The Expression of Slow Myosin During Mammalian Somitogenesis and Limb Bud Differentiation

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Abstract. The developmental pattern of slow myosin expression has been studied in mouse embryos from the somitic stage to the period of secondary fiber formation and in myogenic cells, cultured from the same developmental stages.

The results obtained, using a combination of different polyclonal and monoclonal antibodies, indicate that slow myosin is coexpressed in virtually all the cells that express embryonic (fast) myosin in somites and limb buds in vivo as well as in culture.

On the contrary fetal or late myoblasts (from 15-d-old embryos) express in culture only embryonic (fast) myosin. At this stage, muscle cells in vivo, as already shown (Crow, M. T., and F. A. Stockdale. 1986. *Dev. Biol.* 113:238–254; Dhoot, G. K. 1986. *Muscle & Nerve.* 9:155–164; Draeger, A., A. G. Weeds, and R. B. Fitzsimons. 1987. *J. Neurol. Sci.* 81:19–43; Miller, J. B., and F. A. Stockdale. 1986. *J. Cell Biol.* 103:2197–2208), consist of primary myotubes, which express both myosins, and secondary myotubes, which express preferentially embryonic (fast) myosin. Under no circumstance neonatal or adult fast myosins were detected. Western blot analysis confirmed the immunocytochemical data.

These results suggest that embryonic myoblasts in mammals are all committed to the mixed embryonic-(fast) slow lineage and, accordingly, all primary fibers express both myosins, whereas fetal myoblasts mostly belong to the embryonic (fast) lineage and likely generate fibers containing only embryonic (fast) myosin. The relationship with current models of avian myogenesis are discussed.

THE expression of phenotypic heterogeneity among myogenic cells and the muscle fibers they generate both in situ and in tissue culture has attracted a renewed interest in recent years. In fact, recently accumulated data challenge the long held dogma that muscle is composed of a homogeneous population of plastic cells, which express different phenotypes according to extracellular signals such as nerve-derived messages, hormones, etc. (24). The in vitro expression of differences in morphology, growth requirements, sensitivity to tumor promoters together with differential expression of specific isoforms of contractile proteins have all been considered evidence for intrinsic heterogeneity among myogenic cells (3–10, 18–21, 26, 32). As a result of these studies, it is now possible to divide myogenic cells in three major developmentally regulated classes: (*a*) early or embryonic myoblasts, which appear in the somite and limb buds and are supposed to give rise to the first generation of fibers; (*b*) late or fetal myoblasts, which appear later and likely give rise to secondary fibers; and (*c*) satellite cells, which are mainly responsible for postnatal muscle growth and regeneration (7, 27). Of particular relevance to muscle histogenesis is the heterogeneity of myosin heavy chain isoform expression in embryonic and fetal myoblasts (2, 29). A considerable number of myosin heavy chain isoforms are encoded by a multigene family (23). The fast isoforms are de-

velopmentally regulated and differentially expressed in different muscles (1, 5, 6, 11, 13, 14, 16, 22, 30, 31). While it is clear that nerve and hormones do influence the transitions of these isoforms, the initial commitment to fast or slow myosin expression, as well as the regulation of slow myosin expression during embryogenesis are presently not understood.

In avian development, it has been shown that embryonic myoblasts are composed of three different lineages committed to the generation of three different types of muscle fibers that synthesize fast, slow or both myosins. Conversely, the great majority of chick fetal myoblasts are rather committed to the fast lineage (with a minute proportion of cell expressing both slow and fast myosin). This conclusion is based on experimental work done with two monoclonal antibodies, one that recognizes only slow myosin and a second one that recognizes embryonic, neonatal, and adult fast myosins, which are therefore all considered "fast" myosins (6, 18–20, 25).

In mammals, the early expression of slow myosin has not been investigated, and consequently it is not clear whether the mammalian classes of myogenic cells correspond to those present in birds. It should be noted however that the first generation of muscle fibers in mammals uniformly express both slow and fast (embryonic) myosins (11, 13, 22).

In this study, we have examined the expression of slow my-

osin at the earliest developmental stages of muscle formation and report here the simultaneous expression of slow and embryonic (fast) myosin in all the embryonic muscle cells examined in situ or in culture. Conversely, myotubes derived from fetal myoblasts express in culture only embryonic (fast) myosin.

Materials and Methods

Cell Cultures

Cells were cultured from somites and limbs of mouse embryos of different ages as described before (10, 28). Briefly, the somites or the limbs were isolated from the embryos, digested with 0.05% trypsin (Difco Laboratories Inc., Detroit, MI) in PBS for 15 min at 37°C with occasional shaking. After the proteolytic digestions, the tissues were fragmented by repeated pipetting, the debris were removed by filtration through a sterile nylon gauge, and the cells were collected by centrifugation. All cultures were grown in Dulbecco's basal medium supplemented with 15% horse serum and 5% embryo extract.

As an operational criterion, we define as "embryonic" the myoblasts isolated from 10 to 13-d-old mouse fetuses (since they express an early or embryonic phenotype in vitro; 7, 27). Conversely, we define as "fetal" the myoblasts isolated from 15-d or older fetuses (since they express a late or fetal phenotype in vitro; 7, 27). Indeed, the exact time-course of the disappearance of embryonic myoblasts and of the appearance of fetal myoblasts has not been precisely defined in mammals.

Immunocytochemistry

At the indicated periods of culture, cells were fixed with 50% ethanol/50% acetone at −20°C for 10 min, washed in PBS, and incubated with the antimyosin monoclonal or polyclonal antibodies (at appropriate dilutions for 30 min at 25°C) and then with a goat anti-mouse Ig conjugated with rhodamine or with a goat anti-rabbit Ig conjugated with fluoresceine (at 1:30 dilution for 30 min at 25°C; both from Cappel Laboratories, Malverne, PA), or, in the case of double immunofluorescence, with both second antibodies. Since fixation with paraformaldehyde abolished or strongly reduced staining, frozen sections of unfixed embryos (or isolated somite streaks and limbs) were reacted with the antibodies according to the same protocol. For this reason, the morphology of the earlier stage, where the tissues are minute and soft, was of poor quality and the spatial relationships of different cells were not always correctly preserved.

The following antibodies were used in this study: MF20, MF14, and MF30 monoclonal antibodies raised against chicken skeletal myosin (1); a monoclonal antibody raised against slow rabbit myosin (4); and polyclonal rabbit antibodies against the different isoforms of rat MHC (14). MF20 recognizes all sarcomeric myosin, MF14 only adult fast, and MF30 recognizes adult and neonatal fast myosins. These three antibodies cross react with mouse tissue. The anti-slow monoclonal antibody recognizes exclusively slow myosin and cross reacts with mouse tissue. The polyclonal antibodies specific for slow, neonatal, or fast myosins were prepared and characterized on mouse tissues as described elsewhere (14). The anti-common antibody was obtained by immunization of rabbits with rat myosin and is not affinity purified. It reacts with all skeletal and cardiac myosins but not with smooth muscle and nonmuscle myosins.

Immunoblots

Actomyosin was prepared from tissues or cultures by high salt extraction (0.3 M KCl, 50 mM phosphate buffer, pH 7.4, and 1 mM each of phenylmethylsulfonil fluoride [PMSF], benzamidine, soybean trypsin inhibitor, and leupeptin, as protease inhibitors). The extracts were centrifuged at 10,000 rpm for 30 min and the supernatants dyalized overnight against 10 mM phosphate buffer, pH 6.8. The precipitate was collected by centrifugation (10,000 rpm for 30 min) and analyzed on 8% SDS polyacrylamide gels. After the electrophoretic separation, the proteins were transferred to nitrocellulose paper at 0.15 amp for 3 h with 0.05 M Tris, 0.2 M glycine, 0.05% SDS, and 20% methanol. After the transfer, the nitrocellulose was stained with Ponceau red (0.3%) in 3% TCA, the area corresponding to myosin heavy chains was cut, soaked in 1% BSA in PBS, and incubated with either MF20 or the anti-slow mcab for 3 h at 25°C. After the incubation, the nitrocellulose paper was washed and incubated with a horseradish per-
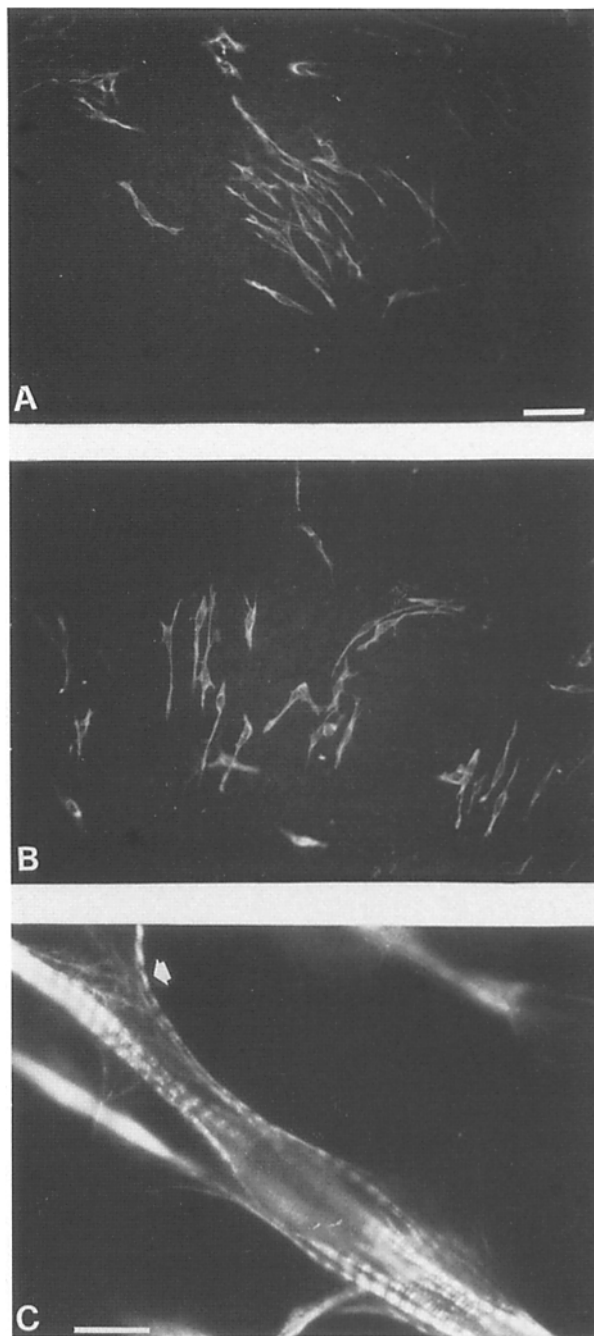

Figure 1. Indirect immunofluorescence analysis (3 d after plating) of cultured myogenic cells isolated from somites of 10-d-old embryos reacted with the anti-common (*A*) and anti-slow (*B*) polyclonal antibodies. In *C* a high magnification of a myogenic cell, stained with anti-slow antibody is shown. The arrow shows cross-striation originating along a pattern of fibers resembling stress fibers. Bars: (*a* and *b*) 10 μm; (*c*) 2.5 μm.

oxidase goat anti-mouse Ig antibody (at 1:600 dilution) for 1 h at 25°C, washed, and reacted with diaminobenzidine and peroxide according to standard procedures.

Results

The somites (plus the neural tube) and the limb buds were isolated from 10-d-old mouse embryos. The cells were dis-
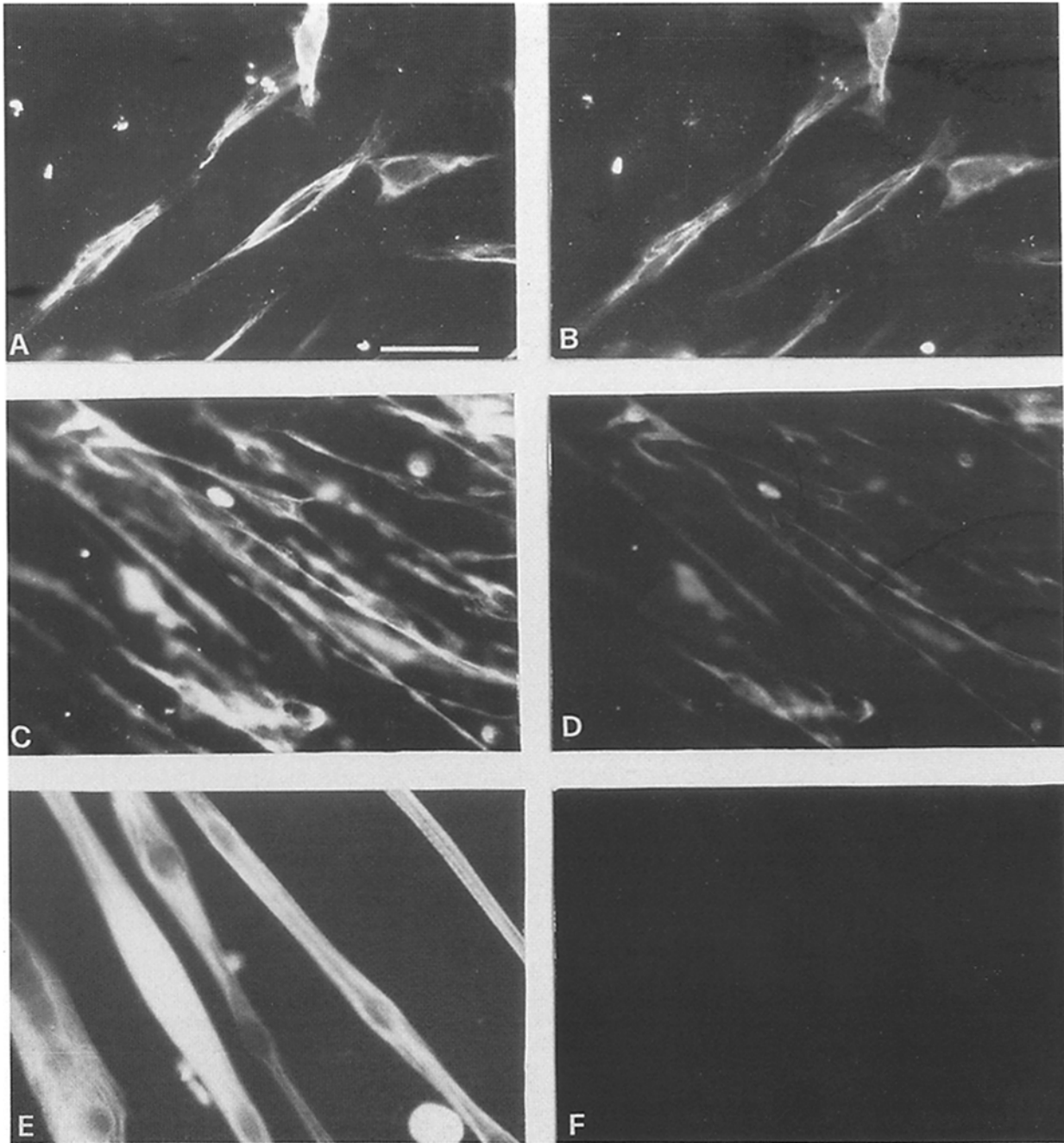
Figure 2. Indirect double immunofluorescence analysis (5 d after plating) of cultured myogenic cells isolated from somites of 10-d-old embryos (*A* and *B*) and limbs of 13- (*C* and *D*) and 15- (*E* and *F*) d-old fetuses reacted with the anti-common polyclonal (*A*, *C*, and *E*) and the anti-slow monoclonal (*B*, *D*, and *F*) antibodies. Bar, 10 μm.

sociated and cultured for different periods (from 1 to 5 d) then fixed and stained with several different antimyosin heavy chain antibodies, directed against either slow, neonatal, adult fast, or common epitopes. Whether poly or monoclonal, the antibodies consistently gave the same staining pattern; i.e., in each culture (limb or somite) and at each day of culture examined (1, 3, and 5 d), the same number of cells per microspot culture stained with both the anti-slow and the anti-common antibodies. No cells ever stained with antibodies specific for neonatal or adult fast myosins, thus showing that the anti-common antibody recognizes only embryonic

myosin in these cells. Fig. 1 shows somitic cells stained with different polyclonal antibodies. The great majority of the fluorescent cells were mononucleated and exhibited a variable morphology from polygonal to spindle shaped and, at low magnification, they appeared in clusters. In a few of these cells (Fig. 1 *C*), initial sarcomerogenesis was observed by staining with either anti-slow or anti-common antibodies. As suggested for cardiac myocytes (12), cross-striation appears along filaments that are reminiscent of stress fibers. Fig. 2 shows a double immunofluorescence of the same cells stained with the anti-common polyclonal (Fig. 2 *A*) and the
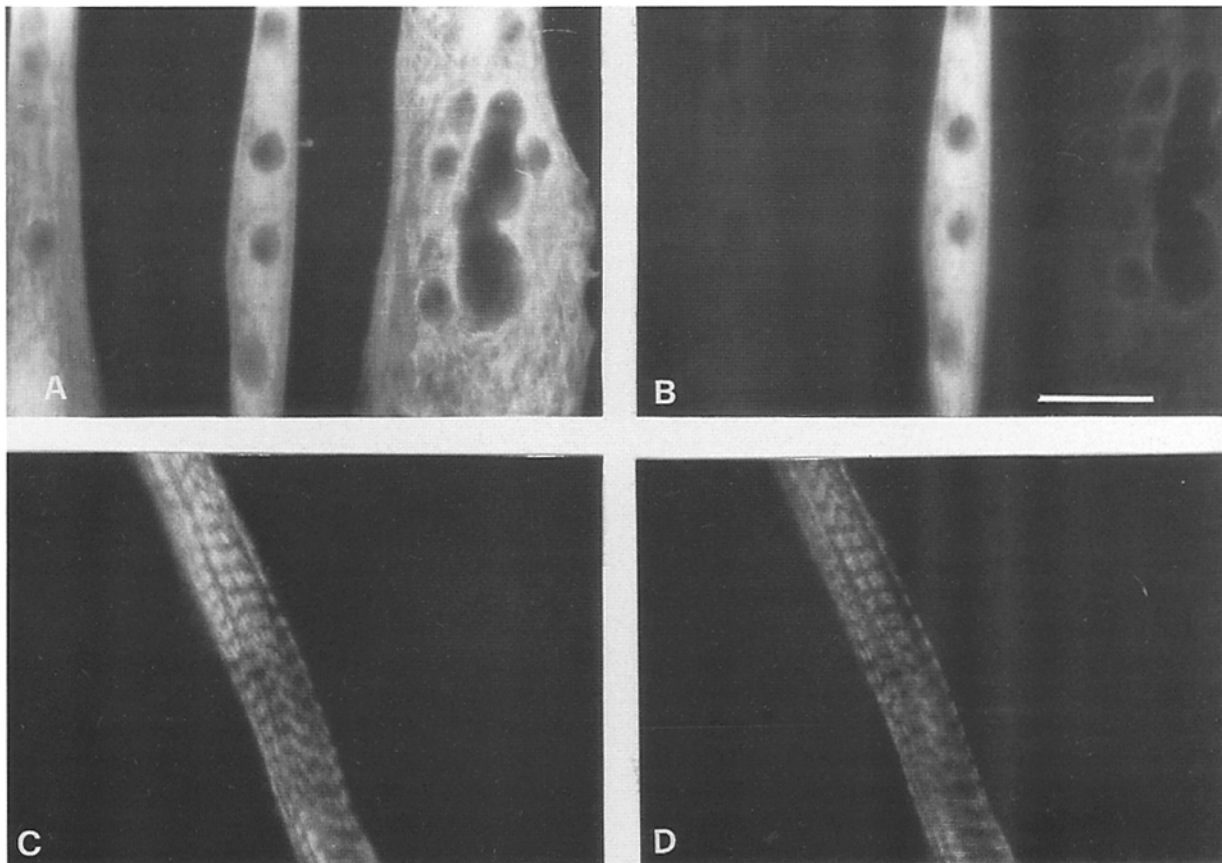
Figure 3. Indirect double immunofluorescence analysis (20 d after plating) of cultured myogenic cells from limbs of 15-d-old fetuses reacted with the anti-common polyclonal (*A* and *C*) and the anti-slow monoclonal (*B* and *D*) antibodies. Bar, 10 μm.

anti-slow monoclonal (*B*). It is obvious from the figure that the same cells stained with both antibodies, and the pattern of fluorescence was identical with both antibodies. Removal of the neural tube, before cell dissociation, did not change the results obtained (data not shown).

When cultures were prepared from limbs of embryos with ages ranging from 10 to 12 d, the results obtained were similar to those obtained with somitic cells; namely, all of the myogenic cells in culture expressed both slow and embryonic (fast) myosin (data not shown).

Starting at day 13 of fetal development, the results obtained with the double labeling became different. Fig. 2, *C* and *D*, shows a culture of myogenic cells isolated from the limb of a 13-d-old fetus. Many thin elongated myotubes stain brightly with the anti-common (Fig. 2 *C*) but poorly with the anti-slow myosin antibody (Fig. 2 *D*). Interestingly however, the same cells appear to be labeled by both antibodies, although with different intensity.

When cultures of fetal myoblasts from a 15-d-old fetus were labeled with both antibodies, the totality of the cells stained only with the anti-common antibody (Fig. 2, *E* and *F*). Other experiments with different antibodies (MF20, MF14, MF30, anti-neonatal, and anti–adult fast polyclonal antibodies) confirmed that only embryonic (fast) myosin was present in these cultures (data not shown).

It has been shown that in long term cultures of quail fetal myogenic cells, a new population of muscle cells appear that

also express slow myosin (25). Similarly, after 20 d in culture, a small but significant percentage (∼5%) of murine fetal myoblasts expressed slow myosin (Fig. 3, *A* and *B*). When expressed in the same myotube (Fig. 3, *C* and *D*) the two myosins appear to colocalize within each sarcomere but with different fluorescence intensity, suggesting variable relative proportion in the intermixing of the two forms.

Double immunofluorescence experiments, performed on frozen sections of mouse embryos, showed that all the cells of somites and early limb buds that stained with the anti-common, also stained with the anti-slow monoclonal, even though the staining with the latter antibody was significantly weaker (Fig. 4, *A* and *B*). Basically the same situation is observed at day 13 (Fig. 4, *C* and *D*), while, as already described by others (11, 13, 22), at day 15 the primary generation of fibers stained with both antibodies, whereas the second generation of smaller fibers stained only with the anti-common antibody.

Similarly to the situation in culture, we never observed staining with any of the antibodies directed against neonatal or adult fast myosins up to the stage of fetal development examined.

To confirm the immunohistochemical data, we isolated crude actomyosin fractions from somites and limbs of the corresponding stages, as well as from cultures derived from them. To compare both total amount and relative proportions of the different myosins, the same proportion of the original
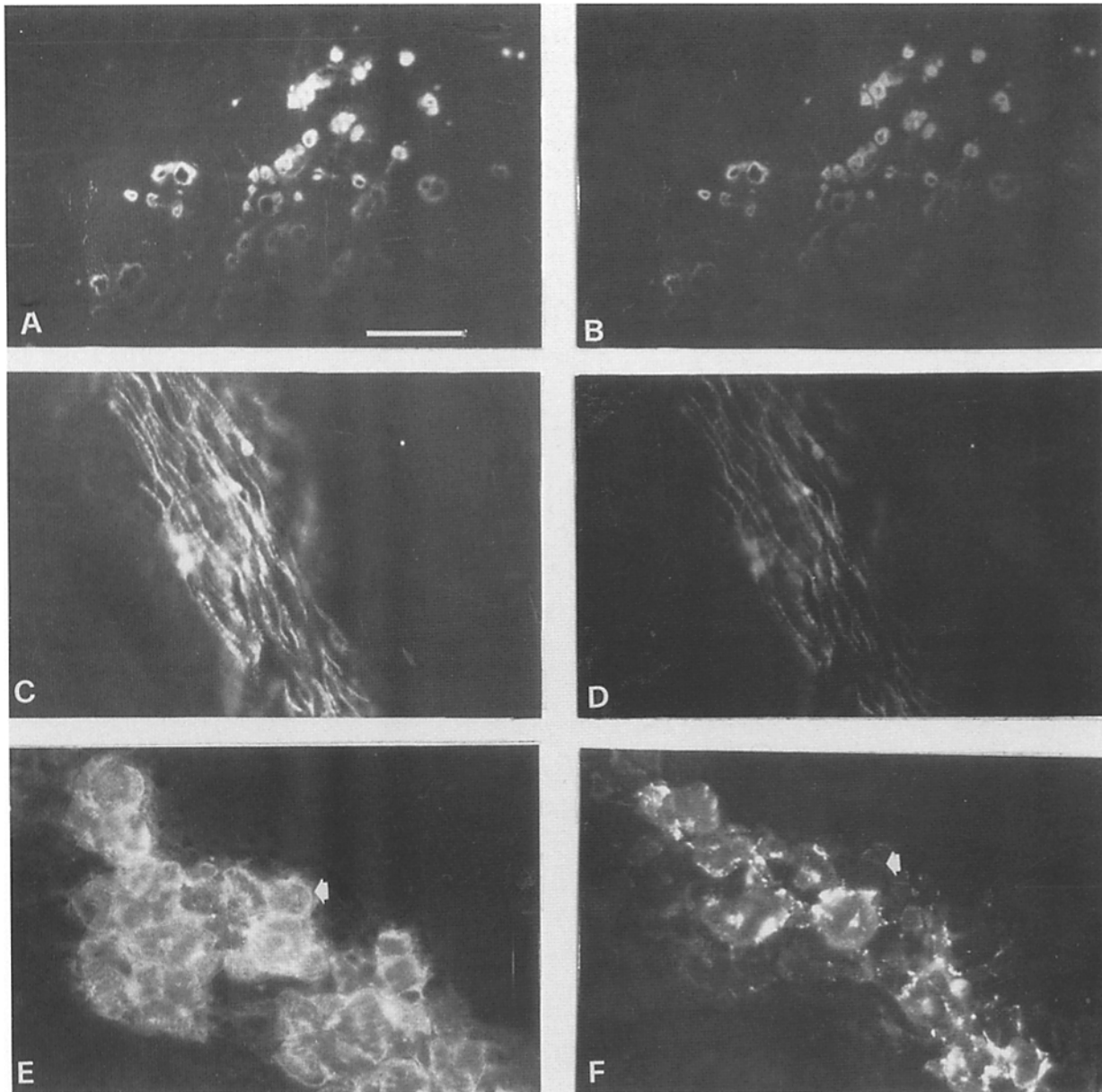
Figure 4. Indirect double immunofluorescence analysis of frozen sections of 10-d-old mouse embryos through the somites (*A* and *B*) and hind limbs of 13- (*C* and *D*) and 15- (*E* and *F*) d-old embryos reacted with the anti-common polyclonal (*A, C,* and *E*) and the anti-slow (*B, D,* and *F*) monoclonal antibodies. The arrow shows a secondary fiber, stained only by the anti-common antibody. Bar, 25 µm.

tissue weight was applied to each lane of the gel, which was then transferred to nitrocellulose paper and blotted either with the anti-slow myosin monoclonal or with MF20. Fig. 5 *A* shows the results obtained with MF20 (which recognizes all sarcomeric myosins): as expected there is a progressive accumulation of myosin throughout development both in vivo and in culture. On the other hand, Fig. 5 *B* shows that slow myosin is clearly detectable in the somite and its amount remains roughly constant during development in vivo but is not present in cultures of fetal myoblasts (Fig. 5, lane *b*). The simplest interpretation of these data is that embryonic myoblasts, which express slow myosin in vivo and in vitro, fuse to form the primary generation of fibers in vivo (and therefore slow myosin is constantly present in vivo), whereas fetal myoblasts, which appear after the 13th day of development,

do not express slow myosin, thus explaining its absence in cultures from later stages.

Discussion

Myogenesis is an asynchronous process in higher vertebrates, extending throughout most of embryonic and fetal development. Embryonic myoblasts, which are present in the mesoderm at an early developmental stage, will eventually generate primary muscle fibers endowed with a specific muscle program. Once these fibers are formed, they are surrounded by other (fetal) myoblasts that will generate secondary fibers, likely endowed with a different program. Furthermore, neural influences, hormones, mechanical load, and other factors might impose an adaptive response on a preex-
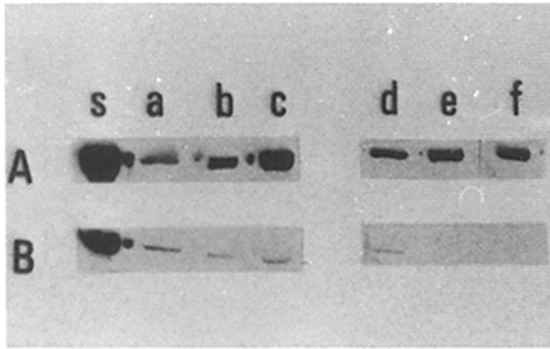
Figure 5. Immunoblot of MF20 (*A*) and anti-slow monoclonal (*B*) antibodies using crude actomyosin extracts of somites from 10-d-old mouse embryos (lane *a*), limbs from 13- (lane *b*) and 15- (lane *c*) d-old embryos, and from cultures at 5 days after plating, prepared from the corresponding developmental stages (lanes *d*, *e*, and *f*). The soleus muscle of an adult mouse is shown in *s* as a control.

isting program of gene expression (24). Since it is not presently possible to identify a muscle cell by the myosin heavy chain isoform expressed and to follow its fate during further development, the hypotheses on which myogenic cell generates which muscle fiber must rely on indirect criteria.

Given these limitations, it is nevertheless important in the absence of better lineage markers to identify different classes of myogenic cells on the basis of the myosin heavy chain that they express in tissue culture and in vivo.

In this paper, we report that as early as myogenic cells appear in the mouse embryo, they all coexpress both slow and embryonic (fast) myosins. It remains to be investigated whether the slow myosin expressed by somitic cells represent the same gene product expressed in adult slow muscles. Furthermore, all the myoblasts that migrate to the limb, form myotubes that also express both types of myosin. Thus, mouse (and rat, as indicated by our unpublished experiments) embryonic myoblasts appear homogeneous with respect to the expression of myosin heavy chain. Consistent with this observation is the presence of both slow and embryonic (fast) myosin in all of the first generation of rat muscle fibers (11, 13, 22), which likely originate from these myoblasts. On the contrary avian embryonic myoblasts, which are divided in three classes (fast, slow, and mixed), will generate three types (fast, slow, and mixed) of primary fibers (6).

The first generation of fibers is supposed to develop autonomously from innervation (6, 17). Similarly, the expression of slow myosin during early muscle development is certainly independent from innervation. However, precocious influence of the neural tube cannot be ruled out, even though removal of the neural tube before culturing somitic cells from 9 or 10-d-old embryos did not influence slow myosin expression.

Fetal mammalian myoblasts appear identical to their avian counterparts, not only because they are all committed to the "fast" lineage, but also because with time in vitro they give rise to a minor subpopulation of muscle cells that express both slow and embryonic (fast) myosins. It is conceivable that fetal myoblasts will produce the second generation of fibers in vivo.

However, since innervation occurs at this time (15), it becomes more difficult to extrapolate the developmental program of the second generation of fibers by either tissue culture or in vivo immunocytochemical studies. Narusawa et al. (22) suggest that the progressive diversification of isomyosin expression in different muscle primordia might depend on different generation of myogenic cells but also might depend on regional patterns of specialization, likely imposed by innervation. Our study is limited to an earlier period of development, even though we do observe fiber heterogeneity (with respect to slow myosin expression) at 15 d of development in the mouse.

In conclusion our data emphasize that the overall developmental program of mammalian myogenic cells is a complex, multistep process with similarities but also clear differences with the program expressed in avian development.

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