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Developmental origins of skeletal muscle fibers: Clonal analysis of myogenic cell lineages based on expression of fast and slow myosin heavy chains

(myoblast/myotube/muscle cell culture/chicken embryo/fast and slow muscle fibers)

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ABSTRACT **A clonal analysis was used to show that skeletal muscle myoblasts are committed to distinct cell lineages during development. Myoblasts taken from embryonic chicken hindlimb muscles of different ages were cultured at clonal density. The content of fast and slow classes of the myosin heavy chain isoforms in the myotubes of the resulting muscle colonies was determined immunocytochemically with specific monoclonal antibodies that served as markers for the different fiber types. The muscle colonies formed by cloning myoblasts from early hindlimbs (days 4–6 *in ovo*) were of three types: the most numerous type, in which all myotubes in a colony contained only the fast class of myosin heavy chain; a less numerous type, in which all myotubes in a colony contained both the fast and slow classes of myosin heavy chain isoforms; and a rare type, in which all myotubes in a colony contained only the slow class of myosin heavy chain. The muscle colonies formed by cloning myoblasts from later hindlimbs (days 10–12 *in ovo*) were, however, all of one type, in which every myotube in a colony contained only fast myosin heavy chain. Thus, myoblasts in the early embryo (days 4–6 *in ovo*) were a heterogeneous population committed to three myogenic lineages: fast, mixed fast/slow, and slow, whereas myoblasts from the later embryo (days 10–12 *in ovo*) were only in the fast myogenic lineage. These results suggest that muscle fiber formation is rooted in two developmental phases—an early phase in which diverse fiber types are formed from intrinsically diverse populations of myoblasts and a later phase in which fibers are formed from a single population of myoblasts.**

The analysis of cell lineage is a central problem in developmental biology. To understand the molecular and cellular mechanisms underlying differentiation of a particular cell type, it is necessary to identify and isolate the immediate precursors of the differentiated cell of interest. One system in which the analysis of cell lineage has presented a long-standing problem is the formation of the three muscle cell types of skeletal muscle: the fast, mixed fast/slow, and slow muscle fibers which are described below. Two models—one requiring a single myogenic lineage and a second requiring multiple myogenic lineages—could explain muscle fiber diversification (1). It has usually been assumed that all muscle fibers arise from a single myoblast population, with diversification specified later by the type of innervation the fibers received. The experimental justification for this assumption has not, however, been compelling, and the alternative model, that fast, mixed fast/slow, and slow fibers form from separate myoblast populations, has remained an open possibility (1). Recent results led us to reexamine myogenic lineages in muscle fiber diversification.

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Adult muscles in birds and mammals contain a heterogeneous population of muscle fibers with fast, mixed, and slow properties. Although first defined physiologically, these fiber types can now be defined biochemically by the presence of fast, slow, or both fast and slow classes of the myosin heavy chain (MHC) isoforms or other contractile proteins (2–4). The fast and slow classes of the MHC isoforms ($M_r \approx 200{,}000$) can be distinguished by electrophoresis and antibody binding (5–8) and serve as markers for the different fiber types. Recent studies of developing avian muscles have shown that embryonic muscle fibers, as well as adult fibers, are also heterogeneous with regard to myosin isoform content (9–11). Results from ATPase histochemistry (12–16) and immunohistochemistry with monoclonal antibodies to fast and slow MHC (11) show that different types of embryonic fibers exist. One major embryonic fiber type contains only a fast MHC, whereas a second type contains a mixture of a fast MHC and a slow MHC. These different embryonic fibers appear important in the development of muscle structure and function, because the distribution of the different embryonic fibers anticipates the future function of the muscle. For instance, embryonic fibers with slow myosin are most numerous in developing muscles that will be predominantly slow in the adult (11).

Previous work has demonstrated that diversification of fiber types in the early embryo does not require innervation (11, 12, 16–18), and we have recently shown (7) by immunochemical analysis of MHC content that three types of myotubes, with only fast, only slow, and both fast and slow MHC, are formed from early embryonic chicken myoblasts in high-density cell culture in the absence of nerves. The multiple-myoblast model, with distinct populations of myoblasts committed to each of the muscle fiber lineages, provided one possible explanation for these results. The existence of multiple myoblast populations could, however, only be proven by analysis of myotube formation from cloned myoblasts. The methods and conditions for cloning of myoblasts from early avian embryos have been developed and studied by Hauschka and his colleagues (19–21) for the developing limbs. We have, therefore, cloned myoblasts from the embryonic chicken hindlimb by these methods and analyzed the types of fibers they form by determining the class of MHC found in individual myotubes of the resulting muscle fiber colonies. The results show that myoblasts in the early embryonic leg are committed to three myogenic lineages: fast, mixed fast/slow, and slow. In contrast, myoblasts in the later embryo are only in the fast lineage. Therefore, there appear to be two distinct phases—based on different myoblast lineages—in the formation of fast, mixed, and slow muscle fibers in the developing chicken hindlimb.

Abbreviations: MHC, myosin heavy chain; ED*n*, embryonic day *n*.

MATERIALS AND METHODS

Tissues, Cell Culture, and Nomenclature. Myoblasts are mononucleated, highly proliferative cells that do not contain skeletal muscle MHC. Myotubes are nonmitotic, differentiated cells that do contain skeletal muscle MHC and are either mononucleated (also known as myocytes) or multinucleated. Differentiated myotubes (muscle fibers) of fast, mixed fast/slow, or slow types are the ultimate differentiated cell in a myogenic lineage, the proliferative myoblast being the immediate precursor cell in a lineage. A myoblast within a particular myogenic lineage is defined by the type of myotube it produces.

For these experiments, single-cell suspensions containing myoblasts were prepared from the hindlimb bud of embryonic day 4 (ED4, stages 23–25) (22), ED5 (stages 27–28), and ED6 (stage 29) or the whole thigh of ED8, ED10, and ED12 White Leghorn chicken embryos by treatment of the muscle tissue with trypsin (23). Myoblasts were grown on gelatin-coated plates at clonal density (2–20 cells per cm^2) so that fewer than 25 muscle colonies formed on each 60-mm dish or at high density (2×10^4 cells per cm^2). Culture medium, used at 0.2 ml/cm^2, consisted of Ham's nutrient mixture F-10, 15% horse serum, 5% chicken embryo extract, penicillin, streptomycin, and Fungizone. For all experiments, fresh culture medium was mixed with an equal amount of conditioned medium (19, 24). The statistical method of myoblast cloning by culture at limiting dilution as used here was developed and validated by Konigsberg (25) and extended by Hauschka and colleagues (19–21, 24). With this method, muscle colonies have been shown to form from the progeny of single myoblasts (25).

Immunocytochemistry. After 10 days in culture, the colonies were fixed with 3.7% formaldehyde in phosphate-buffered saline for 5 min and 100% ethanol for 5 min. Muscle colonies were located with a dissecting microscope, circled, and numbered. The cultures were incubated with 1:10 dilutions of hybridoma supernatants for 1 hr at room temperature. The bound antibodies were visualized by incubating the cultures with biotinylated horse anti-mouse IgG (5 μg/ml) and an avidin–biotin-horseradish peroxidase complex with diaminobenzidine as the substrate as described by the manufacturer (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). The myotubes in each of the previously circled colonies were then scored for staining with each antibody. Double immunofluorescence analysis of single muscle colonies was carried out as described (7). In brief, monoclonal antibodies S58 and F59 (see below) were purified and biotinylated. Cultures were fixed as above and incubated sequentially with biotinylated S58, fluorescein-conjugated avidin, biotin, biotinylated F59, and rhodamine-conjugated avidin. Fluorescence was observed and photographed with a Zeiss photomicroscope equipped for epifluorescence.

Immunoelectrophoresis. $NaDodSO_4$/PAGE was performed as described (26) in 5% gels, and proteins were transferred to nitrocellulose (27). Myosin extracts were prepared (7, 9) and the slow and fast classes of MHC isoforms were detected on the same transfer with a double-labeling technique that is a modification of our previous methods (7). Briefly, nitrocellulose transfers were incubated with monoclonal antibody S58 (specific for slow MHC) and antibody binding was visualized with the avidin–biotin-horseradish peroxidase-linked reagent described above. The reaction product of diaminobenzidine and horseradish peroxidase is brown. The same transfers were then incubated for 30 min with biotin at 50 μg/ml, followed by incubation with monoclonal antibody F59 (specific for fast MHC). Binding of F59 was visualized with an avidin–biotin-glucose oxidase-linked reagent (Vectastain ABC-GO, Vector Laboratories). The reaction product of glucose oxidase and nitro blue tetrazolium is blue.

Monoclonal Antibodies. The preparation and properties of monoclonal antibodies S58 and F59 have been described in detail (7, 8, 10, 11). Immunohistochemical analysis of skeletal muscle fibers in embryonic, neonatal, and adult birds showed that S58 and F59 reacted specifically with muscle fibers containing slow and fast MHC, respectively (7, 10, 11). The reaction of these antibodies on immunoblots of MHC extracted from embryonic, neonatal, and adult chicken muscle is shown in Fig. 1. A combination of $NaDodSO_4$/5% PAGE and immunoblotting was used to show that S58 reacted only with the less rapidly migrating slow class of MHC isoform(s) found in embryonic (Fig. 1, lanes a and b) and neonatal (lane c) thigh muscles and the adult anterior latissimus dorsi (a slow muscle; lane d), whereas F59 reacted only with the more rapidly migrating fast MHC isoforms found in embryonic (lanes a and b) and neonatal (lane c) thigh muscles and in the adult pectoral muscle (a fast muscle; lane e). The epitope recognized by S58 was always on the MHC isoform that migrated more slowly, and the epitope recognized by F59 was always on the MHC isoform that migrated more rapidly during electrophoresis. Neither monoclonal antibody reacted with the MHC isoform of the opposite mobility, irrespective of the age of the bird from which the MHC was extracted. Thus, the epitopes recognized by S58 and F59 define slow and fast classes of MHC isoforms that are found at all developmental ages. Each class of MHC contains an unknown number of developmentally regulated isoforms (5, 28–32). In the discussion that follows, therefore, designation of a MHC as either fast class or slow class is understood to mean specifically that the MHC under consideration has a particular electrophoretic mobility and immunological reactivity that is shared by a number of isoforms within the class.

RESULTS

Hindlimb buds from early chicken embryos (ED4 to -6) and whole thighs from older embryos (ED8, -10, and -12) were used in these studies. Single-cell suspensions from these hindlimbs were prepared by enzymatic digestion (23), and the cells were plated at high density (7) or at clonal density (19–21). Immunocytochemical and immunoblotting analyses using monoclonal antibodies S58 and F59 showed that myotube formation in high-density cultures of hindlimb myoblasts was identical to myotube formation in high-density cultures of breast muscle myoblasts (7). Specifically, in

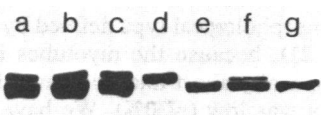

FIG. 1. Properties of monoclonal antibodies S58 and F59. Extracts of MHC were subjected to $NaDodSO_4$/5% PAGE and transferred to nitrocellulose. The reactivity of monoclonal antibodies S58 and F59 was determined with a sequential double-labeling immunoblot technique utilizing a horseradish peroxidase-linked system to visualize S58 binding to slow MHC (brown stain) and a glucose oxidase-linked system to visualize F59 binding to fast MHC (blue stain), as described in *Materials and Methods*. Antibody binding was analyzed with extracts of embryonic (ED6, lane a; ED12, lane b) and neonatal (14-day post-hatch, lane c) leg muscles, as well as with extracts from the predominantly slow, adult anterior latissimus dorsi (lane d) and predominantly fast, adult pectoralis major (lane e) muscles. MHC was also analyzed by immunoblots of extracts of the myotubes formed in 1-week-old, high-density cultures of ED5 (lane f) and ED12 (lane g) hindlimb myoblasts. In all samples, S58 reacted only with the less rapidly migrating slow class of MHC and F59 reacted only with the more rapidly migrating fast class of MHC isoforms.

high-density cultures of ED5 hindlimb myoblasts, three types of myotubes formed, which contained only fast, only slow, or both fast and slow classes of MHC isoforms; in high-density cultures of ED12 hindlimb myoblasts, only a single type of myotube formed, containing only the fast class of MHC (cf. ref. 7). Likewise, immunoblotting of extracts of these high-density cultures showed that both of the electrophoretically distinguishable, fast and slow classes of MHC (see *Materials and Methods*) were synthesized by myotubes in ED5 hindlimb high-density cultures (Fig. 1, lane f), whereas only the fast MHC class was synthesized by myotubes from the ED12 hindlimb high-density cultures (Fig. 1, lane g).

To determine whether the differences in MHC expression among different myotubes (muscle fibers) formed in hindlimb high-density cultures were due to multiple myogenic lineages, we analyzed myotubes formed from cloned myoblasts. Single-cell suspensions containing myoblasts were prepared from the hindlimb bud of ED4, -5, and -6 embryos or whole thigh of ED8, -10, and -12 embryos and cultured at clonal density. The cultures were grown for 10 days, during which time the myoblasts doubled 10–15 times (20) and muscle colonies with MHC-containing myotubes were formed by the progeny of single myoblasts (25). The initial number of myoblasts seeded was adjusted so that fewer than 25 muscle colonies formed in each 60-mm culture dish. The MHC content of the myotubes in each muscle colony was determined immunocytochemically with monoclonal antibodies S58 and F59, which are specific for slow and fast classes of MHC isoforms, respectively.

The muscle colonies formed from cloned ED4 and ED5 hindlimb myoblasts fell into three groups as defined by the reaction of the myotubes within a colony with monoclonal antibodies S58 and F59. This conclusion follows from the quantitative immunocytochemical analysis presented in Table 1. For instance, when colonies were formed from ED5 myoblasts, the myotubes in 23% of the muscle colonies reacted with S58 and the myotubes in 97% of the colonies reacted with F59. Thus, in 77% of the colonies the myotubes appeared to contain only fast MHC, in 20% of the colonies the myotubes contained both fast and slow MHC, and in 3% of the colonies the myotubes contained only slow MHC. Analysis of colonies formed from ED4 hindlimb myoblasts gave similar results (Table 1). When colonies formed from myoblasts taken from all ages of embryos were incubated with both S58 and F59, all myotubes were stained in all colonies. Examples of colonies formed from cloned early hindlimb myoblasts that contained slow or fast MHC are shown in Fig. 2 *a* and *b*. These colonies, which formed from ED5 hindlimb myoblasts, were of the "early" morphological type defined by Hauschka and colleagues (20, 21), because the myotubes in the colonies were characteristically short and contained few nuclei and the fusion index was low (≈30%). We have also observed muscle colonies formed from ED5 hindlimb myoblasts that have slow MHC-containing myotubes and a "non-early" morphology (20) in which the myotubes were somewhat longer and the fusion index was 50–70%.

Table 1. Content of fast and slow classes of MHC isoforms in muscle colonies

Source of myoblasts	Monoclonal antibody probe	Number of colonies examined	Percentage (number) of stained colonies
ED4 leg bud	S58	397	24 (96)
	F59	245	96 (235)
ED5 leg bud	S58	232	23 (54)
	F59	296	97 (287)
ED6 thigh	S58	136	11 (15)
	F59	154	99 (152)
ED8 thigh	S58	162	2 (3)
	F59	175	100 (175)
ED10 thigh	S58	125	0 (0)
	F59	133	100 (133)
ED12 thigh	S58	187	0 (0)
	F59	221	100 (221)

Myoblasts were isolated from the indicated age of embryo, seeded at clonal density, and cultured for 10 days. The resulting muscle colonies were incubated either with monoclonal antibody S58 (specific for the slow class of MHC isoforms) or with monoclonal antibody F59 (specific for the fast class of MHC isoforms). Antibody binding was visualized with a horseradish peroxidase-linked system, and the muscle colonies were observed microscopically to determine whether the myotubes within the colony were stained. The staining of myotubes in 1–2% of the colonies was uninterpretable because the staining was very light (neither clearly positive nor clearly negative), and these colonies were excluded from the analysis.

The age of the embryo from which myoblasts were isolated for cloning was critical in determining whether a single type or multiple types of muscle colonies were formed. Myoblasts from muscles of older embryos (ED10 or -12 thigh) formed only a single type of colony, in which all the myotubes contained only the fast class of MHC. The myotubes in all colonies formed from ED10 or -12 thigh myoblasts stained with F59, but none stained with S58 (Table 1). This single type of muscle colony, in which all the myotubes contained only the fast MHC class, was found even though the myoblasts that produced the colonies were isolated from the muscles composed of the mixed group of fast and slow muscle fibers found in the ED10 and -12 thigh (9, 11). Thus, unlike the colonies formed from myoblasts of the early embryonic limb, colonies formed from myoblasts of the later limb contained only fast MHC. The muscle colonies formed from ED12 thigh myoblasts were of the "late" morphological type (20, 21), because the myotubes in these colonies were characteristically very long and contained many nuclei and the fusion index was high (Fig. 2*c*). When myoblasts from the ED6 and ED8 hindlimb were cloned, the myotubes in 11%

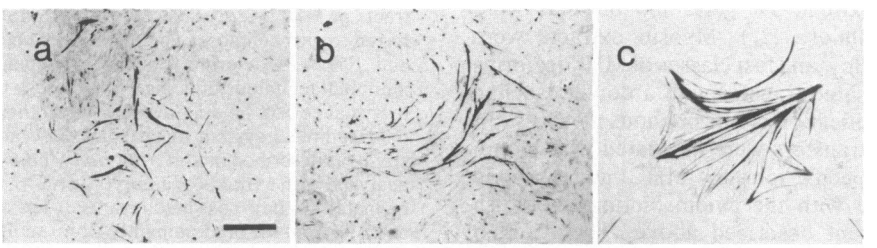

FIG. 2. MHC content in muscle colonies. Myoblasts were prepared from ED5 leg bud (*a* and *b*) and ED12 thigh (*c*) and cultured at clonal density. After 10 days in culture, the resulting muscle colonies were fixed and the MHC content in the myotubes of the colonies was determined by incubating the colonies with monoclonal antibody S58 (*a*, specific for the slow class of MHC) or monoclonal antibody F59 (*b* and *c*, specific for the fast class of MHC). Antibody binding was visualized with a horseradish peroxidase-linked system. Slow MHC was found in the myotubes of muscle colonies formed by myoblasts taken from the ED5 hindlimb bud, but not in the myotubes of muscle colonies formed by myoblasts of the ED12 thigh muscles. See Table 1 for a quantitative analysis. [Bar = 250 μm (*a* and *b*) or 500 μm (*c*).]

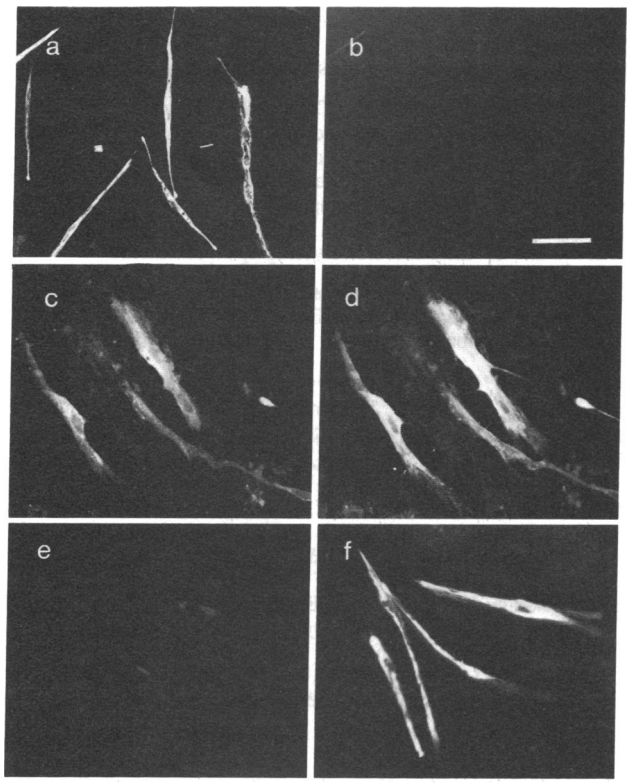

FIG. 3. Double-immunofluorescence analysis of MHC content in single muscle colonies. Myoblasts were prepared from the ED5 hindlimb bud and cultured at clonal density. After 10 days in culture, the resulting muscle colonies were fixed and processed for double immunofluorescence as described in *Materials and Methods*. (*a*, *c*, and *e*) Fluorescein fluorescence, representing slow MHC distribution in myotubes of the muscle colonies. (*b*, *d*, and *f*) Rhodamine fluorescence of the same fields, representing fast MHC distribution in the same myotubes. Only a portion of each colony is shown. [Bar = 25 μm (*a* and *b*) or 10 μm (*c–f*).]

and 2% of the colonies, respectively, contained slow MHC (Table 1). Thus, myoblasts that formed colonies with myotubes containing slow MHC became rare at the developmental age when early myoblasts are known to be replaced by late myoblasts in the hindlimb (19, 20).

The formation of three types of muscle colonies (exclusively fast, mixed fast/slow, and exclusively slow) from three different early myoblast types was confirmed with a double-label immunofluorescence analysis. ED5 hindlimb myoblasts formed three types of colonies in which all the myotubes within a colony reacted with only S58 (Fig. 3 *a* and *b*), with both S58 and F59 (Fig. 3 *c* and *d*), or with only F59 (Fig. 3 *e* and *f*). Colonies with myotubes that contained only slow MHC were rare, as expected from the quantitative analysis (Table 1). For instance, in a survey by double immunofluorescence of 300 muscle colonies formed from cloned ED5 hindlimb myoblasts, 3 colonies were found to have myotubes that contained only slow MHC. The exclusively slow colonies also had fewer myotubes than mixed fast/slow and exclusively fast colonies. In the mixed fast/slow colonies, both fast and slow MHC were expressed within every myotube within the colony (Fig. 3 *c* and *d*), although the relative proportions of the fast and slow classes of MHC varied in different myotubes within the colony. All myotubes within a colony, whether mononucleated or multinucleated, were the same in that they all contained the same class or classes of MHC, and no colonies were found with mixtures of the three fiber types. Double labeling of colonies formed from ED12 myoblasts revealed only one type of colony, in which all the myotubes contained fast MHC.

DISCUSSION

Our results show that early embryonic myoblasts form three types of muscle colonies (exclusively fast, mixed fast/slow, and exclusively slow), as judged by reaction with monoclonal antibodies specific for fast and slow classes of MHC isoforms. This finding strongly suggests that early myoblasts are committed to one of three developmental paths before fibers are formed. If commitment had occurred after myotube formation or during the 10–15 cell doublings of the originally cloned myoblast, we would have expected to see muscle colonies with heterogeneous myotube populations, as are seen in high-density cultures (7). We found instead that all the myotubes within a colony contained the same classes of MHC. Thus, at the time of isolation from embryo, the myoblasts from the early embryo were committed to either the fast, mixed fast/slow, or slow fiber type lineage, and this commitment was stably inherited by the progeny of the cloned cell that produced the muscle colony *in vitro*.

The assignment of myoblasts to a specific myogenic lineage ultimately depended on the reactivity of monoclonal antibodies specific for fast and slow classes of MHC isoforms. MHC isoforms expressed in embryonic, neonatal, and adult chicken skeletal muscle can be unambiguously classified into either the fast class or slow class as defined by electrophoretic mobility in $NaDodSO_4$/5% polyacrylamide gels and reactivity with monoclonal antibodies S58 and F59. In this report (Fig. 1) and elsewhere (7, 8, 11), we showed that S58 reacted only with the less rapidly migrating slow class of MHC isoform(s) found in embryonic, neonatal, and adult muscles and that F59 reacted only with the more rapidly migrating fast class of MHC isoforms found in embryonic, neonatal, and adult muscles. A particular MHC found in the developing bird or *in vitro* was assigned to the fast or slow class to indicate that it had a specific combination of electrophoretic and immunological properties. The fast and slow classes of MHC isoforms are each composed of more than one isoform. Although it will be interesting to determine which isoforms are expressed by the different cultured myotube types, it was not necessary for the biological question considered in this paper. Here, it was sufficient to use the fast and slow classes of MHC isoforms, which were previously shown to be markers for different fiber types in both embryonic (11) and adult birds (5), to demonstrate the commitment of myoblasts to different myogenic lineages.

At least four myoblast types have now been defined in developing skeletal muscles of the chicken. Previous work by Hauschka and colleagues (20, 21) showed that early and late myoblasts are in two independent lineages on the basis of colony morphology and the culture medium required for muscle colony formation. The early myoblasts have been shown not to be precursors of the late myoblasts (21). The results reported here show that the early population of myoblasts in the limb is actually composed of three populations of myoblasts in three distinct myogenic lineages (fast, mixed fast/slow, and slow) and that the late population of myoblasts is a single population in a fast myogenic lineage. In addition to these four embryonic myoblast populations, other myogenic lineages may occur during the life of the bird. For example, the satellite cells of adult fast and slow muscles may be in separate lineages (6), as may the precursor cells of the muscle spindle apparatus. Multiple myogenic lineages may thus be the rule rather than the exception in avian skeletal muscles. Whether the fast and slow muscle fibers in mammals and other classes of vertebrates also originate from multiple myoblast populations remains to be determined (cf. ref. 33).

The timing of appearance of the two phases of myoblasts—the multiple early populations followed by a single later population—follows the timing of the two phases of myotube formation in the bird. Anatomical studies have shown that during vertebrate myogenesis, a first or primary generation of fibers forms initially, and secondary myotubes then form parallel to and surrounding the primary fibers (34). In the limb of the chicken embryo, primary myotubes first appear at ED4.5 (35) and secondary myotubes are clearly delineated by ED11–ED12 (14, 16). Fibers of diverse types appear to form during both phases of myogenesis (11, 14–16). Based on this timing, the primary myotubes appear to form from the early myoblast populations (36), which we found to be committed to three myogenic lineages, and the secondary myotubes appear to form from the late myoblast population.

It is particularly striking that cloned myoblasts from the early embryo formed three different types of muscle fibers in the absence of innervation or contact with nonmuscle cells. This result complements and extends studies (11, 12, 16–18) that showed that the formation of different types of primary muscle fibers in the limb *in vivo* occurs in the absence of innervation. Lack of innervation does, however, affect the formation of secondary muscle fibers (14–16). The formation of primary myotubes of different types appears, therefore, to proceed in a manner that is basically different from the formation of secondary myotubes. Based on our results and those of others cited above, we suggest that there is an early phase of myogenesis in which primary fibers with different MHC contents are formed independently of innervation from the distinct types of myoblasts that we identified here and that a later phase of myogenesis occurs in which the proper formation of secondary fibers requires interaction between muscle fibers and neurons.

These observations raise a number of interesting questions about myoblast commitment, muscle fiber formation, and muscle formation from individual fibers during limb development. First, when does commitment of myoblasts to different myogenic lineages occur? Limb myoblasts are thought to arise from a population of cells that migrates from the somites to the presumptive limb bud region before muscle fibers are formed (37). Are these migratory cells a homogeneous stem cell population or are they committed to different myogenic lineages? Second, are the distinct populations of myoblasts functionally independent? Myoblasts are usually thought to be capable of fusing with all other myoblasts (38), yet mixtures of the three types of early myoblasts appear to form distinct types of myotubes *in vivo* (11) as well as *in vitro* (7). Specific recognition systems may exist that prevent fusion between the various myogenic lineages. Third, how are myoblasts and muscle fibers of different types properly positioned in developing muscles? The different primary fiber types are displayed in characteristic patterns in developing thigh muscles, and these patterns are retained throughout development (11). Thus, primary myoblasts and primary fibers of different myogenic lineages must be guided to the proper positions in the developing limb, a process that probably requires interaction with nonmuscle cells or the extracellular matrix. Finally, what is the relationship between myotubes and motoneurons? Although primary myotubes are innervated soon after formation (39), diversification of these primary fibers is clearly independent of innervation (11, 12, 16–18), and we postulate that primary fiber diversification depends on myoblasts committed to different myogenic lineages. Thus, either there must be a matching of prespecified motoneurons with the corresponding type of primary myotube or the motoneuron type must be determined after innervation, based on the type of myotube innervated. A change must occur during development, however, because muscle fiber type in the adult (40) and the late embryo (14–16) appears to be dependent on innervation. Our finding that distinct myogenic lineages exist provides a new experimental approach to these questions.

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