2	Mononucleated myocytes and multinucleated myotubes	5–30%

* The differentiation index was the percentage of all nuclei in a culture that were contained within MHC-expressing (i.e., F59-reactive) myocytes and myotubes after 7 d in differentiation medium. Approximate ranges are given to reflect observations from different experiments with cell lines at different passage numbers.
‡ Schubert et al., 1974.
§ Yaffe and Saxel, 1977; Blau et al., 1985.
‖ In addition to the predominant mononucleated myocytes, a small number of multinucleated myotubes, containing <10% of the nuclei found in differentiated cells, were formed by BC3H-1 cells.
¶ In addition to the predominant multinucleated myotubes, a small number of the differentiated cells formed by these cell lines were mononucleated myocytes.

Table II. Reactivities of Monoclonal Antibodies with Mouse Myosin Heavy Chain Isoforms

Monoclonal antibody	Reaction of mAb with MHC isoform*					
	Embryonic	Perinatal	Type IIA	Type IIB	α-cardiac	Slow/β-cardiac
S22	−	−	−	−	−	++
S46	−	−	−	−	−	++
S58	−	−	−	−	−	++
R11D10	−	−	−	−	−	+++
F47	−	+++	+++	+++	−	−
F59	+++	+++	+++	+++	+++	+++

* MHCs from whole muscles or cultures were subjected to SDS-PAGE in 5% gels, transferred to immunoblots, and tested for reaction with each mAb. Individual MHC isoforms were distinguished by electrophoretic mobility and expression patterns in animals of different ages and in different muscles or cell cultures (cf., Schiaffino et al., 1988, 1989). The embryonic MHC was from BC3H-1 cultures and E14 limb muscle; perinatal MHC was from C_2C_{12} cultures (Silberstein et al., 1986) and P2 limb muscle; Type IIA MHC was from the adult diaphragm and adult soleus; Type IIB MHC was from adult quadriceps; α-cardiac MHC was from P2 ventricle and adult ventricle; and slow skeletal/β-cardiac MHC was from P2 ventricle, adult diaphragm, and adult soleus. Equal amounts of myosin from each sample were analyzed and the intensity of mAb reaction with each sample was compared using the antibody concentrations or hybridoma supernatant dilutions stated in Materials and Methods; +++, strong reaction; ++, moderate reaction; +, weak reaction; −, no detectable reaction.

BC3H-1 myocytes did not react with mAb F47 (Table II, Fig. 5), though earlier work (Miller et al., 1989) suggested that mAb F47 did react very weakly with the rat embryonic MHC isoform. This difference may either be due to the species difference or to a small amount of perinatal MHC expression in the embryonic rat limb muscle (Harris et al., 1989). The differences in electrophoretic mobilities among the mouse MHC isoforms mirrored those found for rat MHCs by Schiaffino et al. (1988, 1989). None of the mAbs listed in Table I reacted with smooth muscle or nonsarcomeric cytoplasmic MHCs (Evans et al., 1988; Miller et al., 1989; and our unpublished observations).

Immunocytochemistry and Electrophoresis

Cultures were fixed for 5 min in 100% ethanol at room temperature, incubated for 1 h at room temperature with mAbs diluted in PBS containing 2% horse serum and 2% BSA; and mAb binding was visualized with a horseradish peroxidase-linked system using diaminobenzidine as substrate (Vectastain ABC kit, Vector Laboratories Inc., Burlingame, CA). For double immunofluorescence, a modification of the method of Wessel and McClay (1986) was used (Miller and Stockdale, 1989). Samples were incubated with the first mAb (F47 or R11D10) followed by sequential incubations with (*a*) a saturating amount (100 μg/ml) of the monovalent fab fragment of fluorescein-conjugated goat anti-mouse IgG (Cappel Laboratories, Malvern, PA); (*b*) the second mAb (F59); and (*c*) 10 μg/ml rhodamine-conjugated goat anti-mouse IgG. Control experiments, using mAbs that did not react with muscle, showed that the observed fluorescence was due to mAb binding to MHC. Immunofluorescence analysis of MyoD1 expression was performed with rabbit anti-MyoD1 serum (Tapscott et al., 1988).

The differentiation index was calculated as the percentage of nuclei in a culture that were contained in differentiated cells (including both mononucleated myocytes and multinucleated myotubes). Cultures were fixed, differentiated cells were identified by immunofluorescent staining with mAb F59, and nuclei were identified by fluorescent staining with Hoechst 33258. Numbers of nuclei in differentiated and undifferentiated cells were then determined from observations of at least five microscope fields. The differentiation index was used to allow comparison of cell lines that formed mononucleated MHC-expressing myocytes (e.g., BC3H-1) with the differentiation of cell lines that formed multinucleated MHC-expressing myotubes (e.g., C_2C_{12}).

Myosin from cultures or tissue samples was prepared as previously (Evans et al., 1988). MHC isoforms were separated by SDS-PAGE in 5% gels, and transferred to nitrocellulose (Rushbrook and Stracher, 1979; Miller et al., 1985; Miller and Stockdale, 1986*a*). The nitrocellulose transfers were incubated for 1–2 h at room temperature with mAbs diluted in PBS containing 2% nonfat powdered dry milk, and mAb binding was visualized using an alkaline phosphatase-linked system (Vectastain ABC-AP kit, Vector Laboratories Inc.). One-dimensional peptide maps of protease-digested MHCs were obtained by modifying the procedures of Cleveland (1983) and Bandman (1985). MHCs were subjected to SDS-PAGE in 5% gels, briefly stained with Coomassie blue, and the regions of the gel containing MHC were excised. The gel pieces were equilibrated in sample buffer, inserted into sample wells of a 15% gel, and partially digested with 250 ng of *Staphylococcus aureus* V8 protease as described (Schafer et al., 1987). After electrophoresis, the partial proteolytic fragments were transferred to nitrocellulose, incubated with mAb, and mAb binding was visualized with the alkaline phosphatase-linked system.

mRNA Analysis

Total RNA was prepared by a modification of the method of Cheley and Anderson (1984). Solubilization buffer consisting of 7.6 M guanidine hydrochloride and 0.1 M potassium acetate (pH 5) was added to PBS-washed cultures at 1 ml/6-cm dish; solubilized cells were collected by scraping; DNA was sheared by vortexing for 30 s; and the homogenates were centrifuged at 13,000 rpm for 5 min. Supernatants were collected, mixed with 0.6 vol 100% ethanol, incubated at least 16 h at −20°C, and RNA pellets were collected by centrifugation at 13,000 rpm for 5 min. After electrophoresis in 1% agarose-formaldehyde gels, the RNAs were transferred and UV cross-linked to a Nytran nylon membrane (Schleicher & Schuell, Inc., Keene, NH). MyoD1 and myogenin cDNA probes were prepared by random primer labeling with [^{32}P]dCTP (Feinberg and Vogelstein, 1983). Eco RI digestion of pEMC11s was used to yield the ~1.8-kb cDNA fragment encoding MyoD1 (Davis et al., 1987) and partial Eco RI digestion of pEMSV-myogenin was used to yield the ~1.6-kb cDNA fragment encoding mouse myogenin (Edmondson and Olson, 1989); both fragments were purified, and 50 ng of each fragment were used as templates in parallel labeling reactions. Specific activities of the MyoD1 and myogenin probes differed by <20%. To compare relative amounts of MyoD1 and myogenin mRNAs, RNA samples were quantitated by OD_{260}; 10 μg of each sample were applied to duplicate wells of a single gel containing two rows of sample wells; electrophoresis and transfer of the gel were performed; and the transfers were cut to provide duplicates. Hybridization with the appropriate probe was in 50% formamide, 5 × SSC, 1 × PE (PE = 50mM Tris, pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, 0.2% polyvinylpyrrolidone, 0.2% ficoll, 5 mM EDTA, 0.2% BSA), 100 μg/ml salmon sperm DNA at 50°C for 16 h; and membranes were washed with 0.1 × SSC, 0.1% SDS at 65°C.

Results

Differentiation of Mouse Muscle Cell Lines

To determine whether different types of mouse muscle cell lines expressed distinct patterns of MHCs, MyoD1, and myogenin, the differentiation patterns of five newly generated and two previously isolated myogenic cell lines were compared. Table I lists characteristics of these lines. C3H mice were the initial sources of the previously isolated C_2C_{12} and BC3H-1 myogenic lines and of the C3H10T1/2 cells from which the new myogenic cell lines were generated (Reznikoff et al., 1973; Schubert et al., 1974; Yaffe and Saxel, 1977; Blau et al., 1985). Myogenic conversion of 10T1/2 cells by 5-azacytidine treatment (Aza2 line), MyoD1 transfection

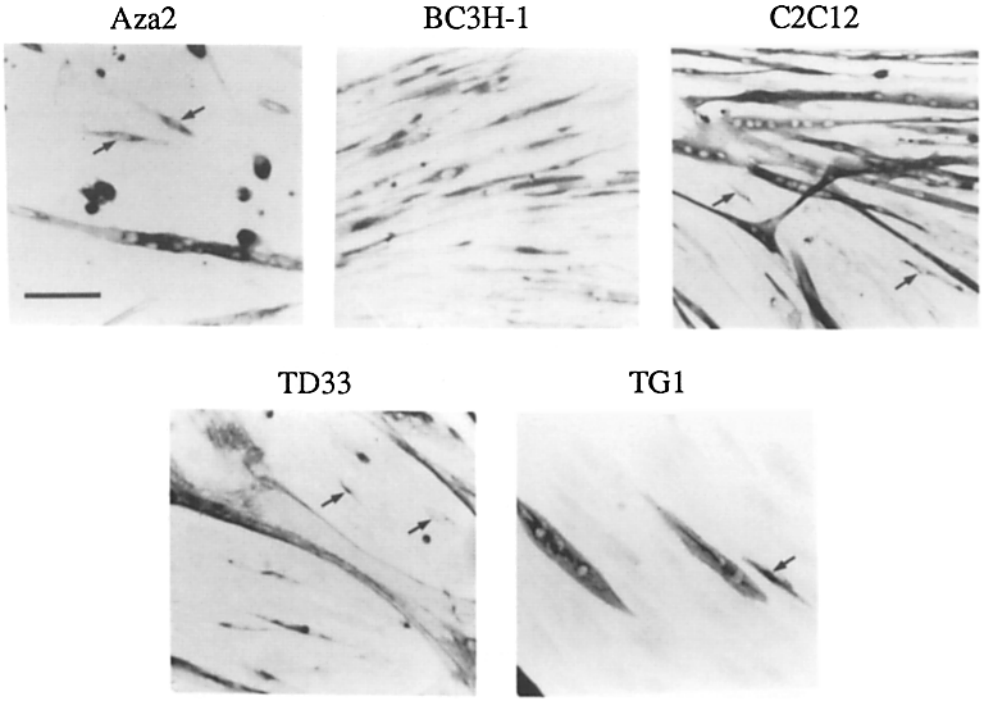

Figure 1. Morphology of differentiated cells formed by mouse muscle cell lines. Aza2, BC3H-1, C_2C_{12}, TD33, and TG1 cells were cultured in growth medium until near confluence and then incubated for a further 7 d in differentiation medium. MHC expressing cells were identified by immunocytochemical staining with mAb F59 using a horseradish peroxidase-conjugated system for detection. Undifferentiated cells that filled the spaces between differentiated cells are not seen well with this bright field image. Arrows indicate mononucleated myocytes in Aza2, C_2C_{12}, TD33, and TG1 cultures. Bar, 100 μm.

(TD33, TD38, and TD45 lines), or myogenin transfection (TG1 line) (Taylor and Jones, 1979; Davis et al., 1987; Edmondson and Olson, 1989) was used to generate the new myogenic cell lines. When maintained below confluence in high mitogen growth medium (15% FCS), cells of each line replicated as undifferentiated myoblasts that did not react with mAb F59. Because mAb F59 reacted with all mouse sarcomeric MHC isoforms (Table I), replicating myoblasts did not appear to accumulate sarcomeric MHC proteins. Upon switching to low mitogen differentiation medium (10% horse serum, 1% FCS), cells of each line formed differentiated cells that reacted with mAb F59 (Fig. 1).

The morphology of the differentiated cells and the percentage of nuclei found in differentiated cells (the differentiation index) varied among the cell lines (Fig. 1, Table I). Most of the cell lines formed multinucleated myotubes as the predominant type of MHC-expressing cell, as shown by immunocytochemical staining with mAb F59 (Fig. 1). In contrast, the MHC-expressing cells of the BC3H-1 cell line were predominantly mononucleated myocytes (Schubert et al., 1974) (Fig. 1), with a small number of myotubes that usually contained less than five nuclei. Though BC3H-1 was the only cell line to form predominantly mononucleated myocytes, such differentiated mononucleated cells were formed to some extent in cultures of each of the cell lines examined (Fig. 1). Mononucleated myocytes were particularly abun-

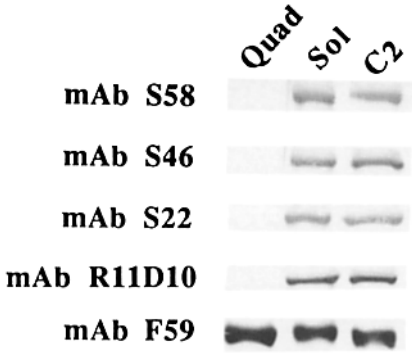

Figure 2. Immunoblot analysis of MHC expression by C_2C_{12} myotubes. MHCs were prepared from adult mouse quadriceps (*Quad*), adult mouse soleus (*Sol*), and C_2C_{12} myotubes (C_2) formed after 7 d in differentiation medium. MHCs were analyzed by SDS-PAGE in 5% gels and immunoblotting with mAbs S58, S46, S22, R11D10, and F59 as indicated. The mAbs specific for the slow MHC isoform expressed in the adult mouse soleus (Table II) also reacted with MHC expressed by C_2C_{12} myotubes.

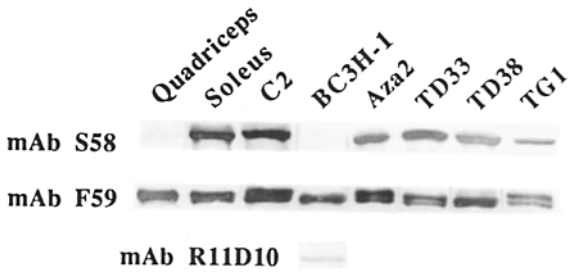

Figure 3. Immunoblot analysis of MHC expression in differentiated cultures of mouse muscle cell lines. Aza2, BC3H-1, C_2C_{12}, TD33, and TG1 cells were cultured in growth medium until nearly confluent and then incubated for a further 7 d in differentiation medium. As indicated, MHCs were prepared from adult mouse quadriceps muscle, adult mouse soleus muscle, and the differentiated cultures, and analyzed by SDS-PAGE in 5% gels and immunoblotting with mAbs S58 and F59. MHC from BC3H-1 cultures was also analyzed with mAb R11D10. Differentiated cells of each cell line, except BC3H-1, expressed MHC that reacted with the slow MHC-specific mAb S58. A small amount of MHC that reacted with mAb R11D10 was detected in BC3H-1 cultures.

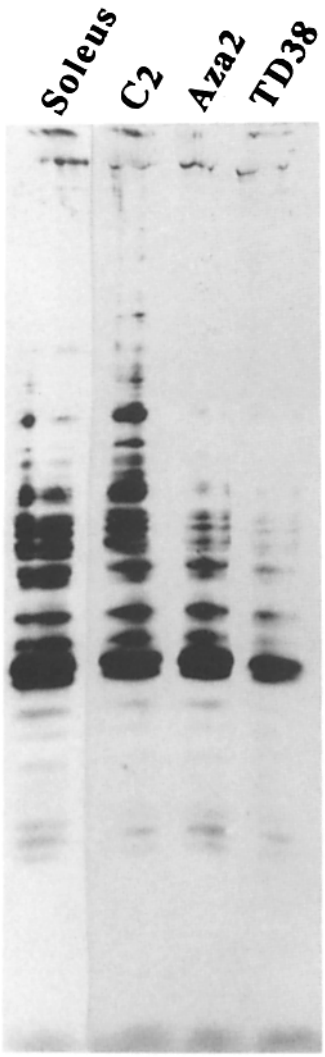

Figure 4. Comparison of slow MHC expressed by the mouse and by mouse muscle cell lines. One-dimensional immunopeptide mapping was used to compare MHCs prepared from adult mouse soleus muscle and from differentiated cultures of the C_2C_{12}, Aza2, and TD33 cell lines. MHCs were partially proteolyzed as described in Materials and Methods, and the resulting MHC fragments were analyzed by SDS-PAGE in 15% gels and by immunoblotting with mAb R11D10 and an alkaline phosphatase–linked detection system. Samples were analyzed on adjacent lanes of the same gel; color development took less time for the soleus than the cell culture MHCs. Slow MHCs from the soleus and differentiated cultures produced identical patterns of immunoreactive peptides.

dant in cultures of the Aza2 and TG1 cell lines, accounting for up to 50% of the nuclei in differentiated, MHC-expressing cells formed by these two lines after 7 d in differentiation medium. In contrast, mononucleated myocytes usually accounted for <5% of the MHC-expressing cells in cultures of C_2C_{12}, TD33, TD38, and TD45 cells after 7 d in differentiation medium. Each of the cell lines, particularly those generated by transfection, tended to have a lower differentiation index after repeated passage and thus accumulated less MHC. The patterns of MHC isoform expression reported below were the same in cells of early and late passage (not shown).

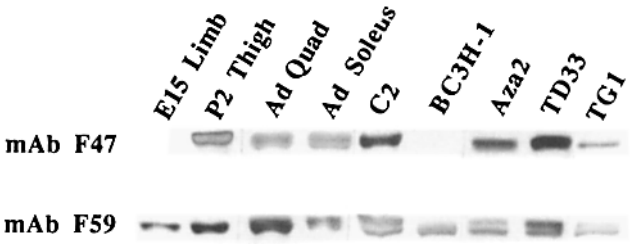

Figure 5. Immunoblot analysis with mAbs F47 and F59 of MHC expression in differentiated cultures of mouse muscle cell lines. Aza2, BC3H-1, C_2C_{12}, TD33, and TG1 cells were cultured in growth medium until near confluence and then incubated for a further 7 d in differentiation medium. MHCs were prepared from E15 mouse limb, P2 mouse limb, adult mouse quadriceps muscle, adult mouse soleus muscle, and the differentiated cultures, and the MHCs were analyzed by SDS-PAGE in 5% gels and immunoblotting with mAbs F47 and F59 as indicated. Differentiated cells of each cell line, except BC3H-1, expressed MHC that reacted with mAb F47.

Expression of Slow MHC Protein

Immunoblotting analysis suggested that myotubes formed from C_2C_{12} cells expressed the slow MHC isoform. A single slow MHC isoform (alternatively known as the slow skeletal or β-cardiac MHC) is expressed at different stages of development in skeletal and heart muscle (Lompré et al., 1981, 1984). Myotubes formed from C_2C_{12} cells after 7 d in differentiation medium expressed MHC that reacted with mAbs S58, S46, S22, and R11D10 (Fig. 2, lane *3*). Each of these mAbs appeared to react with the slow skeletal/β-cardiac MHC found in the adult soleus (Fig. 2, lane *2*), but did not react with the MHC(s) (predominantly Type IIB) found in the adult quadriceps (Fig. 2, lane *1*). Each of these mAbs also reacted with the slow skeletal/β-cardiac MHC isoform when it was expressed in the postnatal day 2 (P2) mouse ventricle, but did not react with the electrophoretically distinct α-cardiac MHC isoform that was coexpressed in the P2 ventricle (Lompré et al., 1981) (Table II). Three additional slow MHC-specific mAbs, NOQ7.5.4D, NA7, and NA8, (Narusawa et al., 1987; Bandman et al., 1990) also reacted with the slow MHC expressed in the soleus and in C_2C_{12} myotubes, but did not react with quadriceps MHC (not shown). Slow MHC from cultured cells and from the mouse had indistinguishable electrophoretic mobilities. The immunoblot band resulting from the reaction of mAb F59 with C_2C_{12} MHC samples was consistently wider than the S58 reaction band (Fig. 2) or was a doublet (cf., Fig. 3), suggesting that, as in previous work (Silberstein et al., 1986; Weydert et al., 1987), C_2C_{12} myotubes expressed more than one MHC isoform (see below).

Slow MHC expression was not limited to C_2C_{12} cells. Cells of the Aza2, BC3H-1, TD33, TD38, and TG1 lines were cultured in low mitogen medium for 7 d, and the MHCs expressed were analyzed by SDS-PAGE in 5% gels and immunoblotting with mAbs S58 and F59 (Fig. 3). Differentiated cells of each line, except BC3H-1, expressed MHC that reacted with mAb S58 (Fig. 3), as well as with mAbs S46, S22, NOQ7.5.4.D, NA7, NA8, and R11D10 (not shown). In four experiments with BC3H-1 cells, no detectable slow MHC expression was found by immunoblotting with mAbs S58, S46, and S22, whereas in each of the four experiments,

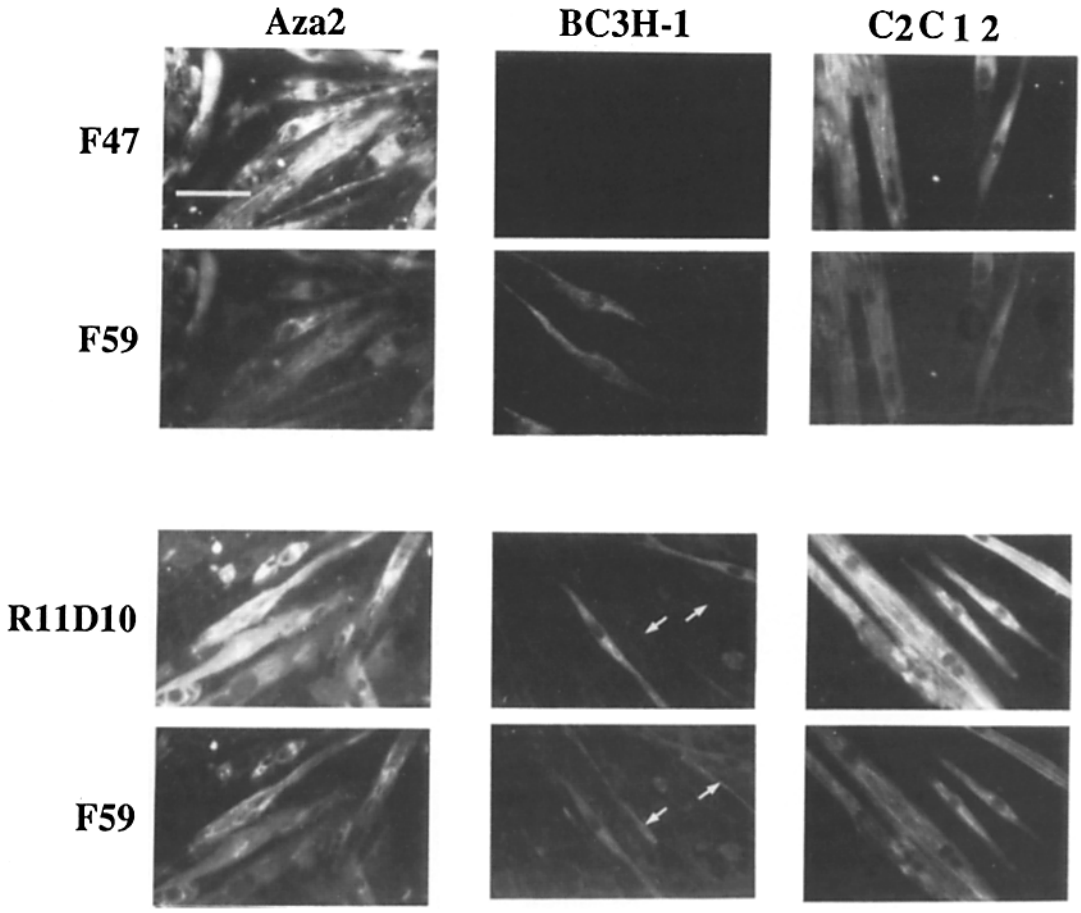

Figure 6. Double immunofluorescence analysis of MHC expression patterns. As indicated, cultures of Aza2, BC3H-1, and C_2C_{12} cells were incubated for 7 d in differentiation medium and analyzed by double immunofluorescence with mAbs F47 and F59 or with mAbs R11D10 and F59. Binding of mAb F59 was detected with a rhodamine-conjugated secondary antibody and binding of mAbs F47 and R11D10 was detected with fluorescein-conjugated secondary antibodies (see Materials and Methods). Note that mononucleated myocytes that reacted with mAbs F47 and R11D10 were found in Aza2 and C_2C_{12} cultures, as well as in BC3H-1 cultures. Arrows indicate BC3H-1 myocytes that reacted with mAb F59 but not with mAb R11D10. Bar, 75 μm.

a barely detectable band was seen using the high affinity mAb R11D10 (Fig. 3). The reaction of mAb F59 with MHC samples from differentiated Aza2, TD33, TD38, and TG1 cells consistently resulted in double immunoblot bands, whereas BC3H-1 MHC produced a single band upon reaction with mAb F59 (Fig. 3). The predominant MHC expressed by BC3H-1 myocytes appeared to be embryonic MHC (cf., Taubman et al., 1989) because it reacted with mAb 59, did not react with mAbs F47 or S58, and had an electrophoretic mobility that was indistinguishable from embryonic MHC and was less rapid than the electrophoretic mobility of slow MHC on double immunoblots (not shown; Miller and Stockdale, 1986*a*; Schiaffino et al., 1988, 1989). Thus, differentiated cells of most lines, like C_2C_{12} myotubes, appeared to express multiple MHCs (see below), whereas BC3H-1 myocytes appeared to express mostly embryonic MHC and a very small amount of slow MHC.

The slow MHC expressed in cultures appeared identical to the slow MHC expressed in the mouse. The structures of the slow MHCs expressed in the animal and in cultures were compared using one-dimensional immunopeptide mapping with mAb R11D10. MHCs were isolated from the adult

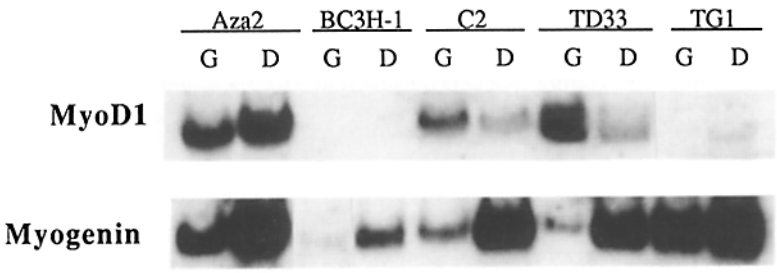

Figure 7. Expression of MyoD1 and myogenin mRNAs in mouse muscle cell lines. Total RNA from Aza2, BC3H-1, C_2C_{12}, TD33, and TG1 cells was prepared from nearly confluent cultures in growth medium (*G*), and from cultures after 3 d in differentiation medium (*D*) as indicated. The levels of MyoD1 and myogenin mRNAs in 10 μg of total RNA were analyzed in parallel Northern blots. All samples were analyzed on the same gel; some lanes were rearranged for presentation.

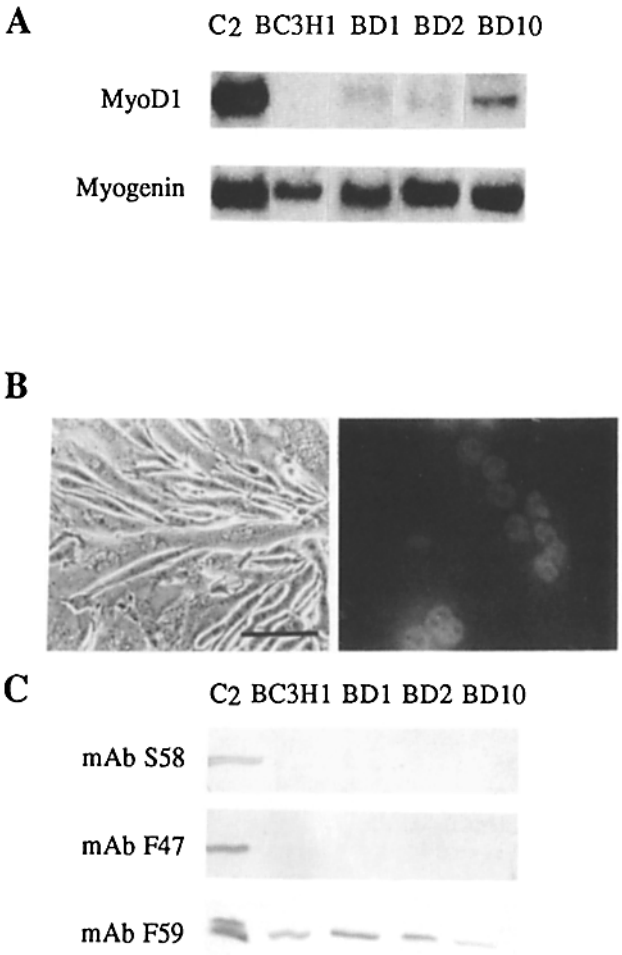

Figure 8. Exogenous MyoD1 expression and differentiation of BD cells lines. The BD1, BD2, and BD10 lines were derived by transfection of BC3H-1 cells with a plasmid on which MyoD1 expression was under control of the β-actin promoter (see Materials and Methods). (*A*) MyoD1 and myogenin mRNA expression were analyzed by Northern blot analysis of total RNA prepared from nearly confluent cultures of C_2C_{12}, BC3H-1, BD1, BD2, and BD10 cells in growth medium. For each cell line, MyoD1 and myogenin mRNAs were analyzed in duplicate gels in the same experiment, but different cell lines were analyzed in different experiments. (*B, left*) Phase microscopy of a living culture showed that BD10 cells formed multinucleated myotubes, and immunofluorescence analysis of a parallel culture (*right*) using polyclonal rabbit anti-MyoD1 and a fluorescein-conjugated secondary antibody showed that MyoD1 protein was expressed and localized to the nucleus of BD10 cells. Bar, (*left*) 100 μm; (*right*) 25 μm. (*C*) Immunoblotting with mAbs F59, F47, and S58 was used to analyze MHCs expressed in cultures of C_2C_{12}, BC3H-1, BD1, BD2, and BD10 cells after 7 d in differentiation medium. Equal amounts of total protein were analyzed in each lane.

soleus muscle and from differentiated cells formed by the C_2C_{12}, Aza2, and TD33 cell lines. Partial proteolytic fragments of the MHCs were prepared, separated by SDS-PAGE in 15% gels, and tested for reactivity with mAb R11D10. MHCs from the soleus muscle and from the differentiated cultures produced identical patterns of MHC fragments that reacted with mAb R11D10 (Fig. 4). An additional experiment showed that MHCs from the adult soleus and P2 mouse ventricle also gave identical immunopeptide maps with mAb R11D10 (not shown); this result was predicted from previous findings that a single slow skeletal/β-cardiac MHC isoform is expressed in the adult soleus and developing heart (Lompré et al., 1984). The immunopeptide mapping results suggested that the slow MHC expressed in the animal was identical to the MHC isoform that reacted with slow MHC-specific mAbs and was expressed in differentiated cultures of the mouse muscle cell lines.

Additional MHC Isoform Expression

Differentiated cultures of each muscle cell line except BC3H-1 expressed at least one MHC isoform, apparently the perinatal MHC, in addition to the embryonic and slow MHC isoforms. For these experiments, cells of the Aza2, BC3H-1, C_2C_{12}, TD33, and TG1 lines were allowed to differentiate in low mitogen medium for 7 d, and the MHCs expressed were analyzed by SDS-PAGE in 5% gels and immunoblotting with mAbs F59 and F47 (Fig. 5). In four experiments, BC3H-1 myocytes did not express detectable MHC that reacted with mAb F47, whereas differentiated cultures of the Aza2, C_2C_{12}, TD33, and TG1 lines did express MHC that reacted with mAb F47 (Fig. 5). The electrophoretic mobility of the MHC that reacted with mAb F47 corresponded to the upper or most slowly migrating of the bands that reacted with mAb F59. This result suggested that the differentiated cultures expressed the perinatal MHC isoform which, as shown for rat MHCs (Carraro and Catani, 1983; Schiaffino et al., 1988, 1989), has a less rapid electrophoretic mobility than the embryonic, IIB, or slow MHC isoforms. Perinatal MHC protein and mRNA expression has been found previously in C_2C_{12} myotubes (Silberstein et al., 1986; Weydert et al., 1987). Based on electrophoretic mobility and mAb reactivity, therefore, the MHC(s) that reacted with mAb F47 and was expressed by all cell lines except BC3H-1 was likely to have been predominantly the perinatal isoform, though additional F47-reactive MHCs (e.g., IIA or IIB) might also have been expressed. The expression patterns of slow MHC and F47-reactive MHC were not changed when cells of the different cell lines were cultured on untreated culture dishes rather than on gelatin-coated dishes. The three TD cell lines, TD33, TD38, and TD45 cells, formed myotubes with similar morphologies and MHC expression patterns.

The MHCs recognized by mAbs F47 and R11D10 were expressed in every differentiated cell of each cell line examined except BC3H-1. Double immunofluorescence analysis of Aza2 and C_2C_{12} cultures that had been in differentiation medium for 7 d showed that every differentiated cell that reacted with mAb F59 also reacted with mAbs F47 and R11D10 (Fig. 6). This same pattern of mAb reactivity was seen with TD33 myotubes. Though the intensity of staining varied among cells in a culture, these results suggested that each differentiated Aza2, C_2C_{12}, and TD33 cell, including mononucleated myocytes (Fig. 6), had initiated slow MHC and perinatal MHC (i.e., F47-reactive MHC) expression by 7 d in differentiation medium. As expected from the immunoblotting results, differentiated BC3H-1 myocytes did react with mAb F59 but did not react with mAb F47 (Fig. 6) or mAbs S58, S46, and S22 (not shown). Some BC3H-1 myocytes did react with mAb R11D10 (Fig. 6), a result that was

in agreement with the small amount of slow MHC detected by mAb R11D10 immunoblotting of BC3H-1 MHC. In one survey by double immunofluorescence, 59 out of 174 (34%) of the BC3H-1 myocytes that reacted with mAb F59 also gave a detectable reaction with mAb R11D10. The MHC(s) expressed by most of the cell lines was not assembled into sarcomeres. After 7 d in differentiation medium, many of the C_2C_{12} myotubes contained striations (e.g., F47-stained myotube in Fig. 6), whereas very few (<10%) of the differentiated Aza2 cells and none of the differentiated BC3H-1 and TD33 cells contained striations. Striations were detected with mAbs F47, F59, and R11D10, suggesting that each MHC isoform expressed was assembled into sarcomeres.

MyoD1 and Myogenin mRNA Expression

MyoD1 mRNA was expressed by cells of each line examined except BC3H-1 (Fig. 7). Total RNA from Aza2, BC3H-1, C_2C_{12}, TD33, and TG1 cells was prepared from confluent cultures in growth medium and from cultures 3 d after switching to differentiation medium. At both stages of culture, cells of each line except BC3H-1 expressed amounts of MyoD1 mRNA that were detectable by this Northern blot analysis, though the amount of MyoD1 mRNA varied among the cell lines (Fig. 7). For the Aza2, C_2C_{12}, and TD33 cell lines, the amount of MyoD1 mRNA stayed constant or decreased upon differentiation, whereas in TG1 cells MyoD1 mRNA levels increased upon differentiation. Previous work also showed that BC3H-1 cells do not express MyoD1 mRNA (Davis et al., 1987; Edmondson and Olson, 1989), and that 10T1/2 cells transfected with myogenin additionally express MyoD1 mRNA that increases in amount upon differentiation (Thayer et al., 1989).

Myogenin mRNA was expressed by cells of each line examined, including BC3H-1 cells. Myogenin mRNA levels varied markedly among the different cell lines, and BC3H-1 cells expressed the lowest levels of myogenin mRNA (Fig. 7). As seen previously for BC3H-1, C_2C_{12}, and rat L6 cells (Wright et al., 1989; Edmondson and Olson, 1989), myogenin mRNA levels were found to be higher in differentiated cultures of Aza2, TD33, and TG1 cells than in growing cultures. After 3 d in differentiation medium, myogenin mRNA levels in all cell lines except TG1 were increased greatly compared to growing cells. Myogenin mRNA was expressed at a relatively high level in growing TG1 cells, due to expression from the transfected pEMSV-myogenin, and this level increased slightly after 3 d in differentiation medium.

The relative amounts of MyoD1 and myogenin mRNAs were markedly different in confluent and differentiating cells. MyoD1 mRNA was generally as abundant or more abundant than myogenin mRNA in growing cells, whereas myogenin mRNA was much more abundant than MyoD1 mRNA after 3 d in differentiation medium (Fig. 7). The exceptions were BC3H-1 cells, which did not express MyoD1, and TG1 cells, in which MyoD1 mRNA was much less abundant than myogenin mRNA at each stage of culture, but increased upon differentiation rather than decreased as in the other MyoD1-expressing cell lines. Cultures in low serum medium contained both cells that expressed MHC and cells that did not express MHC (Fig. 1); and the relative contributions of the differentiated and undifferentiated cells to the changes in MyoD1 and myogenin mRNA levels seen in differentiated cultures remains to be determined. Because myogenin mRNA levels are known to increase in BC3H-1, C_2C_{12}, and rat L6 cells during the first 2–3 d in differentiation medium and then decrease as incubation is prolonged (Wright et al., 1989; Edmondson and Olson, 1989), it is likely that the relative levels of MyoD1 and myogenin mRNAs in Aza2, TD33, and TG1 also will vary with time of incubation in culture.

When exogenous MyoD1 was expressed in BC3H-1 cells, the morphology of the differentiated cells, but not the MHC expression pattern, was changed. BC3H-1 cells were transfected with MyoD1 cDNA under control of the β-actin promoter; and lines that were independently derived from different transfections were cloned, expanded, and termed BD lines. The BD1, BD2, and BD10 cell lines, unlike the parent BC3H-1 line, expressed both MyoD1 mRNA (Fig. 8 *A*), and MyoD1 protein which was correctly localized to the nucleus (Fig. 8 *B*). Confluent cultures of the BD cell lines in growth medium expressed much less MyoD1 mRNA than myogenin mRNA (Fig. 8 *A*). Unlike differentiated BC3H-1 cells, many of the differentiated BD1, BD2, and BD10 cells were multinucleated (Fig. 8 *B*), a result first found by Brennan et al. (1990). In one experiment, only 6% of the nuclei in differentiated (i.e., F59-stained) BC3H-1 cells were in multinucleated myotubes (n = 450; 27 nuclei in myotubes, 423 nuclei in myocytes), whereas >90% of the nuclei in differentiated BD10 cells were in multinucleated myotubes (n = 300; 271 nuclei in myotubes, 29 nuclei in myocytes). In both BC3H-1 and BD10 cultures, ~30% of all nuclei were in differentiated cells after 7 d in differentiation medium. Immunoblot analysis with mAbs F59, F47, and S58 did not reveal any differences in MHC expression between BD cell lines and the parent BC3H-1 cell line (Fig. 8 *C*). Differentiated BD cultures did not appear to accumulate as much striated muscle MHC per mg protein as BC3H-1 or C_2C_{12} cultures (Fig. 8 *A*), but detectable MHC that reacted with S58 or F47 was not found even in overloaded gels of BD myotube MHC (not shown). Thus, although BD cells expressed MyoD1 and formed multinucleated myotubes, the BD myotubes expressed the same simple pattern of MHCs as the parent BC3H-1 myocytes, rather than the complex pattern of MHCs expressed by the morphologically similar myotubes formed by TD cell lines.

Discussion

Different types of mouse muscle cell lines were found to express different patterns of MHC isoforms, MyoD1, and myogenin; but no correlation was found between the pattern of MHC isoform expression and the patterns of MyoD1 and myogenin expression. Differentiated cells of most mouse myogenic cell lines expressed a complex pattern of MHCs, whereas differentiated BC3H-1 and BD cells expressed a simpler pattern of MHCs. Different myogenic cell lines were, therefore, of multiple types that expressed distinct myogenic programs and formed myotubes with different MHC phenotypes.

At least two MHC isoforms, slow MHC and F47-reactive MHC(s), were expressed in distinct patterns by the different cell lines. Slow MHC expression appeared to be quantitatively different in different cell lines. Slow MHC was easily detected with mAbs S58, S46, and S22 in Aza2, C_2C_{12}, and TD myotubes, whereas, in BC3H-1 myocytes, slow MHC

was detectable only in some cells and only with the high affinity mAb R11D10. R11D10 has the same specificity for slow MHC as mAbs S58, S46, and S22, but has a binding constant several orders of magnitude greater than most mAbs (Khaw et al., 1984). On the other hand, F47-reactive MHC expression may have been qualitatively different in different cell lines. No F47-reactive MHC was detectable in BC3H-1 myocytes or BD myotubes, whereas such MHC was easily detectable in myotubes formed by the other cell lines.

Slow MHC was not previously known to be expressed by differentiated cells of mouse muscle cell lines. The identification of the slow skeletal/β-cardiac MHC isoform was based on reaction with slow MHC-specific mAbs, electrophoretic mobility, and immunopeptide mapping. By each of these criteria, the slow MHC expressed by cell lines was indistinguishable from the slow MHC expressed in heart or soleus muscle of the animal. Because seven slow MHC-specific mAbs from four laboratories reacted with the slow MHC expressed by the cell lines, it is unlikely that cross-reaction with MHC, other than slow MHC, was responsible for these results.

The F47-reactive MHC expressed in differentiated Aza2, C_2C_{12}, TD, and TG1 cultures appeared to be predominantly perinatal MHC because it had the same electrophoretic mobility and mAb reactivity as perinatal MHC. Because mAb F47 did not react with the embryonic and slow MHCs but did react with the perinatal, IIA, and IIB MHCs, this mAb was a probe for MHCs that, in the animal, are expressed late in fetal and neonatal development, after the initial stages of myotube formation have occurred (Narusawa et al., 1987; Vivarelli et al., 1988). The predominant sarcomeric MHC expressed by differentiated BC3H-1 cells was probably the embryonic MHC isoform (cf., Taubman et al., 1989) because it reacted with mAb F59, but not with mAbs F47 or S58, and it had the same mobility as embryonic MHC. Previous analyses showed that C_2C_{12} myotubes express embryonic and perinatal MHC proteins, as well as embryonic, perinatal, and IIB MHC mRNAs (Silberstein et al., 1986; Weydert et al., 1987). Further work will determine if the MHC expression patterns change as myotubes formed by different lines mature in culture (Cerny and Bandman, 1986; Silberstein et al., 1986; Weydert et al., 1987; Miller and Stockdale, 1989).

The complex pattern of MHC isoform expression was not dependent on the formation of multinucleated myotubes. The mononucleated myocytes, as well as the myotubes, formed by Aza2, C_2C_{12}, TD, and TG1 cells expressed slow MHC and F47-reactive MHCs, whereas the multinucleated myotubes formed by BD lines did not express detectable amounts of these MHCs. Additionally, exogenous MyoD1 expression in BC3H-1 cells did not lead to accumulation of slow and perinatal MHCs. BD cells expressed both MyoD1 and myogenin, as did Aza2, C_2C_{12}, TD, and TG1 cells, but BD myotubes did not express the complex pattern of MHCs. Because the complex pattern of MHC expression was found in different cell lines that expressed widely varying amounts of MyoD1 and myogenin mRNAs, no simple correspondence appeared to exist between the pattern of MHC isoform expression and the patterns of MyoD1 and myogenin expression.

This analysis did not determine if MyoD1 and myogenin expression might be necessary, though not sufficient, for a complex pattern of MHC expression, or if this pattern of MHC expression might be independent of MyoD1. Myogenic regulatory factors not examined here such as Myf-5, MRF4,MEF1, and MEF2 (Braun et al., 1989*a*, 1989*b*; Buskin and Hauschka, 1989; Gossett et al., 1989; Mueller and Wold, 1989; Rhodes and Konieczny, 1989) might also play a role in differential MHC isoform expression. These possibilities might be tested by using transfection and gene deletion to construct cell lines that express particular combinations of regulatory factors. MyoD1 expression did increase cell fusion and the formation of multinucleated myotubes in the BD lines (Brennan et al., 1990), so the genes required for myoblast fusion may be among those that are transcriptionally regulated by MyoD1 (Braun et al., 1989*a*; Lin et al., 1989; Thayer et al., 1989). Additionally, because nuclei in BD myotubes would be expected to be postmitotic, MyoD1 expression may overcome the inability of BC3H-1 cells to commit to terminal differentiation (Munson et al., 1982; Spizz et al., 1986; Taubman et al., 1989).

Several mechanisms might account for the different patterns of MHC isoforms expressed by different cell lines. One possibility is that the slow and perinatal MHC genes are no longer intact in the BC3H-1 and derived BD lines. It seems unlikely, however, that these two genes, which appear to be on different chromosomes (Mahdavi et al., 1984; Weydert et al., 1985), would both have mutated since the origin of BC3H-1 cells, whereas the embryonic MHC gene in BC3H-1 cells and the embryonic, perinatal, and slow MHC genes in the C_2C_{12} and 10T1/2-derived cells would have remained intact. The small amount of slow MHC detected with mAb R11D10 in BC3H-1 myocytes also suggests that this gene is intact. Another possibility is that BC3H-1 and BD cells may be blocked at particular stages of a single myogenic differentiation pathway, much as different stages of the hematopoietic pathway are represented by cell lines that carry out only a portion of that developmental program. Alternatively, myoblasts from different lines (e.g., BD and TD) might be committed to form myotubes with distinct MHC phenotypes. Multiple types of myoblasts have been identified at different stages of development in birds and mammals, and it seems likely that the different types of myoblasts play distinct roles in the formation and diversification of primary and secondary, fast and slow muscle fibers (White et al., 1975; Rutz and Hauschka, 1982; Miller and Stockdale, 1986*a*, 1986*b*, 1989; Cossu and Molinaro, 1987; Vivarelli et al., 1988; Hoh et al., 1989). The different patterns of MHC isoform expression in mouse myogenic cell lines may provide models for studying the intrinsic differences that distinguish the multiple types of myoblasts and myotubes found in developing animals.

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