MyoD, Myogenin Independent Differentiation of Primordial Myoblasts in Mouse Somites

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Abstract. The accumulation of two myogenic regulatory proteins, MyoD and myogenin, was investigated by double-immunocytochemistry and correlated with myosin heavy chain expression in different classes of myoblasts in culture and during early myogenesis in vivo.

During in vitro differentiation of fetal myoblasts, MyoD-positive cells were detected first, followed by the appearance of cells positive for both MyoD and myogenin and finally by the appearance of differentiated myocytes and myotubes expressing myosin heavy chain (MHC). A similar pattern of expression was observed in cultures of embryonic and satellite cells. In contrast, most myogenic cells isolated from newly formed somites, expressed MHC in the absence of detectable levels of myogenin or MyoD.

In vivo, the appearance of both myogenin and MyoD proteins was only detected at 10.5 d postcoitum (d.p.c.), when terminally differentiated muscle cells could already be identified in the myotome. Parasagittal sections of the caudal myotomes of 10.5-d-old embryos showed that expression of contractile proteins preceded the expression of myogenin or MyoD and, when co-expressed, MHC and myogenin did not co-localize within all the cells of the myotome. In the limb bud, however, many myogenin (or MyoD) positive/MHC negative cells could be observed in the proximal region at day 11. During further embryonic development the expression of these proteins remained constant in all the muscle anlagens examined, decreasing to a low level during the late fetal period. Western and Northern analysis confirmed that the myogenin protein could only be detected after 10.5 d.p.c. while the corresponding message was clearly present at 9.5 d.p.c., strongly suggesting a posttranscriptional regulation of myogenin during this stage of embryonic development.

These data show that the first myogenic cells which appear in the mouse myotome, and can be cultured from it, accumulate muscle structural proteins in their cytoplasm without expressing detectable levels of myogenin protein (although the message is clearly accumulated). Neither MyoD message or protein are detectable in these cells, which may represent a distinct myogenic population whose role in development remains to be established.

DIFFERENT populations of skeletal myoblasts (embryonic, fetal, adult, fast, slow, and mixed) might account in part for the asynchronous generation and the phenotypic heterogeneity of muscle fibers (Stockdale and Miller, 1987; Cossu and Molinaro, 1987; Stockdale, 1989; Vivarelli et al., 1988). Until now, however, it has not been clear how such myoblast heterogeneity might arise and whether it might be in any way relevant to the later phases of muscle development.

The recent identification of four myogenic regulatory genes (MyoD, myogenin, myf-5, and myf-6/MRF4/herculin), belonging to the helix-loop-helix (HLH)[1] family of DNA binding proteins and capable of activating myogenesis upon transfection in non-muscle cells (reviewed in Olson, 1990; Weintraub, 1991), has opened new approaches to the problem of myogenic cell heterogeneity. In fact, the differential expression of one or more of these genes in different populations of myoblasts might be responsible for subtle phenotypic differences observed among these different myogenic cells. In situ hybridization studies on the expression of these factors during mouse embryonic development support this hypothesis, since each of the myogenic factors shows a unique pattern of expression in space and time. Myf-5 is the first of these genes to be detected in the dorsal lip of the der-

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1. *Abbreviations used in this paper*: HLH, helix-loop-helix; rt, room temperature.

mamyotome at eight days of development, but this expression disappears at about 12–13 d (Ott et al., 1991). Myogenin and MyoD transcripts appear, respectively, at 8.5 and 10.5 d in the myotome and at ~11 d in the limb bud and continue to be expressed (Sassoon et al., 1989). Finally, myf-6 is transiently expressed in the myotome but not in the limb buds during the embryonic period and is subsequently expressed in all muscle during fetal and adult life (Bober et al., 1991; Hinterberger et al., 1991).

The recent availability of polyclonal antibodies and mAbs against the protein products of several of these genes allows an immunohistochemical analysis of the expression of these factors. Here we describe the expression of two of these factors, MyoD and myogenin, in different classes of myoblasts in vitro and in vivo.

Materials and Methods

Cell Cultures

Cells were cultured from somites or limbs of mouse embryos and fetuses, ranging in age from 8 to 17 d postcoitum (d.p.c.), and from limbs of adult mice as previously described (Vivarelli and Cossu, 1986; Cossu et al., 1983). Briefly, embryonic tissue was dissociated by gentle pipetting in a Ca-Mg-free PBS, while fetal and adult tissues were digested with 0.05% trypsin (Difco Laboratories Inc., Detroit, MI) in PBS for different periods at 37°C. After the proteolytic digestion, the tissues were fragmented by repeated pipetting, the debris was removed by filtration through sterile nylon gauze, and the cells were collected by centrifugation. Unless otherwise specified, cells were inoculated at an initial cell density of 3×10^5 cells/ml in DME (Flow Laboratories, Irvine, Scotland) supplemented with 15% horse serum (Flow Laboratories) and 3% chick embryo extract.

Immunocytochemistry

The following antibodies were used in this study: (*a*) An anti-MyoD rabbit antiserum (obtained from H. Weintraub; Tapscott et al., 1988). (*b*) Anti-myogenin mAbs, obtained by immunizing mice with rat recombinant myogenin (Wright et al., 1991). The monoclonal F5D reacts in ELISA with recombinant myogenin but not with MyoD, Myf 5, or MRF4. The epitope is located just carboxyterminal to the HLH domain of the myogenin protein. On Western blots the antibody reacts with a pair of ~34-kD polypeptides present in myogenic but not in fibroblastic cell lines. The reaction is abolished by pre-incubating the antibody with recombinant myogenin. Other monoclonals (D12F, A3B), directed against different myogenin epitopes, were also used where indicated. (*c*) MHC antibodies. MF20 is a mAb which recognizes all sarcomeric myosins (provided by D. A. Fischman; Bader et al., 1982). A rabbit antiserum against (MHC) was obtained from Sigma Chemical Co. (St. Louis, MO). In control experiments on tissue culture and cryostat sections, it stained the same cells and subcellular structures stained by MF20, i.e., sarcomeres in skeletal and cardiac muscle cells.

Embryos were fixed in 4% paraformaldehyde in PBS, dehydrated through a series of sucrose solutions (12, 18, and 30%), sectioned on a cryostat, and mounted on gelatin-coated slides. The slides were washed in 1% BSA in PBS for 30 min. Sections were pretreated with goat anti-mouse IgG (Cappel Laboratories, Malvern, PA) at 1:30 dilution to reduce nonspecific fluorescence.

The tissue sections and cultured cells were incubated for 1 h at room temperature (rt) with polyclonal antibodies (at dilutions ranging from 1:50 to 1:500) and mAbs (diluted 1:10); after the incubation, the samples were washed three times in 1% BSA in PBS and then incubated with a fluorescein-conjugated goat anti-rabbit Ig and with a rhodamine-conjugated goat anti-mouse Ig (both second antibodies were obtained from Cappel Laboratories and used at 1:30 dilution for 1 h at rt). Cultures and slides were mounted in PBS at pH 8, supplemented with 75% glycerol, and observed under an epifluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Western Blot Analysis

Tissues were homogenized in 0.4 M NaCl, 50 mM Tris-Cl, pH 7.4, 5 mM $MgCl_2$, and 1 mM each of the protease inhibitors benzamidine, PMSF, leupeptin, and soybean trypsin inhibitor (all from Sigma Chemical Co.). The homogenate was centrifuged at 100,000 *g* and the supernatant was separated on a 10% SDS-PAGE, transferred to nitrocellulose, and incubated with anti-myogenin antibodies at 1:10 or 1:50 dilution for 2 h at rt. The reaction was revealed by the immunogold–silver staining kit (Amersham Corp., Arlington Heights, IL) using an auroprobe BLplus goat anti-mouse second antibody according to manufacturer's instructions. Under identical conditions 1–2 ng of recombinant myogenin (Wright et al., 1991) could be detected.

Northern Blot Analysis

Total RNA was prepared from somites of 9.5- or 11.5-d-old mouse embryos or from differentiating C2C12 cells by acid phenol extraction (Chomczynsky and Sacchi, 1987). Gel electrophoresis, RNA transfer, and hybridization conditions have previously been described (Bober et al., 1991). The probes used for Northern blot analysis were a DNA fragment corresponding to the 3′ UTR of the myogenin cDNA (as described for cRNA probes used for in situ hybridization) and the pA1 plasmid which recognizes β-actin (Ferrari et al., 1990). DNA was labeled to a specific activity of $1–3 \times 108$ cpm/μg using the multiprime labeling kit from Amersham.

Preparation and Pre-hybridization of Tissue Sections

CD1 mouse embryos were fixed and embedded as described for immunocytochemistry. Restriction fragments, which specifically detect myogenin transcripts, were subcloned in the Bluescript transcription vector (Stratagene, La Jolla, CA) and grown in *Escherichia coli* TG1. The probe corresponds to the 3′ terminal 700 bp of the rat myogenin cDNA (Wright et al., 1989). The cRNA transcripts were synthesized according to the manufacturers instructions and labeled with ^{35}S-UTP (>1,000 Ci/mmole; Amersham Corp.). cRNA transcripts were partially hydrolyzed to a mean size of 70 nucleotides for efficient access to in situ mRNA.

Hybridization and Washing Procedures

Hybridization and washing procedures were performed as described in Lyons et al. (1990). Slides were dipped in undiluted NTB-2 nuclear track emulsion (Eastman Kodak Co., Rochester, NY) and exposed for 1 wk at 4°C. Slides were developed in Kodak D-19 and analyzed using light and dark field optics on an Axioplan microscope (Carl Zeiss).

Nomenclature

Throughout this paper, the following nomenclature (as described in Stockdale and Miller, 1987) will be adopted: (*a*) Myoblasts are myogenic, mononucleated, mitotic cells that are considered "committed" to the myogenic lineage but do not express skeletal muscle MHC in their cytoplasm. (*b*) Myotubes are postmitotic, oligo (>2), or multinucleated cells that express different MHC proteins in their cytoplasm. (*c*) Myocytes are postmitotic mononucleated cells that express different MHC proteins in their cytoplasm. (*d*) The embryonic period of development, in which major morphogenetic events take place in the developing organism, lasts from gastrulation (6.5 d.p.c.) to 12 d.p.c. in the mouse. (*e*) The fetal period, corresponding to a period of growth in the already formed organism, lasts from day 13 to birth. (*f*) Embryonic myoblasts are isolated from muscle anlagens of mouse embryos ranging in age from 10 to 12 d.p.c. and correspond to embryonic myoblasts isolated from chick E5 stage (Stockdale and Miller, 1987) and to early muscle colony-forming myoblasts (Rutz and Hauschka, 1982): they form oligonucleated myotubes (hence, referred to as embryonic myotubes) which co-express fast embryonic and slow MHC and differentiate in the presence of TPA (Cossu et al., 1988). (*g*) Fetal myoblasts are isolated from fetal muscles of mouse fetuses ranging in age from 15 to 17 d.p.c. and correspond to fetal myoblasts isolated from chick day 12 embryos and to late muscle colony-forming myoblasts: they form large multinucleated myotubes (fetal myotubes) that initially express only fast embryonic MHC.

Results

In Vitro Studies

Fetal myoblasts, isolated from limb muscles of 16 d.p.c. mouse fetuses, were fixed after different days in culture and incubated with different combinations of antibodies directed against MyoD, myogenin, or MHC. Fig. 1 shows fetal myo-

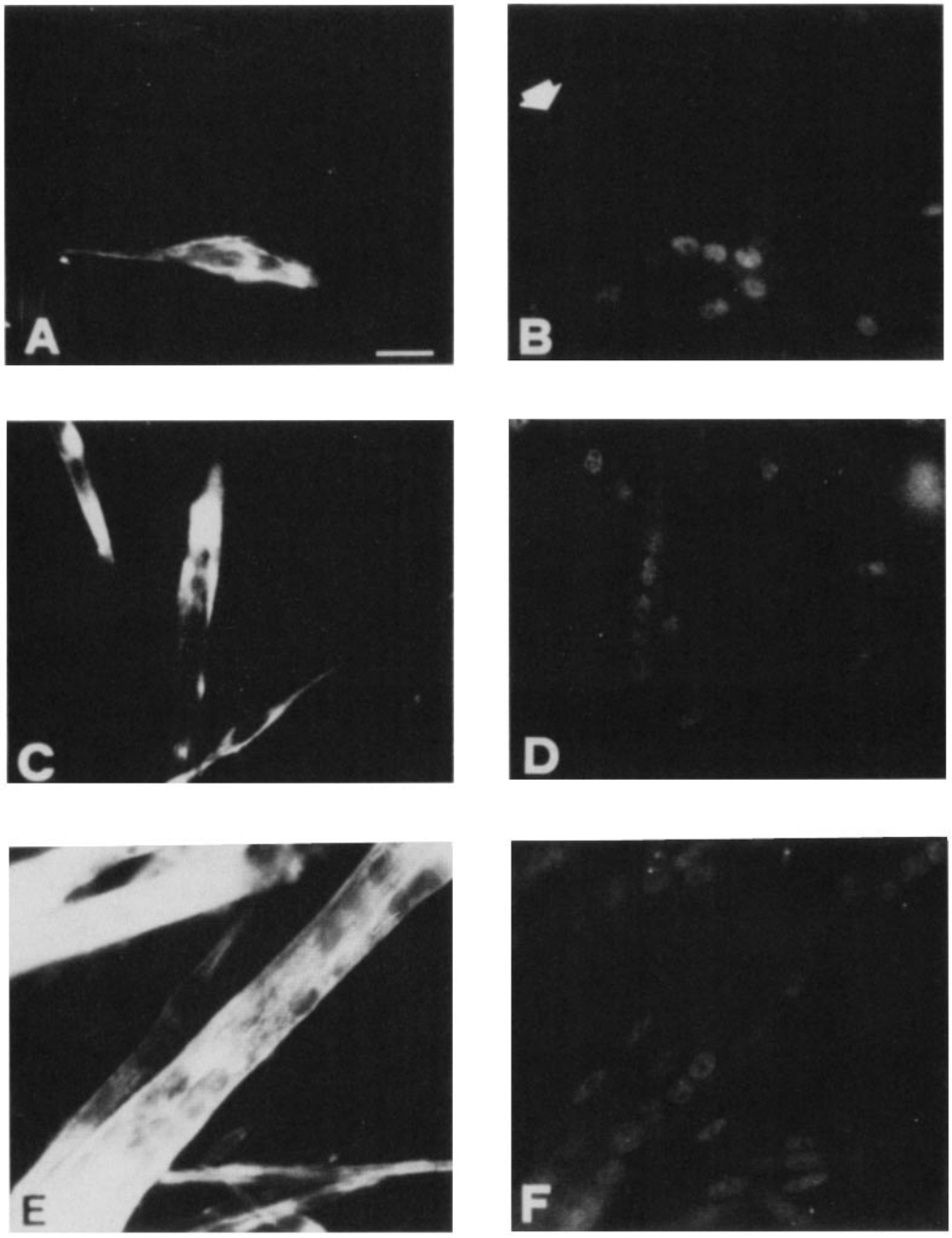

Figure 1. Double immunofluorescence of fetal myoblasts cultured from 16-d.p.c. mouse fetal limbs. The cells were stained with MF20 mcab (*A*, *C*, and *E*) and anti-MyoD pcab (*B*, *D*, and *F*) at day 1 (*A* and *B*), day 3 (*C* and *D*), and day 5 (*E* and *F*) of culture. Arrowhead shows a nucleus stained with anti-MyoD antibody, whose fluorescence was considered threshold in order to quantify positive cells (see Fig. 3). Bar, 10 μm.

blasts double stained with MF20 (which stains all sarcomeric myosins) versus an anti-MyoD polyclonal antibody. At day 1 of culture (Fig. 1, *A* and *B*), many nuclei stained with the anti-MyoD antibody (Fig. 1 *B*) while very few cells stained with MF20 (an example is shown in Fig. 1 *A*). At the onset of fusion (day 3 of culture) many differentiated myocytes and newly formed myotubes stained with both the anti-MyoD and MF20 antibodies, while many cells stained with anti-MyoD but not with MF20 (Fig. 1, *C* and *D*). No MF20+/MyoD− cells could be detected. After fusion was completed (day 5 of culture), the majority of cells stained with both antibodies (Fig. 1, *E* and *F*) but the intensity of staining in myotube nuclei had decreased. The same culture was also double stained with F5D (a mcab specific for myogenin) and anti-MyoD antibody. At day 1 of culture (Fig. 2, *A* and *B*) many more cells stained with anti-MyoD than with anti-myogenin; at day 3, however (Figs. 2, *C* and *D*), the two proteins co-localized in virtually all of the myogenic cells, as they did in the nuclei of myotubes at day 5 (Fig. 2, *E* and *F*) even though, at this stage, fluorescence was weaker in some nuclei. Fig. 3 illustrates the increase in the number of MyoD, myogenin, and MHC positive cells in cultures of fetal myoblasts. When the experiment was repeated on satellite cells, isolated from skeletal muscle of adult mice, essentially the same results were obtained (data not shown).

Embryonic myoblasts, isolated from 11-d.p.c. limb buds, showed a somewhat different pattern of expression of MyoD and myogenin in vitro. On day 1 of culture, many myogenic cells expressed MyoD and myogenin in their nuclei while still negative for the presence of MHC in their cytoplasm. Unexpectedly a minor, but reproducible, fraction of the myogenic population expressed MHC in the cytoplasm but no de-

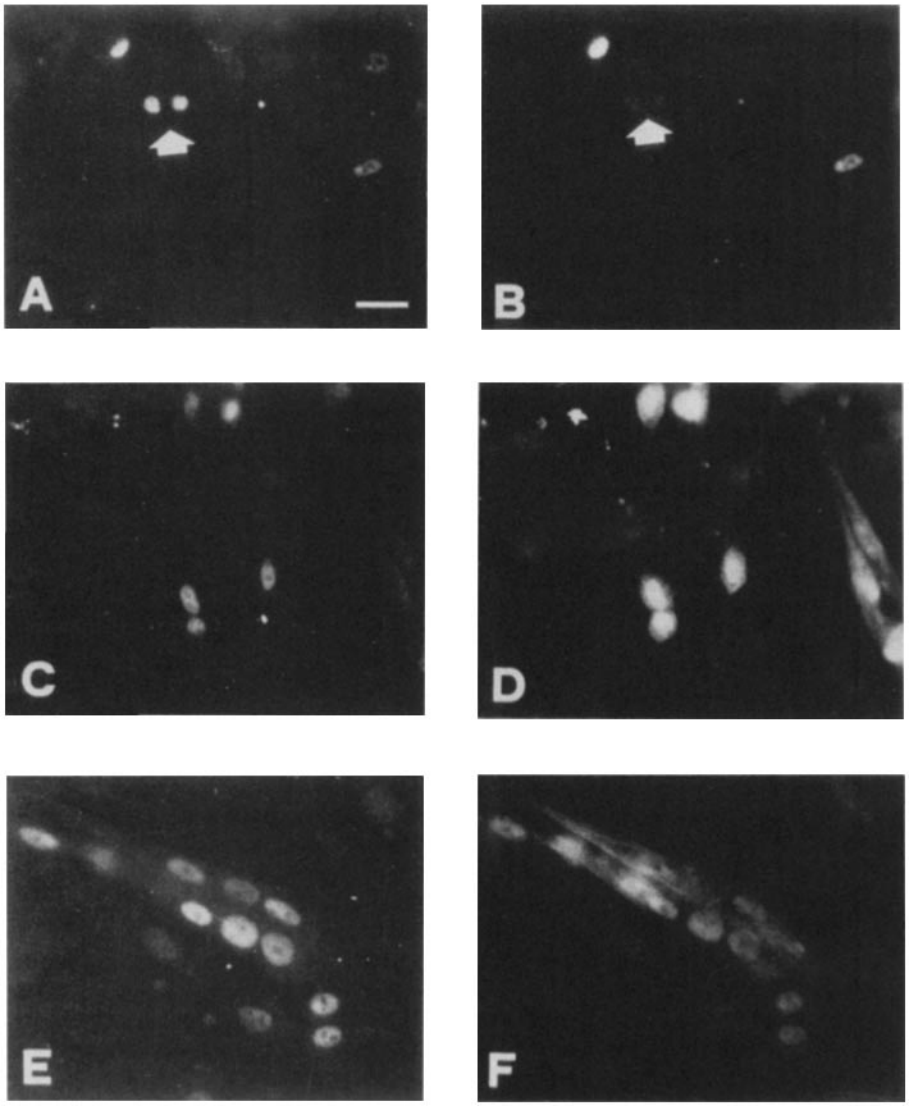

Figure 2. Double immunofluorescence of fetal myoblasts cultured from 16-d.p.c. mouse fetal limbs. The cultures were stained with anti-MyoD pcab (*A*, *C*, and *E*) and F5D (anti-myogenin) mcab (*B*, *D*, and *F*) at day 1 (*A* and *B*), day 3 (*C* and *D*), and day 5 (*E* and *F*) in vitro. Arrows show myoblasts whose nuclei express MyoD but not myogenin. Bar, 10 μm.

tectable amount of MyoD or myogenin in their nuclei. Fig. 4 shows examples of these myocytes, (MHC+/MyoD− in Fig. 4, *A*, *B*, and *MHC+/myogenin−* in *C* and *D*) together with MHC−/MyoD+ or myogenin+ myoblasts and MHC+/MyoD+ or myogenin+ embryonic myotubes. The presence of MHC+-differentiated muscle cells which do not express detectable levels of MyoD or myogenin in their nuclei might depend on transient expression of these gene products in embryonic myogenic cells or on the existence of a subpopulation of cells which can differentiate without ever expressing these proteins (at levels detectable by immunofluorescence). To discriminate between these two possibilities, we analyzed early myoblasts from somites for the expression of MyoD and myogenin during in vitro differentiation. In fact, if MyoD−/myogenin− myoblasts represent a different population, it is conceivable that this population might be an early one (since it is not apparently present among fetal cells) and therefore would predominate at the earliest stages of myogenesis. If, on the other hand, transient expression of MyoD and myogenin was a feature of all embryonic myoblasts, one would expect to find MyoD−/myogenin−-differentiated cells in similar proportions among somitic myoblasts. When myoblasts were explanted from somites of 8.5 d.p.c. mouse embryos and grown in culture, many mononucleated, cross-striated, differentiated (MHC-positive) myocytes appeared during the first days in vitro (Fig. 5, *A* and *B*). None of these myocytes (out of 565 myocytes scored in three separate cultures) expressed detectable levels of MyoD or myogenin in their nuclei during the first day of culture. After 2 d in culture, several MyoD and myogenin positive cells appeared but none of these cells yet expressed MHC (Fig. 5, *C* and *D*). Fig. 6 illustrates the increase in the number of MyoD, myogenin, and MHC positive cells in micromass cultures where an initial inoculum of 10^5 somitic and neural tube–derived cells were plated: only MHC positive (MyoD−/myogenin−) cells increase in number during the first day of somitic cultures, while myogenin+ (or MyoD+) cells begin to accumulate during the second and third day in vitro. Thus this pattern is radically different from that observed in cultures of fetal myoblasts, where only MHC− (MyoD+/myogenin+) cells increase in number during the first days in vitro (Fig. 3). It should be noted that MHC+ cells represent a very small

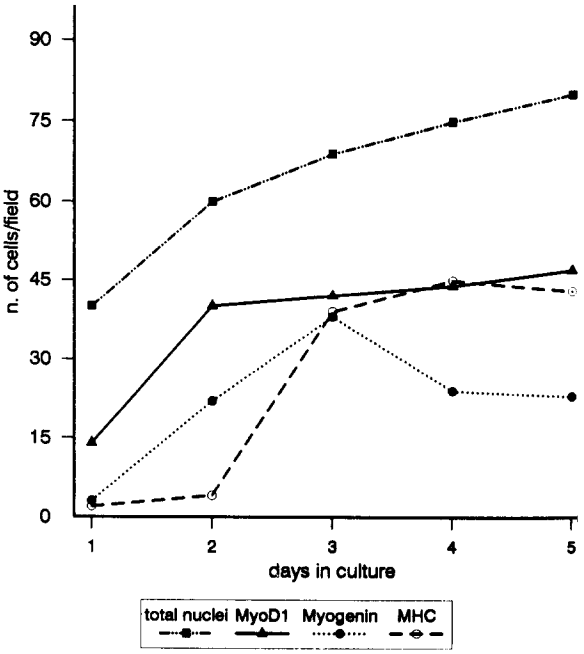

Figure 3. Time course of appearance of cells positive for MyoD (—▲—) myogenin (···●···) or MHC (---o---)/microscopic field (scored at 400×), during culture of fetal myoblasts. (-·-·-■-·-·-): total nuclei/microscopic field. 10^5 cells were plated in 30-mm dishes in 1.5 ml of complete medium, fixed at daily intervals, stained with the different antibodies, and the number of positive nuclei (for MyoD) and cells (for MHC) was counted. Since fluorescence varied from cell to cell, we considered positive those nuclei whose fluorescence could be distinguished over cytoplasmic background (an example of such nuclei is shown in Fig. 1 *B*). When myotubes appeared, each nucleus within the myotube was counted as a separate cell. On day 5 of culture, ~50–70% of nuclei were in myotubes.

fraction (<5%) of the total cell population, since the great majority of cells, cultured from these early stages, do not express any marker which might allow immunocytochemical identification. When the experiment was repeated with 9.5-d.p.c. embryos, the results were similar, except that MyoD+/myogenin+ myoblasts appeared after the first day of culture and in larger numbers, suggesting that their appearance is somehow timed in the embryo at ~10 d.p.c. (see below). To investigate whether these MyoD−/myogenin− myogenic cells might give rise to MyoD+/myogenin+ myoblasts we cloned cells isolated from 9.5-d.p.c. somites. After 7 d of clonal culture, the dishes were similarly stained with the various combinations of antibodies. Fig. 7 shows examples of these cells, the majority of which appeared as single, nonclonogenic differentiated MHC+ myocytes. Approximately 60% of these single myocytes were myogenin+, the others myogenin− (Fig. 7, *A* and *B*). No MHC−/myogenin+ cells were observed. A minority of small (2–8 nuclei) differentiated MHC+ clones were observed: in 22 of these clones, all the nuclei were myogenin+. Other 5 MHC+ differentiated clones were composed of two nuclei which were myogenin− (one of these clones is shown in Fig. 7, *E* and *F*). The results of three separate experiments are reported in Table I. In no case were myogenin+ and myogenin− cells present in the same clone.

In Vivo Studies

To investigate when and where these "primordial" (MHC+/MyoD−/myogenin−) muscle cells might appear during embryonic development in vivo, we incubated serial sections (both transverse and parasagittal) of mouse embryos, ranging in age from 8.5 to 13 d.p.c., with the same combinations of antibodies used for the in vitro study. Fig. 8 shows a double immunofluorescence of 9.5 (*A* and *B*) and 10.5 d.p.c. (*C* and *D*) embryos incubated with both anti-MyoD (*A* and *C*) and anti-myogenin (*B* and *D*) antibodies. It is clear from the figure that no nuclei can be labeled by either antibody in a transverse section of 9.5-d.p.c. embryos (which shows the neural tube with adjacent somites), while many myotomal nuclei were labeled by both antibodies at 10.5 d.p.c. In more than 50 different sections through many different 8.5- to 9.5-d.p.c. embryos (ranging from 5 to 30 somites) we never detected any nucleus positive for either MyoD or myogenin. Once fluorescence became detectable in myotomes at 10 d.p.c. (>40 somites), the great majority of nuclei co-expressed both MyoD and myogenin. Similarly, the majority of the cells present in the proximal region of the forelimb bud at 11.5 d.p.c., co-expressed MyoD and myogenin (Fig. 8, *E* and *F*). Since this is the developmental period when terminally differentiated muscle cells appear in the myotomes (Furst et al., 1989; Babai et al., 1990), we compared the appearance of MHC with that of both the MyoD and myogenin proteins. To this purpose, we incubated transverse and parasagittal sections of 10.5-d.p.c. embryos with the various antibodies. By taking advantage of the cranio–caudal gradient of muscle differentiation, we could observe both MHC positive and negative somites in a cranio–caudal succession through the same section. Fig. 9 (*A* and *B*) shows an example of two adjacent MHC+/myogenin+ somites, where positive nuclei are usually located in the central area of the myotome, whose edges are characterized by elongated MHC+/myogenin− cells. Fig. 9 (*C* and *D*) shows a more caudal region of the embryo, where the cranial somite appears to co-express MHC and myogenin, while the next caudal somite is exclusively composed of MHC+/myogenin− cells. In a transverse section, MHC+ cells are observed throughout the myotome but myogenin+ nuclei appear only in a proportion of these cells (Fig. 9, *E* and *F*). Identical results were obtained co-staining parallel sections with anti-MyoD and anti-MHC (data not shown). At 11 d.p.c., all the myotomes expressed MyoD, myogenin, and MHC. Infrequently, small areas in the lateral edge of the myotome did not express detectable levels of myogenin (an example is shown in Fig. 10, *A* and *B*). In the developing limb of the same embryo, the pattern of expression was radically different from that observed in the somites. Fig. 10 (*C* and *D*) shows a group of myogenin+/MHC− myoblasts, located in the intermediate region of the limb bud; the figure also shows a single MHC+ myocyte which does not express detectable levels of myogenin in the nucleus. During further embryonic development, virtually all of the newly formed primary fibers, present in both the body wall and in the limbs of a 13-d.p.c. mouse embryo co-express MyoD and myogenin in their nucleus, but the intensity of labeling is usually decreased (data not shown).

The data reported above are in apparent contrast with the results of in situ hybridization where the presence of myogenin transcripts in somites of 8.5-d.p.c. mouse embryos was demonstrated (Sassoon et al., 1989) while MyoD transcripts

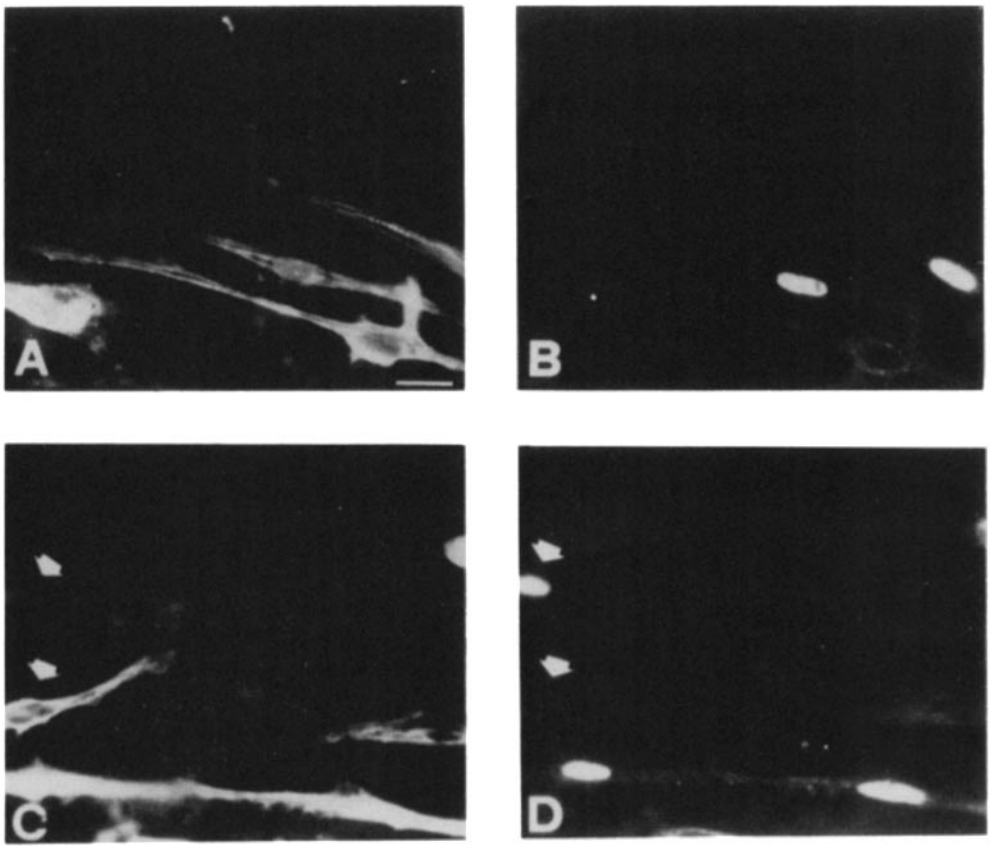

Figure 4. Double immunofluorescence of embryonic myoblasts cultured from 11-d.p.c. mouse embryo limb buds. After 3 d in culture, the cells were double stained either with MF20 mcab (*A*) and anti-MyoD pcab (*B*) or with anti-MHC pcab (*C*) and F5D mcab (*D*). Arrows show MHC+/myogenin− and MHC−/myogenin+ cells. Bar, 15 μm.

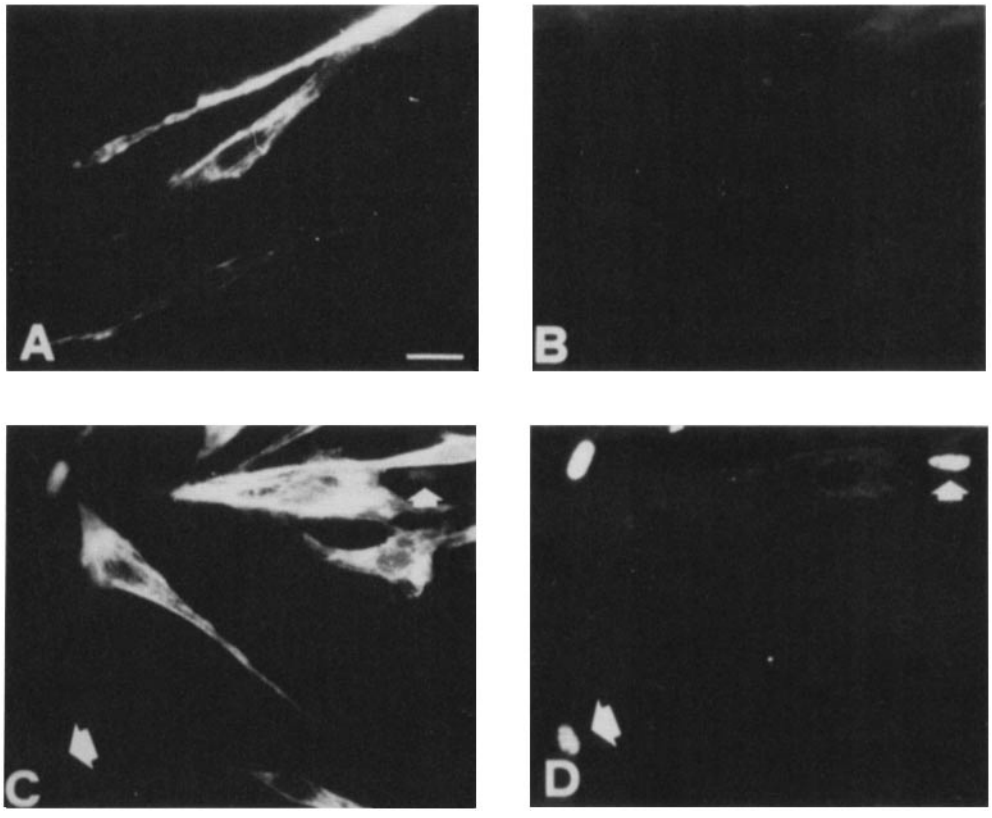

Figure 5. Double immunofluorescence of myoblasts cultured from 8.5-d.p.c. mouse somites. The cultures were stained with anti-MHC pcab (*A* and *C*) and F5D mcab (*B* and *D*) at day 1 (*A* and *B*) and day 3 (*C* and *D*) in vitro. Arrows show MHC−/myogenin+ cells. Bar, 15 μm.

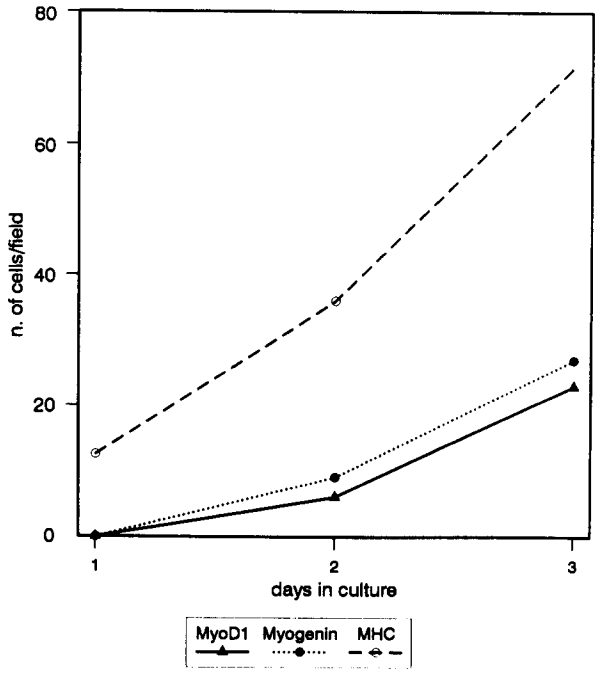

Figure 6. Time course of appearance of cells positive for MyoD (—▲—), myogenin (···●···), or MHC (---o---) during culture of somitic cells. Numbers refer to positive cells/microscopic field. 4×10^4 cells were plated in 100 μl of complete medium as a microspot in the center of 30-mm dishes, fixed at daily intervals, and stained with the different antibodies. In these cultures, <10% of total cells express any myogenic marker.

could not be detected until 10.5 d.p.c. Although a perfect temporal correspondence exists between the appearance of the MyoD transcript at 10.5 d.p.c. and of the corresponding protein, the appearance of the myogenin transcript apparently precedes the appearance of the corresponding protein by about two days. To verify that this might not depend upon trivial reasons, such as different strains of mice or different staging of the embryos, we prepared serial parasagittal cryostat sections of 10.5-d.p.c. embryos: one section was incubated with anti-myogenin and anti-MHC antibodies, the next was hybridized with the same riboprobe specific for the myogenin transcript described before (Sassoon et al., 1989). Fig. 11 shows the abundance of myogenin transcripts (Fig. 11 *B*) and the absence of the corresponding protein (Fig. 11 *C*) in myotomes which already express MHC (Fig. 11 *D*).

To rule out that the mcab used (F5D) reacts with an epitope which might be masked (e.g., phosphorylated, methylated, etc.) at this developmental stage, we repeated the experiments using a variety of anti-myogenin mcabs directed against epitopes located within different domains of the protein. In all cases, the results obtained were identical to those obtained with ID5F7 (data not shown).

Biochemical Analysis of the Myogenin Transcript and Protein

To attempt a biochemical comparison between message and protein, we isolated somites (still connected to neural tubes) from 193 9.5-d.p.c. mouse embryos (selected at a stage between 15 and 35 somites), divided these somites into two randomly selected groups and extracted RNA and high salt soluble proteins from them. Fig. 12 *A* shows a Northern blot analysis of total RNA isolated from these somites (lane *a*) and of the corresponding somites of 11-d.p.c. embryos (lane *b*); fig. 12 *B* shows a Western blot analysis of proteins extracted from the same tissues (somites from 9.5 and 11 d.p.c.; *d* and *e*, respectively). It is clear from the figure that comparable amounts of myogenin message are present at both 9.5 and 11 d.p.c., while the myogenin polypeptide can only be detected at 11 d.p.c. Preliminary experiments using recombinant myogenin (Wright et al., 1991) had shown that the sensitivity of the method used would reveal ~1–2 ng of protein (data not shown). Therefore, assuming that the antibody would recognize with similar affinity both recombinant and natural myogenin, we can tentatively conclude that in 50 μg of our somite extracts (which correspond to ~100 μg of total homogenate), there is <1–2 ng of myogenin (<0.002% of total proteins) at 9.5 d.p.c. and ~10 ng of myogenin (0.01% of total proteins) at 11 d.p.c.

Discussion

This paper describes the early appearance and spatial distribution of MyoD and myogenin proteins in the post-implantation mouse embryo and their expression in myogenic cells which were cultured from muscles at different stages of development. The results show that myoblasts isolated from limbs at all developmental stages (from 11 d.p.c. to limbs of adult mice) express MyoD and myogenin in vitro according to a pattern which has already been described (at the mRNA level) for many myogenic cell lines (reviewed in Olson, 1990; Weintraub et al., 1991). MyoD is the first protein to be expressed in vitro; myogenin rises at the onset of cell differentiation, immediately followed by the appearance of myosin and other muscle products. These results suggest that the phenotypic differences among different myogenic cells and the fibers they form in vivo or in vitro do not depend on differential expression of MyoD or myogenin. They might be related to differential expression of other members of this gene family such as Myf 5 or MRF4 or to different regulatory genes yet to be identified.

A substantial departure from this pattern is found in a population of myogenic cells, which we term "primordial". These cells are isolated from newly forming somites and terminally differentiate in vitro without expressing detectable levels of MyoD or myogenin proteins in their nuclei. If myogenic cells are isolated from late somites or early limb buds, a second population appears in vitro which co-express both MyoD and myogenin in their nuclei before the onset of terminal differentiation. These probably represent "embryonic myoblasts".

The lineage relationship of primordial and embryonic myogenic cells is unclear. Clonal analysis suggests that "primordial" myogenic cells divide little or not at all in culture: 80% of the "clones" of MHC+/myogenin− cells were single myocites and the five remaining ones were doublets. Furthermore BUdR labeling of micromass cultures revealed that all primordial myocytes were post-mitotic (G. Cossu, unpublished observations). No evidence for a common precursor was obtained, since no mixed colonies were found and all 17 clones containing three or more nuclei were composed of MHC+/myogenin+ cells. On the other hand culture condi-

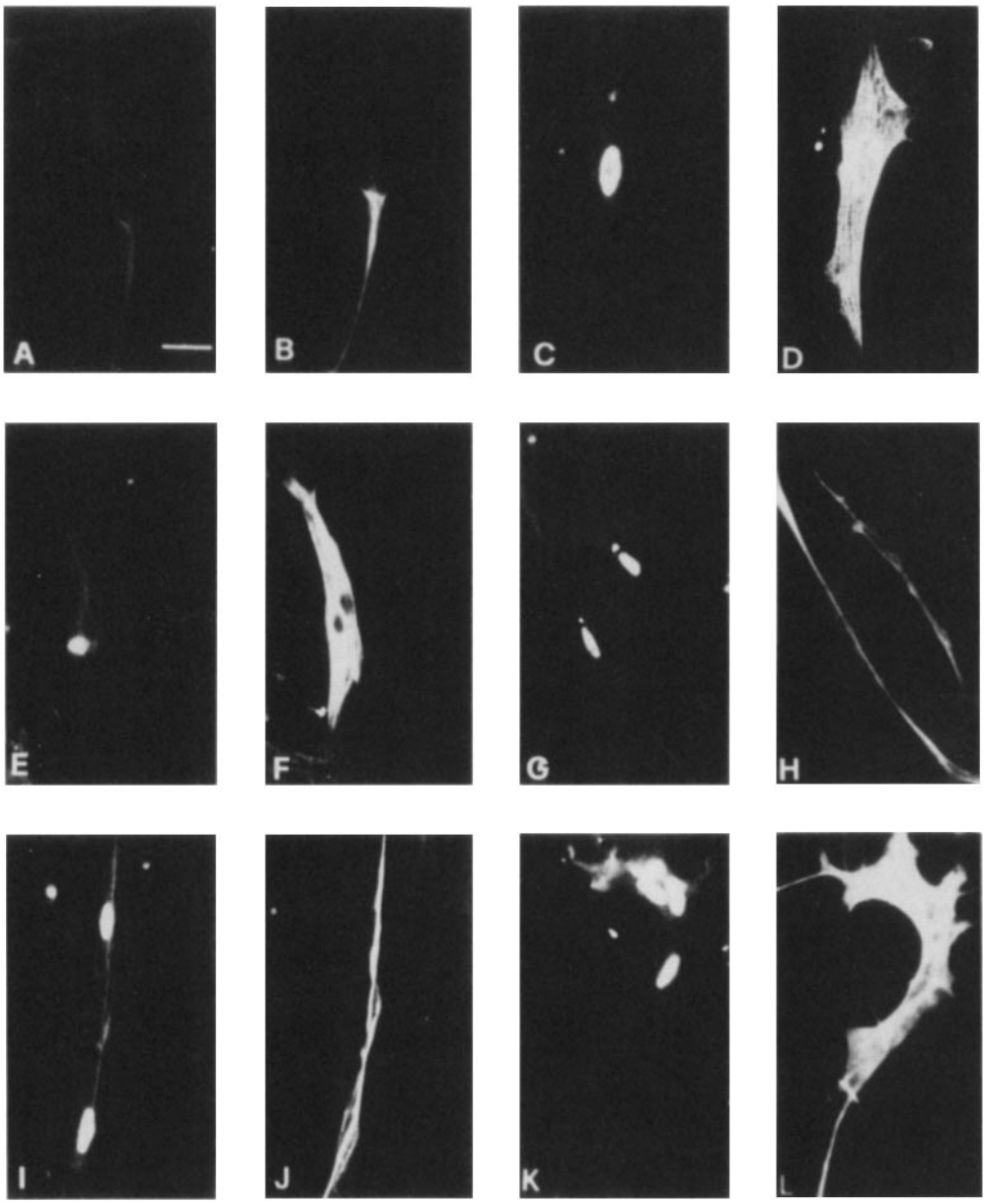

Figure 7. Examples of clones of somitic myocytes from 9.5-d.p.c. mouse embryos, double-stained with F5D mcab (*A*, *C*, *E*, *G*, *I*, and *K*), and anti-MHC pcab (*B*, *D*, *F*, *H*, *J*, and *L*) after 7 d in vitro. Note the absence of mixed (*myogenin−/myogenin+*) clones (in *E* a spot of fluorescence is seen, outside the dark shadows of the nuclei). Bar, 10 μm.

Table I. Survival and Differentiation of Myoblasts from Somites of 9.5-d.p.c. Embryos under Clonal Culture Conditions

Number of MHC+ clones	Number of cells/clone	Number of MHC+/ myogenin+ cells/clone	Number of MHC+/ myogenin− cells/clone
33	1	1	0
26	1	0	1
8	2	2	0
5	2	0	2
8	3	3	0
6	4	4	0
1	5	5	0
2	8	8	0

Cells were cloned by progressive dilution on a STO feeder layer and grown for 7 d in DME supplemented with 20% FCS and 5% E. E. and then fixed and double stained with anti-MHC and ID5F7 antibodies as described in Materials and Methods. No MHC−/myogenin+ cells or mixed clones containing both myogenin+ and myogenin− cells were detected under these conditions.

tions may simply not have been permissive for the division of primordial myogenic cells.

During this period of development (from 8 to 11 d.p.c.) the embryo undergoes a dramatic increase in size, and moreover, the muscle forming areas increase from a small portion of the somite to the majority of the available mesodermal space (Rugh, 1990). This means that the actual number of muscle forming cells and already differentiated muscle undergo an explosive growth during this period and, if the MyoD−/myogenin− myocytes do not increase in number (as discussed above), these cells would be lost among the multitude of embryonic and fetal myoblasts. Even so, it is likely that "primordial" precursors migrate to the limb, since MyoD−/myogenin− differentiated cells can be identified (although as a very minor fraction) among myogenic cells cultured from early forming limb buds and also because rare myogenin−/MHC+ myocytes can be identified in developing limb buds in vivo. Co-staining for MHC and myogenin

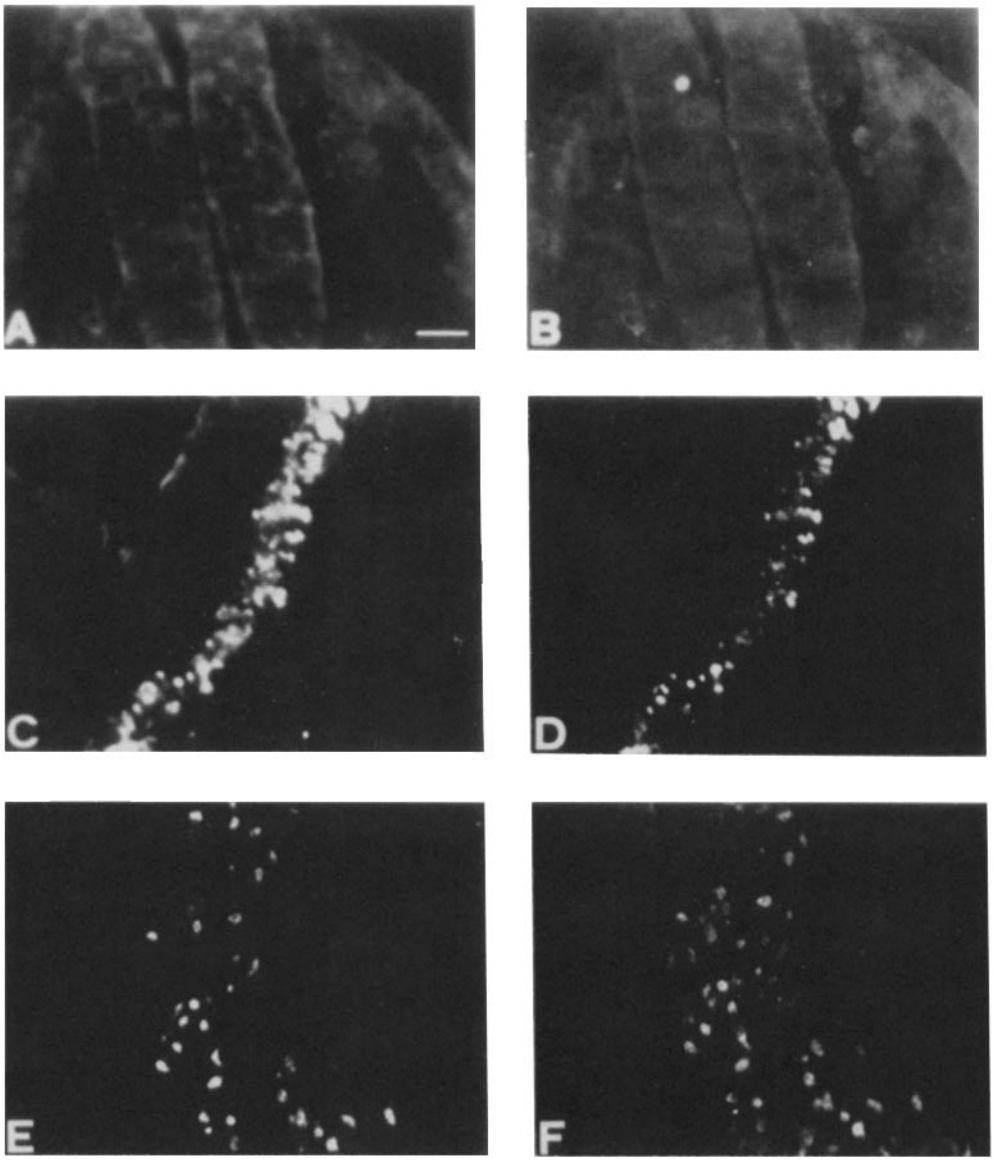

Figure 8. Double immunofluorescence of a transverse section of the trunk of 9.5 d.p.c. (*A* and *B*) and 10.5 d.p.c. (*C* and *D*) and of the limb bud of 11.5-d.p.c. (*E* and *F*) mouse embryos, stained with anti-MyoD pcab (*A*, *C*, and *E*) and F5D (*B*, *D*, and *F*). *A* and *B* were deliberately over exposed to show absence of nuclear staining in somites (*A* and *B*). Note co-staining of virtually all of the nuclei (*C* and *D*) of the myotomes and of the limb bud (*E* and *F*). Bar, 50 μm.

reveals that both these proteins are present in the forming myotomes of a 10-d.p.c. mouse embryo: both proteins appear in an expected cranio–caudal succession, but MHC expression precedes myogenin expression so that we frequently observed MHC+/myogenin− myotomes but never a MHC−/myogenin+ myotome. Furthermore, even when co-expressed in the same myotome, myogenin positive cells are typically located in the center but not at the edge of the myotome where MHC+ (primordial?) myocytes are present. Even at a later stage of myotome maturation, minor lateral areas of MHC+/myogenin− cells can be observed.

It could be argued that myogenin is not an abundant protein and therefore its presence at early stages might be undetectable by techniques such as Western blotting or immunofluorescence, which might nonetheless show the expression of an abundant protein such as MHC. However, a comparison of the pattern of expression of these proteins in developing limb buds argues against such an interpretation: many myogenin+/MHC− myoblasts can be detected in the limb buds of the same embryo where myogenin−/MHC+ myotomes were observed. In any case, the level of nuclear expression which can be revealed by immunofluorescence obviously reflects a considerable accumulation of the protein within the nucleus so that lower levels of expression might be functionally important but below the threshold of detectability. This consideration is particularly relevant to the unexplained discrepancy between the early detection of the myogenin message and the relatively late detection of its protein product. This is striking when compared to the simultaneous appearance of MyoD message and its protein product in the myotomes as well as to the appearance of both messages (Sassoon et al., 1989) and related proteins in limb buds. In the case of myogenin in the somites, the simplest interpretation of the data is a translation block or decreased half-life of the protein in 9-d.p.c. embryos. In fact, even considering the possible increased sensitivity of in situ hybridization over immunofluorescence, comparison of the relative abundance of message and protein, by both morphological and bio-

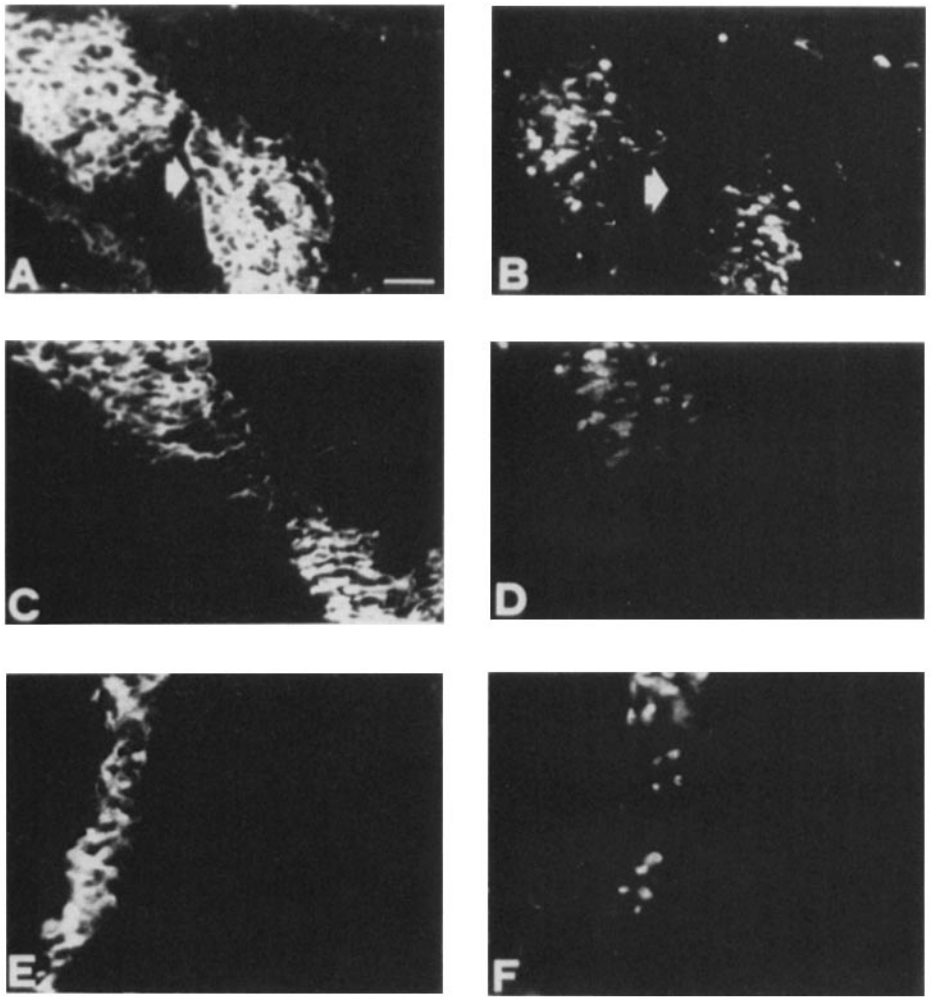

Figure 9. Double immunofluorescence of parasagittal (*A–D*) or transverse (*E* and *F*) sections of 10.5-d.p.c. mouse embryo, stained with anti-MHC pcab (*A*, *C*, and *E*), and F5D (*anti-myogenin*) mcab (*B*, *D*, and *F*). Arrows show myogenin− cells at the edge of the myotome whose center contain myogenin+ cells. Bar, 30 μm.

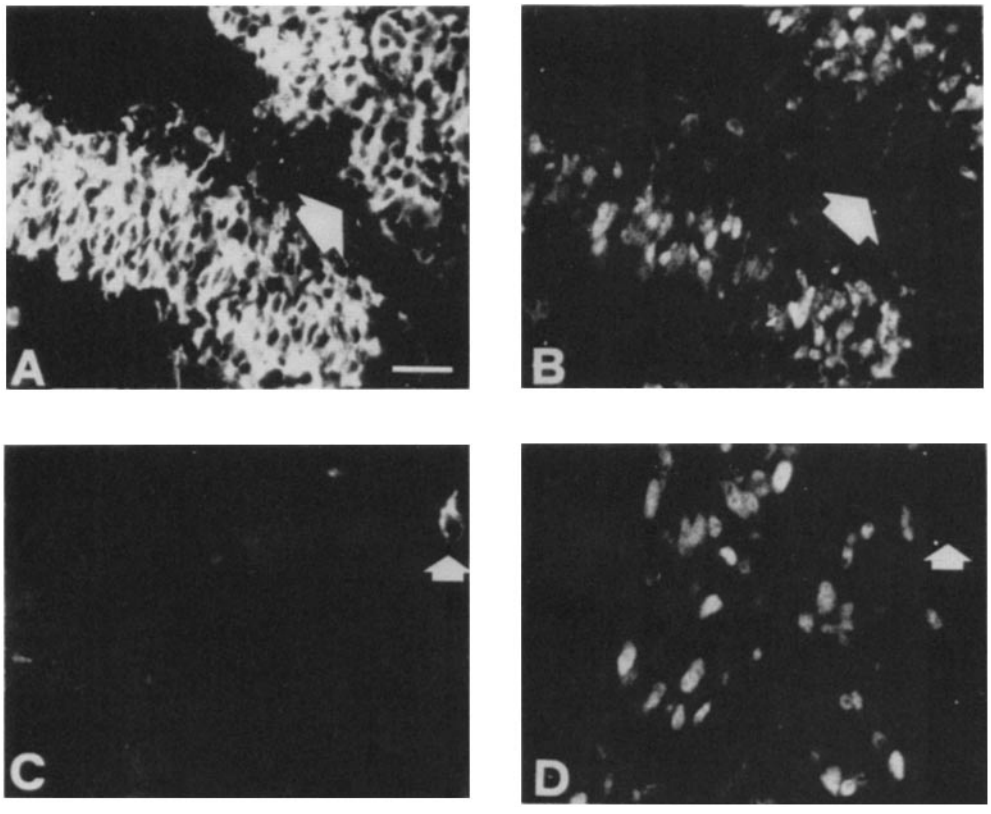

Figure 10. Double immunofluorescence of transverse sections of 11.5-d.p.c. mouse embryo, stained with anti-MHC pcab (*A* and *C*) and F5D (*anti-myogenin*) mcab (*B* and *D*). Arrows show myogenin−/MHC+ cells in the myotome (*A* and *B*) and a single myogenin−/mHC+ myocyte in the proximal region of the limb bud (*C* and *D*). Bar, 20 μm.

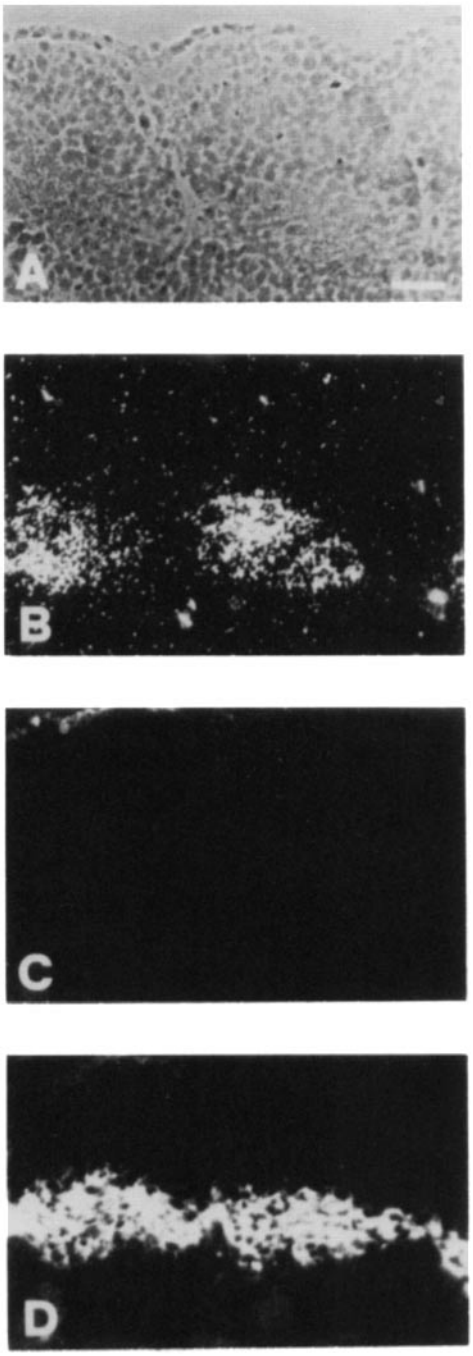

Figure 11. In situ and double immunofluorescence on serial parasagittal sections of 10.5-d.p.c. mouse embryos. One section (phase contrast in *A*) was hybridized to myogenin probe (dark field in *B*). The next section was double-stained with F5D (*C*) and with anti-MHC pcab (*D*). Bar, 20 μm.

chemical methods, clearly shows that a similar amount of message is accompanied by a striking increase in myogenin protein between day 9 and 11 of mouse embryonic development. A translational block has been postulated for other transcription factors such as Growth Hormone Factor 1 whose message is detected at the earliest stages of pituitary formation, while the corresponding protein can only be detected two days later, when the adenohypophysis has completed differentiation (Dollè et al., 1990). Furthermore, messages for two contractile proteins, namely the neonatal myosin heavy chains (Lyons et al., 1990) and the cardiac troponin I (Ausoni et al., 1991), are expressed early in rodent development while the corresponding proteins can be detected only several days later. In all these cases, information on the molecular basis of this phenomenon has been difficult to obtain, since little material is available for biochemical analysis. Control of polyadenylation, such as was found for maternal messages (Paris and Richter, 1990), does not seem to be involved (Bouchè, M., and G. Cossu, unpublished observations).

In the case of myogenin, a sucrose gradient analysis revealed that the myogenin message is already associated with the polysome fraction at 9.5 d.p.c. (Bouchè, M., unpublished observations), thus suggesting that the protein may be translated, but does not accumulate to a level detectable by antibodies. Since many posttranslational modifications are required to transport proteins to the nucleus (Silver, 1991), inefficiency of one of these enzymatic processes might prevent nuclear accumulation of myogenin and eventually increase its rate of cytoplasmic degradation. Whatever the mechanism underlying the posttranscriptional control of myogenin accumulation in "primordial" myogenic cells, low levels of myogenin protein do not prevent terminal differentiation of these cells, either in vivo or in vitro. Therefore "primordial" myogenic cells differentiation is either dependent upon very low levels of myogenin or upon the expression of other, as yet unidentified, regulatory gene products (see below).

To understand the possible developmental significance of these primordial myogenic cells in muscle histogenesis, it is necessary to obtain a detailed knowledge of their appearance and distribution in vivo. This is not an easy task, especially because these cells have been characterized by the absence rather than the presence of an early marker, and therefore can be identified only by the relatively late appearance of MHC in their cytoplasm. Recent data on myf-5 transcript expression in mouse embryogenesis (Ott et al., 1991), suggest that this gene product, being the first to be detected in early forming somites, might selectively regulate differentiation of these primordial myogenic cells. Unfortunately, to date, mAbs produced against human recombinant myf-5 have failed to react with the homologous rodent protein and therefore we have not been able to extend this immunohistochemical analysis to this gene product. Myf-6 transcripts are also present in early myotomes (Bober et al., 1991; Hinterberger et al. 1991), although antibodies are not yet available. These two myogenic factors may substitute for myogenin and MyoD at this stage. The initial observations on transcript accumulation were puzzling in that myf-6 appeared transiently in early myotomes but was not detectable in the pre-muscle masses of the limb. In the limb, myf-5 transcripts are present but only very briefly prior to the expression of muscle structural genes. These observations can now be interpreted in terms of the presence of both MyoD and myogenin proteins in most cells in the pre-muscle masses of the limb, while the absence of both MyoD and myogenin proteins in the early somite, may explain why both myf-5 and myf-6 are expressed abundantly there. This would be in keeping with a scheme in which at least two myogenic regulatory sequences are required for triggering muscle differentiation. In muscle cell lines myogenin is invariably present (Braun et al., 1990); perhaps myf-6 substitutes for it in the early somite. However, it is also possible that none of the known HLH family of genes might be responsible for the differentiation of these primordial myogenic cells.

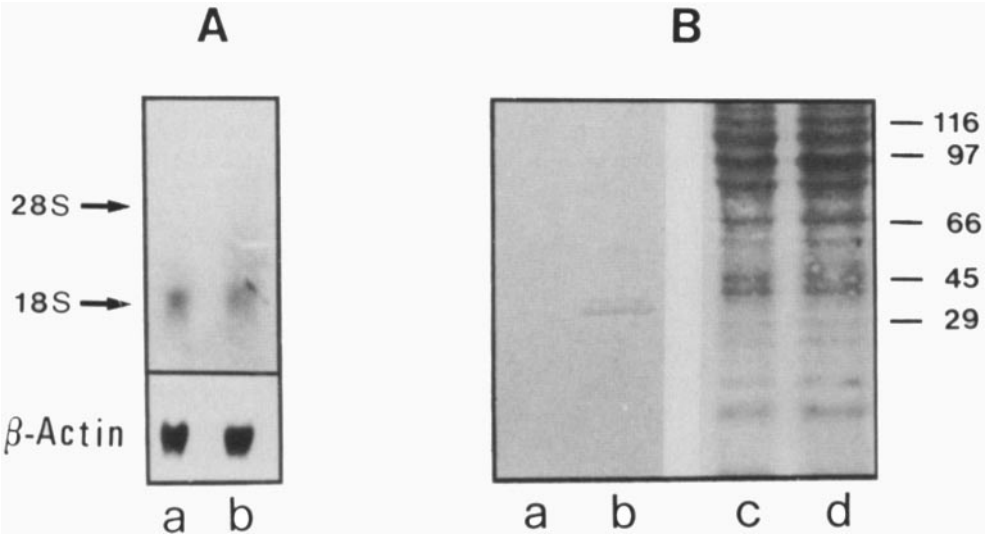

Figure 12. Comparison of myogenin message and protein in developing myotomes. (*A*) Northern blot of RNA extracted from 9.5 (*a*) and 11.5-d.p.c. (*b*) mouse embryo somites and hybridized to a myogenin complementary cDNA; the same filter was rehybridized to a β-actin probe (*inset*) to confirm that similar amounts of RNA had been loaded for both samples. Arrows indicate the migration of 28 and 18S RNA. (*B*) Western blot or proteins extracted from 9.5 (*a*) and 11.5 (*b*) mouse embryo somites and reacted with F5D mcab. The corresponding total proteins stained with amido-Schwartz are shown in lanes *c* and *d*, respectively. Molecular weight standards were: β-galactosidase (116 kD), phospholipase (97 kD), bovine albumin (67 kD), ovo-albumin (45 kD), and carbonic anhydrase (29 kD).

As to their possible developmental role, we can only speculate that these cells, which terminally differentiate earlier than primary muscle fibers and appear to be post-mitotic, may play in mammals a similar function to the role played by pioneer myoblasts in invertebrates (Jensen, 1990). In the grasshopper (Ho et al., 1983) or in the leech (Jellies and Kristan, 1988), for example, the first myogenic cells that form in the embryo are disposed in a metameric pattern and attach to the overlaying ectoderm to define territories where successive populations of myoblasts will later migrate. Recently muscle precursors expressing a MyoD analog in a founder cell-like pattern (Michelson et al., 1990; Paterson et al., 1991), have been identified in Drosophila (Bate, 1990). By analogy, we like to think that pioneer myoblasts might be needed in vertebrates as well, to define territories where later embryonic and fetal myoblasts will migrate. In the absence of a specific marker, and given the size and complexity of higher vertebrate embryos compared to the very limited number of these primordial myoblasts, they could have escaped previous investigations (Jacob et al., 1979; Krenn et al., 1988).

Further investigation and new immunological reagents are required to define the precise phenotype and developmental role of these "primordial" myoblasts.

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