Developmental Regulation of the Multiple Myogenic Cell Lineages of the Avian Embryo

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Abstract. The developmental regulation of myoblasts committed to fast, mixed fast/slow, and slow myogenic cell lineages was determined by analyzing myotube formation in high density and clonal cultures of myoblasts isolated from chicken and quail embryos of different ages. To identify cells of different myogenic lineages, myotubes were analyzed for content of fast and slow classes of myosin heavy chain (MHC) isoforms by immunocytochemistry and immunoblotting using specific monoclonal antibodies. Myoblasts from the hindlimb bud, forelimb bud, trunk, and pectoral regions of the early chicken embryo and hindlimb bud of the early quail embryo (days 3–6 in ovo) were committed to three distinct lineages with 60–90% of the myoblasts in the fast lineage, 10–40% in the mixed fast/slow lineage, and 0–3% in the slow lineage depending on the age and species of the myoblast donor. In contrast, 99–100% of the myoblasts in the later embryos (days 9–12 in ovo) were in the fast lineage. Serial subculturing from a single myoblast demonstrated that commitment to a particular lineage was stably inherited for over 30 cell doublings. When myo-blasts from embryos of the same age were cultured, the percentage of muscle colonies of the fast, fast/slow, and slow types that formed in clonal cultures was the same as the percentage of myotubes of each of these types that formed in high density cultures, indicating that intercellular contact between myoblasts of different lineages did not affect the type of myotube formed. An analysis in vivo showed that three types of primary myotubes—fast, fast/slow, and slow—were also found in the chicken thigh at day 7 in ovo and that synthesis of both the fast and slow classes of MHC isoforms was concomitant with the formation of primary myotubes. On the basis of these results, we propose that in the avian embryo, there is an early phase of muscle fiber formation in which primary myotubes with differing MHC contents are formed from myoblasts committed to three intrinsically different primary myogenic lineages independent of innervation and a later phase in which secondary myotubes are formed from myoblasts in a single, secondary myogenic lineage with maturation and maintenance of fiber diversity dependent on innervation.

THE diversification of muscle fibers into the fast, mixed, and slow types found in vertebrate skeletal muscle has been thought to be specified by the pattern of innervation. Thus, muscle fibers in the embryo were thought to be formed from a single lineage of myoblasts, with diversification in fiber type occurring later due to the type of innervation received. The possibility that different fiber types arose from multiple myoblast lineages had not been tested (49). Recently, however, both ATPase histochemistry (11, 24, 30, 31, 39) and immunohistochemistry with monoclonal antibodies specific to fast and slow myosin isoforms (14) have been used to show that muscle fiber diversification in the early chicken embryo (days 5–8 in ovo) occurs in the absence of functional innervation.

These results appear to be explained by our finding that three distinct types of myoblasts, committed to either the fast, mixed fast/slow, or slow lineage, exist in the early embryo (32, 33). We used monoclonal antibodies specific to the fast and slow classes of myosin heavy chain (MHC)[1] iso-forms as markers to determine the fiber types formed from myoblasts in vitro. MHC isoforms are markers for fast, mixed, and slow fibers in vivo in both embryonic and adult birds (14, 32, 43), and the fast and slow classes of the MHC isoforms are distinguishable by electrophoresis (2, 32, 33, 43), thus allowing unambiguous biochemical confirmation of immunocytochemical data. We showed (32) that when myoblasts were taken from embryonic days 5 and 6 (ED 5–6) chicken pectoral regions and cultured at high density, three types of myotubes formed that contained only the fast class of MHC, both the fast and slow classes of MHC, or only the slow class of MHC isoforms. Myoblasts from older embryos (ED 8 or 12), however, formed a single type of myotube that contained only a fast class of MHC. These results were shown (33) to be due to distinct myoblast types because cloned myoblasts from ED 5–6 chicken hindlimb formed three types of muscle colonies in which all the myotubes in a colony contained only the fast class of MHC, both the fast

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1. *Abbreviations used in this paper:* CMR, conditioned medium requiring; ED, embryonic day; FMS, fresh medium sufficient; MHC, myosin heavy chain.

and slow classes of MHC, or only the slow class of MHC isoforms. Unlike myoblasts from the early embryonic hindlimb, cloned myoblasts from ED 10–12 hindlimb formed muscle colonies in which the myotubes contained only the fast class of MHC. Thus, it appears that muscle fiber diversification in the early chicken embryo is not dependent on innervation but is based on myotube formation from intrinsically different populations of myoblasts committed to distinct myogenic cell lineages.

Several questions arose from these initial studies of multiple myogenic lineages. For instance, when during development do the different myoblast types appear and how during development do the relative numbers of myoblasts in each lineage change? Do all myoblast types appear in all developing muscle groups, or are they restricted to specific muscles? How do the myoblasts in the lineages we identify relate to previous classifications of avian myoblasts (6, 44, 54)? Do myoblasts remain in specific lineages during subcloning? What is the relationship between myotube formation by myoblasts in clonal versus high density cultures? Finally, how do the results in vitro correspond to the formation of diverse fiber types and the expression of fast and slow MHC isoforms in muscle fibers in vivo? We address these questions in this paper and use the results to propose a model for muscle fiber diversification in the avian embryo.

Materials and Methods

Cell Culture

Myoblasts were isolated from White Leghorn chicken or Japanese quail embryos and cultured as described (38). Muscle regions were identified, dissected, minced, dissociated with trypsin, and cultured on gelatin-coated dishes at 2.0×10^4 cells/cm^2 for high density cultures or 2–20 cells/cm^2 for clonal cultures. When somites or trunk muscles were used, tissue was taken only from the region between the forelimb buds and hindlimb buds, and the neural tube, visceral structures (including heart), and lateral mesoderm were removed before dissociation and culture. Culture medium was used at 0.2 ml/cm^2 and consisted of 80% Ham's F-10, 15% horse serum that was pre-selected to support clonal growth, 5% chicken embryo extract, penicillin, streptomycin, and Fungizone. Unless stated otherwise, fresh medium and conditioned medium (53) were mixed in equal amounts. Medium was replaced every 3 d with high density cultures, but was not changed during clonal cultures. The age of the embryos used in these experiments is reported according to days of incubation in ovo. For the chicken, days of incubation corresponded to the stages of Hamilton and Hamburger (18) as follows: embryonic day 3 (ED 3, stages 20–22), ED 4 (stages 23–25), ED 5 (stages 26–27), and ED 6 (stages 28–29). For the quail, the stages of Zacchei (56) were used and corresponded to days of incubation as: ED 4 (stages 18–19), ED 5 (stages 20–21), and ED 6 (stages 22–23).

Subcloning of chicken and quail muscle colonies was performed as described by Rutz and Hauschka (44). Primary muscle colonies, which formed from myoblasts incubated at clonal density for 7–10 d and were well separated from neighboring colonies, were located and marked. Sterile glass cloning cylinders with a 1-cm internal diameter were coated with silicone grease on one end and placed over the previously located colonies. The colonies were washed with 2 × 0.1 ml of Hanks' buffered saline solution and dissociated by a 5-min incubation in 0.1 ml of 0.025% trypsin at 37°C. The cells from each dissociated primary muscle colony were divided into three portions and cultured in three 18-mm wells of a 12-well cluster plate. After 7–10 d these wells were covered with the daughter cells of a single myoblast. The cells in one of the three wells were dissociated and cultured in two 18-mm and one 60-mm dish for 7–10 d until tertiary cultures were formed. By the tertiary subculture, the clonal lines had typically expanded to reach or nearly reach confluence on the 60-mm dish, thus providing sufficient material for analysis of MHC content by immunoblotting.

Immunostaining and Microscopy

Fixed mass or clonal myotube cultures and cryostat sections were used to determine the MHC content of individual myotubes as described (14, 32, 33). Cultures were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 5 min and 100% ethanol for 5 min. Sections were fixed in 100% ethanol for 5 min. For clonal cultures, muscle colonies were located with a dissecting microscope, circled, and numbered. Cultures or sections were incubated with 1:10 dilutions of hybridoma supernatants for 1 h at room temperature. The bound antibodies were visualized by incubating the cultures for 1 h with 5 μg/ml of biotinylated horse anti-mouse IgG and an avidin-biotin-horseradish peroxidase complex for 1 h, with diaminobenzidine as the substrate as described by the manufacturer (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). To determine the percentage of muscle colonies stained by a particular monoclonal antibody (mAb), the myotubes in the previously identified colonies were scored for staining using the bright-field optics of an inverted microscope. To determine the number of myotubes/cm^2 in a high density culture that stained with a particular mAb, a square of known size was drawn on the bottom of each culture well and the number of stained myotubes was counted within the square. Myotubes were counted in an area that contained at least 400 stained myotubes or in an area of at least 2.5 cm^2 for sparsely stained cultures.

Double immunofluorescence analysis of the content of myosin heavy chain isoforms in individual myotubes was performed with mAbs S58 and F59, which are specific for the slow and fast classes of MHC isoforms, respectively, as described below. mAb S58 is an IgA and mAb F59 is an IgG1. Cultures or cryostat sections prepared as above were incubated sequentially with a 1:10 dilution of mAb S58 supernatant, a 10 μg/ml solution of fluorescein-conjugated rabbit anti-mouse IgA (Zymed Laboratories, South San Francisco, CA), a 1:10 dilution of mAb F59 supernatant, and a 10 μg/ml solution of rhodamine-conjugated goat anti-mouse IgG (gamma chain specific, Zymed Laboratories). Incubations were for 1 h at room temperature and reagents were diluted in PBS containing 2% bovine serum albumin, 2% horse serum, 1% normal rabbit serum, and 1% normal goat serum.

Electrophoresis and Immunoblotting

Myosin was extracted from cultures or from embryos and analyzed by SDS PAGE in 5% gels as described previously (14, 32). After electrophoresis, proteins were transferred to nitrocellulose (9) and the transfers were incubated with mAb S58 or mAb F59. Antibody binding was visualized by incubating the transfers with 5 μg/ml of biotinylated horse anti-mouse IgG (heavy and light chain specific) and an avidin-biotin-enzyme complex. The biotin-linked enzyme was horseradish peroxidase, glucose oxidase, or alkaline phosphatase as noted, and the enzyme reactions were performed as described by the manufacturer (Vectastain ABC kits, Vector Laboratories).

Monoclonal Antibodies

The preparation and properties of mAbs S58 and F59 have been described in detail (13, 14, 32, 33, 50). In summary, immunohistochemical analysis showed that in embryonic, neonatal, and adult birds, mAb S58 reacted specifically with muscle fibers containing slow MHC, and mAb F59 reacted specifically with fibers containing fast MHC (13, 32, 50). In addition, a combination of SDS PAGE in 5% gels (2, 43) and immunoblotting showed that mAb S58 reacted only with the less rapidly migrating slow class of MHC isoform(s) found in the adult anterior latissimus dorsi (a slow muscle) and in the muscles of the embryonic, neonatal, and adult hindlimbs, and that mAb F59 reacted only with the more rapidly migrating fast class of MHC isoforms found in adult breast muscle (a fast muscle) and in the muscles of embryonic, neonatal, and adult hindlimbs (14, 32, 33, 50). Thus, the epitopes recognized by mAbs S58 and F59, each of which are on the S1 (head) region of the respective MHC isoform (14), define electrophoretically distinguishable, slow and fast classes of MHC isoforms that are found at every developmental age. Each class of MHC isoforms contains an unknown number of developmentally regulated isoforms (1, 2, 25, 28, 43, 46, 51). In the discussion that follows, therefore, designation of an MHC as fast class or slow class indicates that it has a particular electrophoretic mobility and immunological reactivity that is shared by a number of isoforms within the class.

Results

Source of Myoblast Donor and MHC Expression in High Density and Clonal Cultures

We first determined when myoblasts in the three early lineages appeared in the developing embryo. Previously (32), we qualitatively analyzed MHC expression by double immunofluorescence only in myotubes formed from ED 5 and ED 12 myoblasts. We have extended these results by using a quantitative double immunofluorescence analysis with mAb F59 (specific for the fast class of MHC isoforms) and mAb S58 (specific for the slow class of MHC isoforms) to determine the percentage of myotubes containing only fast, both fast and slow, or only slow MHC isoforms. Analysis was performed on myotubes formed in high density culture by day 6 of incubation using myoblasts obtained from hindlimb buds of ED 3 (when the limb buds are first recognizable), ED 4–6 embryos, and from the whole thigh of ED 8, 10, and 12 embryos. Multiple types of myotubes were formed in cultures of myoblasts isolated from chicken embryo hindlimbs as early as ED 3 and as late as ED 6 (Fig. 1 a). Fast myotubes, the predominate type of myotube at all ages, became more common as the age of the donor embryo increased, whereas fast/slow and slow myotubes became rarer. Myotubes of the fast/slow and slow types were almost never seen when myotubes were formed from myoblasts obtained from ED 8 or older chicken embryo thighs; myotubes in such cultures almost always contained only the fast class of MHC. Occasionally, a few mononucleated myotubes (myocytes) that reacted with mAbs F59 and S58 or only with mAb S58 were seen in 6-d-old high density cultures of ED 10, 12, and 14 chick thigh myoblasts, but such cells amounted to <0.1% of the total number of myotubes.

Within individual myotubes of the fast/slow type, the fast and slow classes of MHC were usually, but not always, identically distributed. Double immunofluorescence demon-

strated that in >95% of fast/slow myotubes, the distributions of the two classes of isoforms were superimposable (Fig. 2, a and b). In <5% of the fast/slow myotubes, however, the distribution of the fast and slow classes of MHC only partially overlapped (Fig. 2, c and d). We observed areas within fast/slow myotubes that contained the fast, but not the slow class of MHC, as well as areas that contained the slow, but not the fast class of MHC. Typically, it was in the extremities of the myotubes that the distribution of the two MHC classes did not overlap.

Myotubes in high density cultures could form by fusion of the daughter cells of more than one primary myoblast type, whereas myotubes in clonal cultures can only form by fusion of the progeny of a single myoblast type. We used a quantitative analysis, therefore, to compare the relative numbers of fast, fast/slow, and slow myotubes formed by myoblasts in high density and in clonal cultures.

The percentages of muscle colonies in which the myotubes were of the fast type, fast/slow type, or slow type were similar to the percentages of myotubes of each of these types that formed in high density cultures when the myoblasts used for cloning and high density cultures were from the same age of chicken embryo. In a previous analysis (33) we used single-label immunocytochemistry to examine the myotube types formed in clonal cultures of myoblasts from the ED 4, 5, 6, 8, 10, and 12 chicken hindlimb. We augmented these results by using double immunofluorescence to quantitate the myotube types in several hundred more muscle colonies formed from myoblasts taken from each age of embryo and by extending the analysis to include myotube formation in clonal cultures of ED 3, 7, 9, and 14 chicken hindlimb myoblasts. Myoblasts that form muscle colonies when cloned are first found in the chicken embryo at ED 3 (6). The percentages of the three myotube types formed were the same in high density (Fig. 1 a) as in clonal (Fig. 1 b) cultures. When myoblasts from ED 5 chicken hindlimb buds were cloned, 75%
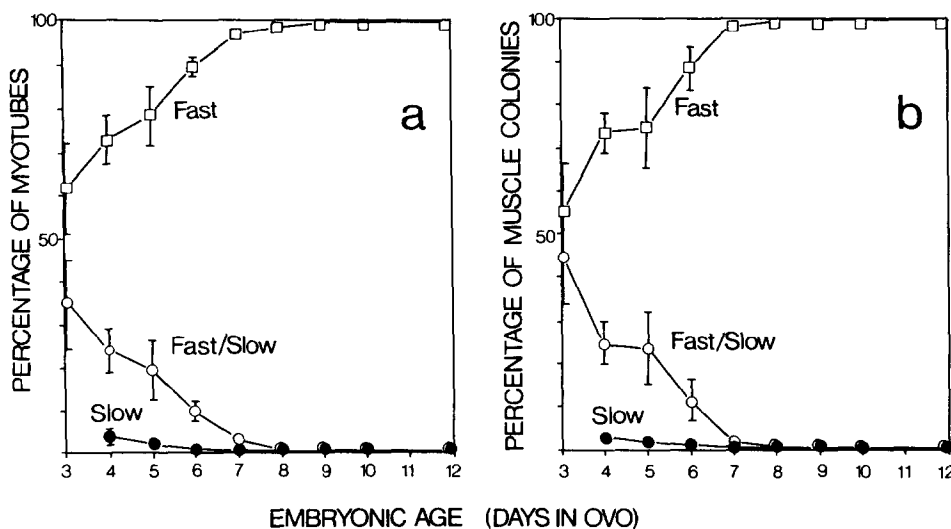

Figure 1. Effect of age of the myoblast donor on formation of three types of myotubes in high density and clonal density cultures. Myoblasts were obtained from hindlimbs of the indicated ages of chicken embryos and cultured in parallel dishes at (a) high density for 6 d or (b) clonal density for 7–10 d. A quantitative double immunofluorescence analysis of the MHC content of the resulting myotubes was performed with mAbs F59 and S58 as described in Materials and Methods. At least 300 myotubes in high density cultures (except n = 85 for ED 3) or 200 muscle colonies in clonal density cultures (except n = 76 for ED 3) were observed for each point. Myotubes in high density cultures that were stained only with mAb F59 contained only the fast class of MHC and were classified as fast; myotubes that reacted with F59 and S58 contained both fast and slow classes of MHC and were classified as fast/slow; and myotubes that reacted only with S58 contained only the slow class of MHC and were classified as slow. The muscle colonies that formed in clonal density cultures were classified as fast, fast/slow, or slow on the basis of the MHC content of the myotubes within the colony. Error bars represent the range of measurements from cultures made on different days (n = 2, ED 3; n = 4, ED 4–12).
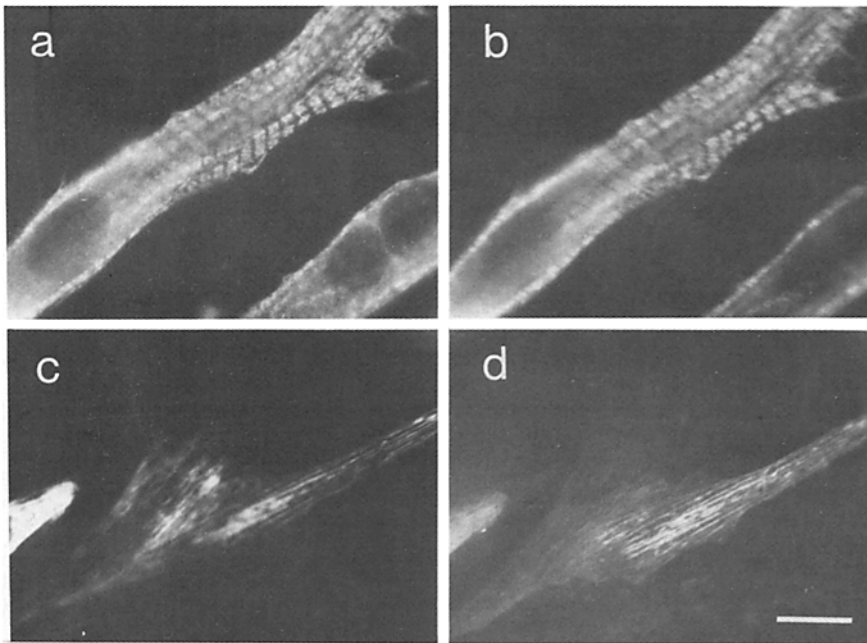
Figure 2. Distribution of fast and slow classes of myosin heavy chain isoforms in myotubes formed in high density cultures. Myoblasts were obtained from ED 5 chicken hindlimb and cultured for 6 d. MHC distribution in the resulting myotubes was determined by double immunofluorescence with mAbs F59 and S58 as described in Materials and Methods. Rhodamine fluorescence, representing the distribution of the fast MHC class, is shown in *a* and *c*. Fluorescein fluorescence of the same fields representing the distribution of the slow MHC class is shown in *b* and *d*. The distributions of fast and slow classes of MHC are coincident in *a* and *b*, but only partially overlapping in *c* and *d*. Bar: (*a* and *b*) 15 µm; (*c* and *d*) 35 µm.

of the resulting muscle colonies contained myotubes with only the fast class of MHC, 24% of the colonies contained myotubes with both the fast and slow MHC classes, and 1% of the colonies contained myotubes with only the slow class of MHC. In high density cultures of ED 5 chicken hindlimb myoblasts, 80% of the myotubes were of the fast type, 18% of the myotubes were of the fast/slow type, and 2% of the myotubes were of the slow type. When hindlimb myoblasts from ED 9, 10, 12, and 14 chicken embryos were cloned, no myotubes that contained slow MHC were found among the more than 400 colonies examined.

To determine if myoblasts in multiple myogenic lineages were restricted to the developing hindlimb or were found in all developing muscle regions, we prepared high density and clonal cultures of myoblasts from different regions of the chicken embryo and determined the class of MHC expressed in the resulting myotubes. Myoblasts were used from the trunk muscles of ED 4 or ED 12 donors and the wing and pectoral region muscles of ED 5 or ED 12 donors. When ED 4 trunk or ED 5 wing muscle myoblasts were incubated for 6 d in high density cultures, fast, fast/slow, and slow types of myotubes formed, just as was found when ED 5 thigh or pectoral myoblasts were used (32, 33). When ED 12 myoblasts from the wing, trunk, or pectoral muscle regions were incubated in high density culture only a fast type of myotube was formed. The same results were obtained with clonal cultures. When myoblasts were incubated for 8–10 d at clonal density to allow muscle colony formation, the percentage of muscle colonies with myotubes that reacted with mAb S58 was 30% (*n* = 113) when ED 4 trunk muscle myoblasts were used, 22% (*n* = 32) when ED 5 pectoral myoblasts were used, and 19% (*n* = 80) when ED 5 wing muscle myoblasts were used. In contrast, no myotubes that reacted with mAb S58 were found in muscle colonies that formed from ED 12 chicken myoblasts taken from any of the different muscle regions. Thus, myoblasts committed to distinct myogenic lineages were found throughout the developing muscle regions of the early chicken embryo, whereas myoblasts

committed only to a single fast lineage were found in developing muscles of the later embryo or fetus.

To determine if multiple myogenic lineages were found in other avian species, we also examined myotube formation by myoblasts prepared from different ages of quail embryos (7, 8, 16, 23, 34). Using the same quantitative double immunofluorescence methods described above, we found that quail myoblasts formed different proportions of fast, fast/slow, and slow types of myotubes in high density and clonal cultures depending on the age of the donor embryo in a manner similar to chicken myoblasts. Two differences were noted, however. First, quail myoblasts that formed muscle colonies with myotubes expressing only the slow class of MHC were rarer than such chicken myoblasts of comparably aged donor membranes. For example, only one of 325 muscle colonies formed by myoblasts from the ED 5 quail embryo reacted with mAb S58 and not with mAb F59. Second, a small population of myoblasts that gave rise to fast/slow myotubes persisted through ED 12 in the quail embryo. From 0.1 to 2.0% of the myotubes in high density cultures and in muscle colonies formed from ED 10 or ED 12 quail myoblasts reacted with both mAbs F59 and S58. Except for these small differences, the formation of myotubes with different MHC contents by quail and chicken myoblasts was quantitatively and qualitatively similar.

Culture Medium and MHC Expression in Muscle Colonies

In the experiments reported thus far, all cultures were grown in a mixture of equal parts of conditioned medium and fresh medium. This medium mixture was used because Hauschka and colleagues showed that myoblasts isolated from early embryos (beginning at ED 3) would not form muscle colonies unless conditioned medium was used (6, 53, 54). These myoblasts were termed conditioned medium requiring (CMR). CMR myoblasts form muscle colonies of both early (short myotubes, low fusion index) and late (long myotubes,
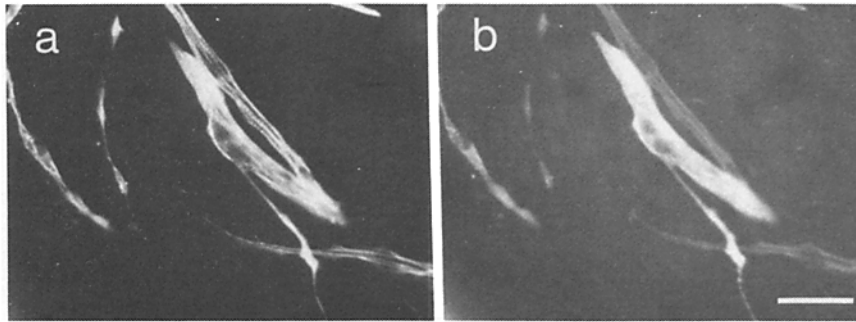
Figure 3. Variability of myosin heavy chain expression among the myotubes in secondary subcultures derived from cloned fast/slow myoblasts. The content of fast and slow classes of MHC isoforms was determined by double immunofluorescence with mAbs F59 and S58 in the myotubes of muscle colonies formed from subcultured ED 5 chick hindlimb myoblasts. Rhodamine fluorescence, representing binding of F59 and distribution of the fast MHC class, is shown in *a*. Fluorescein fluorescence, representing binding of S58 and distribution of the slow MHC class, is shown in *b*. Only a portion of the muscle colony is shown. All myotubes within the colony contained both fast and slow classes of MHC isoforms, but the relative amounts of each isoform varied in different myotubes. Bar, 60 μm.

high fusion index) morphological types (44). A population of myoblasts that does not require conditioned medium for muscle colony formation and was thus termed fresh medium sufficient (FMS) begins to appear at ED 5 and increases to 30–40% of the clonable myoblasts by ED 12 (54). FMS myoblasts form muscle colonies of only the late morphological type. We compared our results with those of Hauschka and colleagues by determining if myoblasts that formed muscle colonies in which the myotubes expressed the slow class of MHC were restricted to CMR myoblasts or were found in both CMR and FMS myoblasts.

We found that muscle colonies containing myotubes that expressed the slow class of MHC were of the CMR class, and that the CMR class is, therefore, a mixture of myoblasts committed to fast, mixed fast/slow, and slow lineages. Myoblasts were obtained from different ages of chicken hindlimbs and were incubated for 10 d at clonal density in either fresh medium or in the standard mixture of fresh medium and conditioned medium. The presence or absence of the slow class of MHC in the resulting muscle colonies was determined immunocytochemically using mAb S58 and the horseradish peroxidase–linked visualization procedure (Table I). We observed muscle colonies of both the early and late morphological types in which the myotubes expressed the slow class of MHC, but these colonies developed only in conditioned medium and were, therefore, formed exclusively by myoblasts of the CMR class. Because myoblasts from ED 4 chicken hindlimbs did not form muscle colonies in fresh medium (Table I), but formed three types of colonies (fast, mixed fast/slow, and slow) when conditioned medium was used (Table I), it was clear that the CMR myoblast population was heterogeneous. FMS myoblasts, in contrast, only formed myotubes containing the fast class of MHC.

Subcloning and Stability of Myoblast Commitment

The finding that cloned early embryonic (ED 3–6) myoblasts formed muscle colonies of different types strongly suggested that myoblasts were committed to distinct myogenic lineages (33). To determine if commitment to distinct lineages remained stable, we serially subcultured the progeny of a single chicken or quail myoblast and determined the class of

MHC isoforms expressed in the myotubes formed at each successive step of the subculturing.

We analyzed myotubes in secondary and tertiary subcultures formed from myoblasts isolated from a single primary muscle colony. Individual primary muscle colonies that had formed from ED 5 chicken hindlimb myoblasts were isolated within a cloning ring (44) and myoblasts were removed from the culture dish with trypsin. These secondary myoblasts were split into three culture dishes and incubated for 7–10 d until myotubes formed in the secondary cultures. Two of these secondary culture dishes were used to determine the reactivity of myotubes with mAb S58 or F59. The secondary muscle cultures in the third dish were removed with trypsin, and these tertiary myoblasts were cultured in three additional culture dishes for 7–10 d until myotubes formed in the tertiary cultures. Each of 24 primary colonies formed from single ED 5 chicken hindlimb myoblasts was subcultured to the tertiary culture stage. Of these, 18 clonal lines contained myotubes that reacted only with mAb F59 in both the secondary and tertiary subcultures and were, therefore, in the fast myogenic lineage. In the remaining six clonal lines, the myotubes reacted with both F59 and S58 in the secondary and tertiary subcultures and were in the fast/slow myogenic lineage.

Table I. Effect of Medium Composition and Age of Myoblast Donor on Expression of the Slow Class of MHC in Muscle Colonies

Source of myoblasts	mAb Probe	Medium*	No. of colonies examined	Percentage (No.) of stained colonies
ED 4 chicken hindlimb bud	S58	Conditioned	124	30% (37)
		Fresh	—‡	—‡
ED 6 chicken hindlimb	S58	Conditioned	165	10% (16)
		Fresh	182	0% (0)
ED 8 chicken thigh	S58	Conditioned	180	1% (2)
		Fresh	171	0% (0)
ED 11 chicken thigh	S58	Conditioned	184	0% (0)
		Fresh	117	0% (0)

* Conditioned medium was a mixture of equal parts of conditioned and fresh medium (see Materials and Methods).
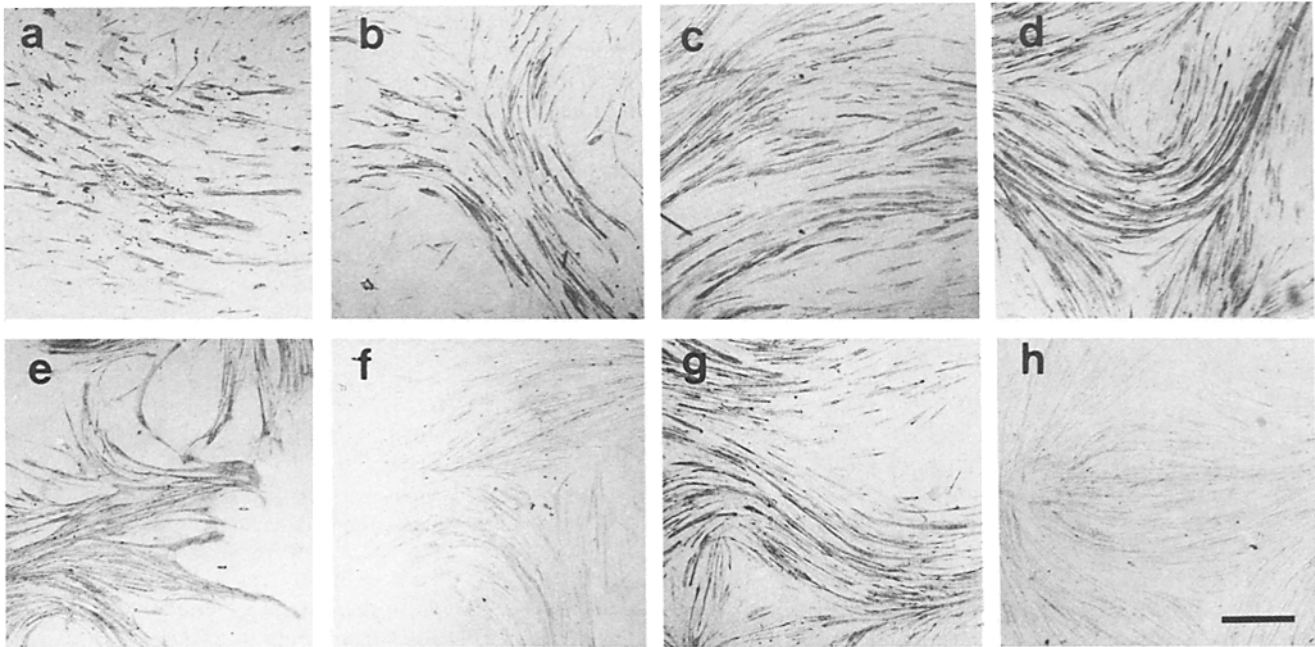‡ No muscle colonies formed in fresh medium from ED 4 myoblasts.

Figure 4. Myosin heavy chain expression in myotubes formed from serially subcultured myoblasts. Primary muscle colonies formed from cloned ED 5 quail hindlimb myoblasts were subcloned and serial subcultured as independent clonal cell lines. After each round of subculturing, the content of fast and slow classes of MHC isoforms was determined in myotubes formed in each of the independent clonal lines by incubating parallel cultures with either mAb F59 or mAb S58, and visualizing antibody binding with a horseradish peroxidase–linked system described in Materials and Methods. Staining of a fast/slow clonal line is shown at the secondary (*a* and *b*) and tertiary (*c* and *d*) stages of subculture, and staining of a fast clonal line is also shown at the secondary (*e* and *f*) and tertiary (*g* and *h*) stages of subculture. Staining with mAb F59 is shown in *a*, *c*, *e*, and *g*, and staining with mAb S58 is shown in *b*, *d*, *f*, and *h*. Bar, 100 μm.

Double immunofluorescence analysis of these subcultures showed that all myotubes in a mixed fast/slow culture reacted with both mAb S58 and F59, but the relative intensity of staining with each mAb varied among the myotubes (Fig. 3). In no instance did a fast/slow clonal line change to an exclusively fast or exclusively slow type of clonal line during serial subculturing, nor did subcultures of a fast culture ever express the slow MHC class. Thus, the pattern of expression of fast and slow classes of MHC isoforms was stable in the myotubes formed from the progeny of more than 30 cell divisions (39) of the originally cloned chicken myoblast.

For biochemical analyses, clonally derived populations of quail myoblasts committed to different lineages were sequentially subcultured and grown into large numbers of myogenic cells committed to a single lineage. Quail myoblasts cloned more readily and these clones formed larger subcultures than chicken myoblasts (16, 34) which made the subcultured quail myoblasts more useful for biochemical analysis. Myoblasts from ED 4, 5, and 6 quail hindlimbs were cloned and 60 of these primary clones were subcultured through the tertiary culture stage. Of these, 44 of the individual clonal lines contained myotubes that reacted with only mAb F59 and were, thus, in the fast lineage, and the remaining 16 clonal lines contained myotubes that reacted with both mAb F59 and mAb S58 and were, thus, in the fast/slow lineage. Each of these quail clonal lines retained its particular pattern of MHC expression to the tertiary culture stage or more than 30 cell doublings. Examples of the morphology and immunocytochemical staining with mAbs F59 and S58 of expanded subcultures of the fast and fast/slow type are given in Fig. 4.

Biochemical analysis of the MHC content of the myotubes formed in independently expanded clonal cultures confirmed the immunocytochemical classification of the muscle colonies and stability of the class of MHC expressed during serial subculture. Tertiary cultures were formed by subculturing individual primary muscle colonies formed from ED 5 quail myoblasts. Myosin was extracted from 60-mm dishes of two tertiary subcultures identified immunocytochemically as fast type and two tertiary subcultures identified as fast/slow type and was analyzed by SDS PAGE in 5% gels and immunoblotting with mAbs F59 and S58. As shown in Fig. 5, MHC of both the fast and slow classes of MHC was found in extracts of clonal lines that were previously identified immunocytochemically as fast/slow type, and MHC of only the fast class was found in extracts of clonal lines previously identified as fast type. The fast and slow classes of MHC found in the subcultures were electrophoretically distinct peptides that had relative mobilities and reactivities with mAbs F59 and S58 that were indistinguishable from the relative mobilities and reactivities of the fast and slow isoforms of MHC found in all ages of quail and chicken (2, 32, 33, 43).

MHC expression was also stable through two subcultures in serially subcultured muscle colonies formed from ED 12 chicken and quail myoblasts. Five independent primary muscle colonies formed from individual ED 12 chicken thigh myoblasts and five formed by individual ED 12 quail thigh myoblasts were subcultured to the tertiary culture stage, and the MHC content of the myotubes formed in cultures of these independent clonal lines was determined at each subculture. All the myotubes in each of the 10 independent clonal lines reacted with mAb F59 and thus contained the fast class of
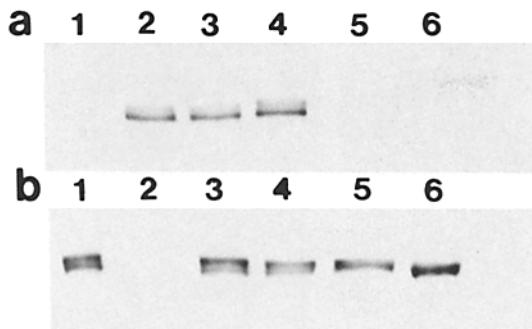
Figure 5. Fast and slow classes of MHC isoforms expressed by myotubes formed from cloned myoblasts. Myoblasts were isolated from primary muscle colonies formed from ED 5 quail hindlimb myoblasts and serially subcultured as described in Results. Extracts were made of the MHC synthesized in tertiary subcultures of each of the independent clonal lines and analyzed by SDS PAGE in 5% gels and immunoblotting. Duplicate gels were run and nitrocellulose transfers were reacted with mAb S58 (*a*) or mAb F59 (*b*), and antibody binding was visualized with an alkaline phosphatase-linked system. As references, fast MHC from adult chicken pectoral muscle was analyzed in lane *1*, and slow MHC from adult chicken anterior latissimus dorsi was analyzed in lane *2*. Extracts from two independent clonal lines previously identified immunocytochemically as fast/slow were analyzed in lanes *3* and *4*, and extracts from two clonal lines identified as fast were analyzed in lanes *5* and *6*. The immunocytochemical identification of the subclone analyzed in lane *3* is shown in Fig. 4, *a-d*, and of the subclone analyzed in lane *5* is shown in Fig. 4, *e-h*.

MHC at each step of the sequential subculturings. In no instance did subcultured muscle colonies appear in which the myotubes within the colonies had switched from the fast type to the fast/slow or slow types. Myotubes in the secondary and tertiary subcultures did not react with mAb S58, except that, in two of the quail lines, very few isolated cells (one or two myotubes per dish) did react with mAbs F59 and S58 or with only mAb S58 at all stages of the subculturing. Biochemical analysis by immunoblotting confirmed that only the fast class of MHC was detectable in each of the clonal lines derived by serial subculturing of ED 12 chicken and quail thigh myoblasts (not shown).

Fast and Slow Classes of MHC In Vivo

To correlate the results in vitro with events in vivo, we determined the distribution of the fast and slow MHC classes in embryonic myotubes by immunohistochemistry of frozen sections of hindlimbs, and we analyzed extracts of developing muscles from different ages of embryos for the presence of the fast and slow classes of MHC isoforms. We used cryostat sections of the ED 7 chicken thigh to determine the percentages of fibers with exclusively fast, mixed fast/slow, and exclusively slow MHC content in the primary fibers of the early chick hindlimb in ovo. The cryostat sections were stained using the double-label immunofluorescence technique with mAbs F59 and S58, and muscle fibers of different types were observed and counted. We found that in the thigh of the ED 7 chick, 60–70% of the fibers contained only fast MHC, 30–40% of the fibers contained both the fast and slow classes of MHC isoforms, and <1% of the fibers contained only slow MHC. Sections of different muscles of the ED 7 thigh showing these three fiber types are shown in Fig. 6.

Myotubes that reacted with both F59 and S58 and thus contained both fast and slow classes of MHC isoforms were located in developing muscles such as the semitendinosus (Fig. 6, *a* and *b*), sartorius, and medial adductor, which contain many slow and mixed fast/slow fibers in the adult. A complete description of the development of fast and fast/slow fibers in the chicken hindlimb is given by Crow and Stockdale (14). The few fibers that reacted only with mAb S58 and thus contained only the slow class of MHC were found in the semimembranosus (Fig. 6, *c–e*) and anterior iliotibialis muscles (Fig. 6, *f–h*), muscles that were otherwise composed entirely of muscle fibers containing only the fast class of MHC.

We also used immunoblots to determine the classes of MHC isoforms expressed in different ages of embryonic muscles. Previous work had shown that fast and slow classes of MHC isoforms are found in the ED 6 chicken hindlimb as analyzed by immunoblots (50) and that myotubes containing the slow class of MHC are detectable by immunohistochemistry in the ED 5 chicken limb bud (14). These results were extended here by showing that both the fast and slow classes of MHC were detectable by immunoblotting of muscle tissue extracts from ED 3 somites (stages 20–22), ED 4 combined leg and wing buds (stages 23–25), ED 5, and ED 6 leg buds (Fig. 7). The extracts were analyzed by SDS PAGE in 5% gels and by immunoblotting with mAbs F59 and S58. Electrophoretically distinct MHCs that reacted with only S58 or only F59 were found at ED 3 and throughout embryonic development.

Discussion

Based on results presented here and previously (32, 33, 50), we propose that fast, mixed fast/slow, and slow muscle fibers originate during development of the bird from distinct myogenic lineages as diagrammed in Fig. 8. In early embryonic muscle (~ED 3–7), three types of myoblasts are present which are committed to fast, mixed fast/slow, and slow myogenic lineages. We term these early lineages the primary fast, primary fast/slow, and primary slow myogenic lineages. We assume that the three myoblast types independently form three types of primary myotubes in the same proportions as the three myoblast types are present in the muscle anlagen. Initial fiber diversification has, therefore, a cellular basis that is rooted in the diversity of myoblasts committed to different lineages. This early fiber diversification in the embryo is independent of innervation (11, 14, 30, 31, 39). During fetal development (after ED 8), myoblasts in a single lineage of the fast type are predominant and myoblasts in fast/slow and slow lineages are vanishingly rare. We term the single late lineage the secondary fast lineage, and assume that myoblasts in this lineage form the secondary myotubes. In this later developmental period, maturation and maintenance of muscle fiber diversity is dependent on innervation (10, 14, 19, 32, 39). We focus our discussion on the evidence for this model.

The presence of myoblasts committed to the different primary myogenic lineages has now been demonstrated in early embryonic (ED 3–6) hindlimb, forelimb, pectoral, and trunk muscles of the chicken and hindlimb muscles of the quail. Myoblasts from early embryos were shown (33) to be committed to distinct myogenic lineages because every myotube formed by the progeny of a cloned myoblast contained the
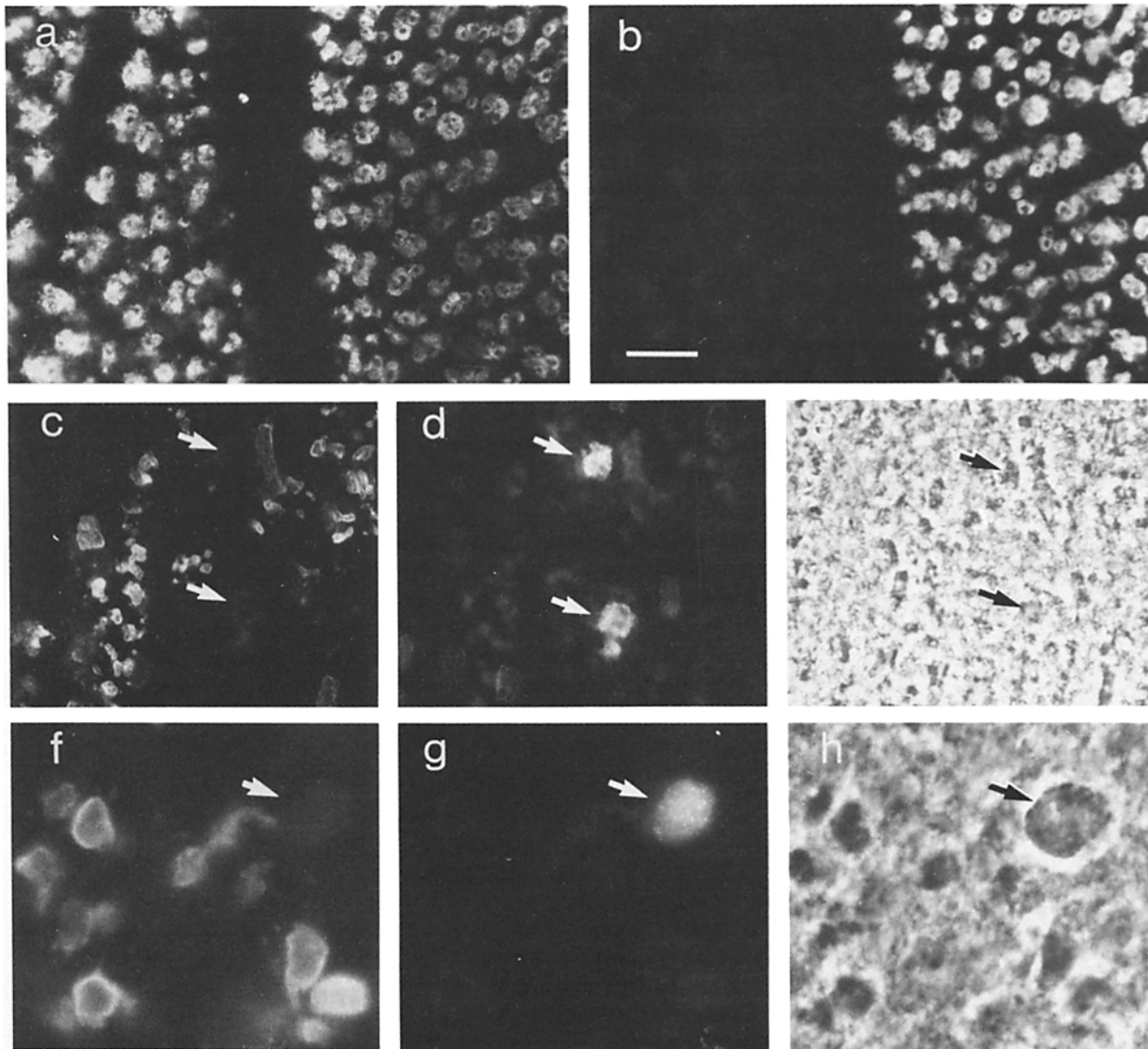
Figure 6. Muscle fiber diversity in the developing chicken thigh. Cryostat sections of ED 7 chicken thighs were prepared and MHC distribution in the myotubes was determined by double immunofluorescence with mAb F59 (*a, c,* and *f*) and mAb S58 (*b, d,* and *g*). In *a* and *b*, the region where the semimembranosus (left part of *a* and *b*) and semitendinosus (right part of *a* and *b*) adjoin is shown. Fibers in the semitendinosus are all of the fast/slow type, whereas fibers in the semimembranosus are of the fast type. A portion of the anterior iliotibialis containing two slow type fibers and many fast type fibers is shown stained with F59 in *c,* S58 in *d,* and by phase in *e.* A portion of the semimembranosus containing one slow type fiber and many fast type fibers is shown stained with F59 in *f,* with S58 in *g,* and by phase in *h.* Bar: (*a–e*) 30 µm; (*f–h*) 10 µm.

same class or classes of MHC isoforms. Commitment to a each lineage was stably inherited for at least the 10–15 cell doublings required for a single myoblast to form a primary muscle colony, and, in the case of myoblasts in the fast and fast/slow lineages, the subculture of such clones showed that commitment was stable for 30 or more cell doublings. We have not yet succeeded in subculturing myoblasts from clones in the primary slow lineage. Because such myoblasts should be very rare (∼1% of the myogenic cells in ED 4–5 chicken embryos), they may have been missed. Alternatively, it may be that myoblasts in the primary slow lineage cannot be subcultured, perhaps due to an intrinsically limited potential for division or to an unknown requirement for growth in culture medium.

Our results showed that myoblasts in the three primary lineages were predominate in the early embryo but were largely or entirely superseded in the later embryo by myoblasts in the secondary fast lineage. The transition occurred during days 6–8 in ovo which corresponded closely to the ages when the early (CMR) and late (FMS) myoblasts defined by Hauschka and colleagues (44, 45, 54) were found in the embryo. In fact, myoblasts in each of the three primary lineages required conditioned medium to form muscle colonies and were therefore CMR myoblasts as defined by White et al. (54). Myoblasts in the secondary fast lineage formed muscle colonies in vitro in fresh medium, thus FMS myoblasts are included in the secondary fast lineage. The CMR myoblasts are not the precursors of the FMS myoblasts (44,
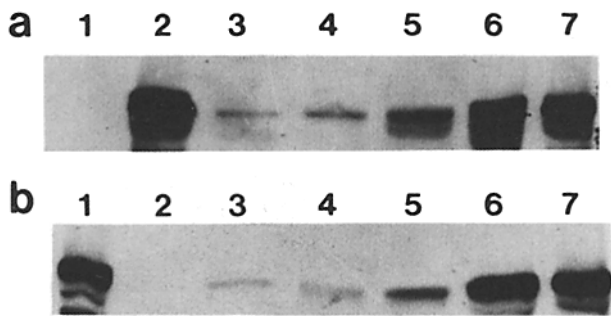
Figure 7. Fast and slow classes of MHC isoforms in developing muscles in vivo. MHC was extracted from muscles of different ages of chicken embryos and analyzed by SDS PAGE in 5% gels and immunoblotting. Duplicate gels were run, nitrocellulose transfers were incubated with mAb S58 (*a*) or mAb F59 (*b*), and antibody binding was visualized with an alkaline peroxidase–linked system. As references, fast MHC from adult chicken pectoral muscle (lane *1*) and slow MHC from adult chicken anterior latissimus dorsi (lane *2*) were analyzed. MHCs from ED 3 somites (lane *3*), ED 4 pooled leg and wing buds (lane *4*), ED 5 leg buds (lane *5*), ED 6 whole leg (lane *6*), and ED 8 thigh (lane *7*) were compared with the reference MHC isoforms.

45, 55). Recently we have found that myotubes formed from secondary quail myoblasts (ED 12) after four or more serial passages express MHC of both fast and slow classes (Schafer, D. A., J. B. Miller, and F. E. Stockdale, unpublished observations). Although we have not explicitly studied MHC isoform expression in the myotubes formed by each of the CMR myoblast types of White et al. (54), at least four myogenic lineages—three early and one late—clearly exist in the avian embryo.

We termed the myogenic lineages "primary" and "secondary" because the time of appearance of myoblasts in these lineages in developing muscles corresponds to the primary and secondary phases of myotube formation recognized from anatomical studies (4, 5, 21). Although we suggest, based on this timing, that myoblasts in the primary myogenic lineages form primary myotubes and myoblasts in the secondary lineage form secondary myotubes, it is unlikely that this distinction is rigidly maintained, in that secondary myoblasts probably fuse with primary myotubes (21).

We assume in our model that myoblasts in the different myogenic lineages form myotubes independently of each other in developing muscles. Independent formation could arise either because the myoblasts in one lineage are intrinsically unable to fuse with myoblasts in a different lineage, or because myoblasts in different lineages are kept spatially separated in embryonic muscles by some extrinsic mechanism. There is indirect evidence that different populations do not fuse with each other. First, the number of myotubes that form and contain the slow class of MHC is the same when primary myoblasts from early embryos and secondary myoblasts from late embryos are cultured together as when myoblasts from the early embryo are cultured alone (32). In addition, as shown here, the relative proportions of fast, fast/slow, and slow myotubes formed from early myoblasts in the primary myogenic lineages are the same in clonal as in high density cultures, although extensive intercellular contacts among myoblasts in different lineages must occur in the high density cultures. A direct determination of the amount of fusion between myoblasts in different lineages is required.

Evidence supporting the idea that early muscle fiber diversification is independent of innervation whereas later myogenesis is dependent on innervation has come from several studies. Myoblasts from the early embryo (ED 3-6) form three types of myotubes (fast, fast/slow, and slow) when cultured aneurally at high density (32) or clonal density (33, this work). In chicken embryos in which functional innervation is prevented by neural tube removal on ED 2 or continuous administration of curare from ED 2 or ED 4, muscle formation and muscle fiber diversification is normal until about
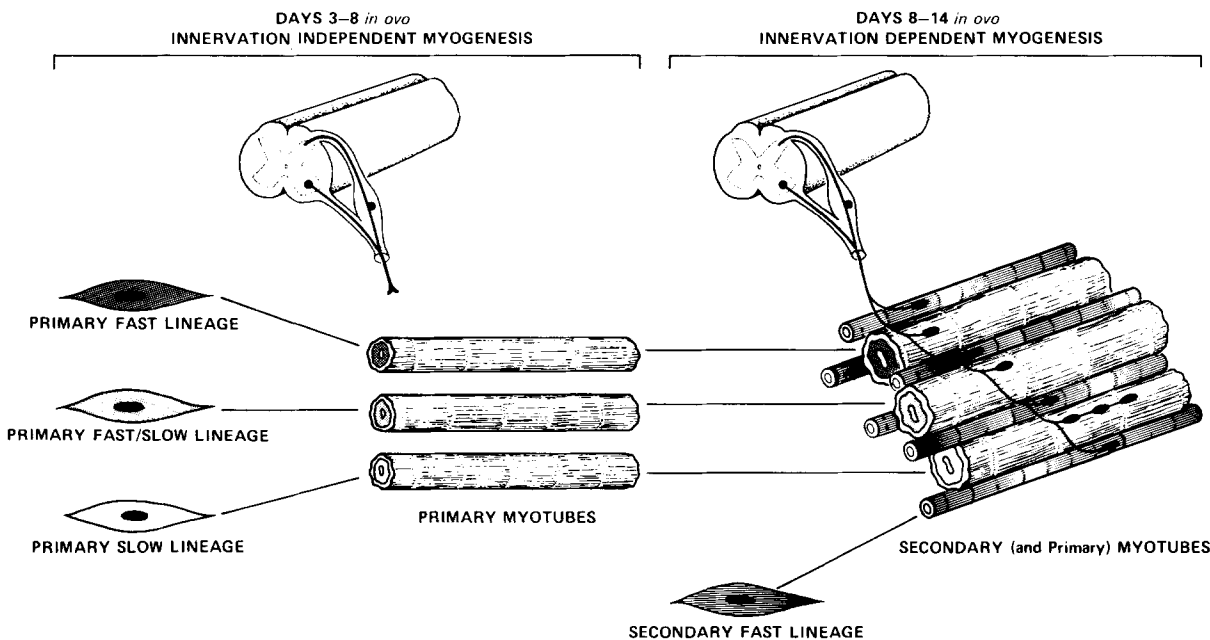

Figure 8. A model for myotube formation by myoblasts committed to multiple myogenic lineages in the avian embryo.

ED 9 (10, 14, 31, 39). In older embryos, however, myogenesis in aneural embryos is markedly altered. The number of muscle fibers is decreased in later (ED 10–12) aneural embryos compared with controls (10, 14, 39), with a selective loss of secondary myotubes (31) and slow MHC-containing fibers (14) in the aneural embryos. Cross-innervation experiments in adult animals have also shown that muscle fiber type is dependent on the type of motoneuron that innervates the fiber (reviewed in reference 19). Thus, during the developmental period when myoblasts in the primary myogenic lineages predominate (ED 3–6), muscle fiber formation and diversification is independent of innervation as assessed both in vivo and in vitro, but during the later developmental period (after ED 8) when myoblasts in the secondary fast myogenic lineage predominate, muscle fiber diversification and maturation is dependent on innervation.

The synthesis of electrophoretically distinguishable fast and slow classes of MHC isoforms is a very early event in avian development that coincides with the formation of the primary generation of muscle fibers. Both the fast and slow classes of MHC isoforms were found by ED 3 in the trunk muscles (somites) and ED 4–5 in the developing limbs of the chicken embryo. Both classes of isoforms were distributed into distinct primary fiber types by ED 5 (14). By ED 7, as shown here, three primary muscle fiber types are found in the thigh, 60–70% of the fast type, 30–40% of the mixed fast/slow type, and <1% of the slow type. These proportions of the different muscle fiber types in the early embryo are similar to the proportion of myoblasts in the corresponding primary myogenic lineages. Fibers were designated as fast, fast/slow, or slow to indicate that they contain MHC isoforms with a specific combination of electrophoretic mobility and reactivity with mAbs F59 and S58, even though at each age of the bird the fiber may contain different MHC isoforms within the fast or slow class. For example, the avian MHC isoforms found in the breast muscle and designated embryonic, neonatal, and adult (1, 2, 25) are all members of the fast class of MHC isoforms (14, 33) because each reacts with mAb F59 and has the electrophoretic mobility characteristic of fast MHC. It will be interesting to determine which isoforms of the fast and slow classes of MHC are expressed in vivo and in vitro and to determine the structure of the epitope that is common to different MHC isoforms within a class.

In our model, myoblasts are committed to qualitatively distinct myogenic cell lineages that express different classes of MHC. An alternative would be a single lineage model where myoblasts differ quantitatively such that they fuse to form a continuum of myotubes expressing different levels of fast and slow MHC. Several pieces of evidence favor the multiple lineage model. That one obtains a reproducible percentage of three myotube types from experiment to experiment when the same aged embryonic donor was used does not exclude the alternative model, but the finding that, when cloned, this myoblast population produces the same percentage of muscle colonies of the three types favors the first model. Likewise, the finding that muscle colonies are composed exclusively of one type of myotube rather than of multiple types is most consistent with the multiple lineage model. Finally, the finding that subclonally derived myoblasts form myotubes of only one type makes the model of discrete myogenic lineages most likely.

The model of muscle fiber diversification during development that we present here may be in its simplest form. The model is based on experiments in which we used the content of fast and slow classes of MHC isoforms as a marker for fast, mixed, and slow fibers. The fast and slow MHC isoforms were chosen because the rate of ATP hydrolysis of the different MHC isoforms correlates well with the maximum contraction velocity of fast, mixed, and slow fibers (3, 25, 26, 40, 46, 52) and because these MHC isoforms have been shown to be markers for different fiber types in both the embryonic and adult bird (13, 14, 32, 43). Other muscle specific proteins such as C-protein (37), troponin (27), tropomyosin (35, 41), and the myosin light chains (12, 17, 36, 41, 48) also have slow and fast isoforms, but not all of these proteins are equally good markers for fast and slow fibers. The slow myosin light chains, for example, are often found associated with fast myosin heavy chain, both in adult (14) and embryonic (12, 48) muscle fibers and in myotubes formed in vitro from embryonic myoblasts (20, 47). To the extent, however, that isoforms specific to fast and slow fibers are coordinately expressed, we would expect that using the isoforms of these other muscle-specific proteins as fiber-type markers would give results similar to those found with MHC isoforms. In denervated and regenerating muscle fibers, however, different muscle-specific proteins are not coordinately expressed (29) and such discoordinate expression, if it occurs in the myotubes formed by embryonic myoblasts in vitro, could indicate further complexity among myogenic lineages.

The finding of multiple myogenic lineages in the avian embryo raises many interesting questions about the mechanisms of myoblast commitment, muscle fiber diversification, and the formation of whole muscles containing multiple fiber types. First, which cells are the precursors of myoblasts in the different lineages? Myoblasts are typically identified by the ability to form myotubes, but assays to identify the precursors of myoblasts are lacking. What molecular mechanisms underlie commitment of myoblasts to distinct myogenic lineages? Cell determination to the myogenic lineage appears to require the action of a small number of genes (22, 38), but the genetics of commitment to the different fast, fast/slow, and slow lineages have not been studied. Does the distribution of primary myoblast types establish the distribution of primary fiber types that subsequently populate a muscle as it grows? From the earliest cleavage of a muscle anlagen in the avian limb, the newly formed muscles have characteristic proportions and spatial distribution of fibers of different types, even when innervation is prevented (10, 14, 39). Such specific fiber formation and localization could arise from specific localization of the myoblasts in different lineages, perhaps by interaction with nonmuscle cells or extracellular matrix components in the developing limb. What is the relationship between MHC expression in primary and secondary myotubes (cf. 42)? Secondary myotubes form in close apposition and are electrically coupled to primary myotubes in the embryo (reviews in references 4, 15, and 21), but can express different MHCs (14, 30, 39, 42). Does the class of MHC isoform synthesized in the primary myotube affect the class of MHC synthesized in the secondary myotube, or is the secondary fiber type determined solely by the type of motor innervation received? Our model for muscle fiber diversification based on multiple myogenic lineages in

the avian embryo provides a new experimental framework to approach these questions.

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