Muscle Fiber Pattern Is Independent of Cell Lineage in Postnatal Rodent Development

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Summary

Muscle fibers specialized for fast or slow contraction are arrayed in characteristic patterns within developing limbs. Clones of myoblasts analyzed in vitro express fast and slow myosin isoforms typical of the muscle from which they derive. As a result, it has been suggested that distinct myoblast lineages generate and maintain muscle fiber pattern. We tested this hypothesis in vivo by using a retrovirus to label myoblasts genetically so that the fate of individual clones could be monitored. Both myoblast clones labeled in muscle in situ and clones labeled in tissue culture and then injected into various muscles contribute progeny to all fiber types encountered. Thus, extrinsic signals override the intrinsic commitment of myoblast nuclei to particular programs of gene expression. We conclude that in postnatal development, pattern is not dictated by myoblast lineage.

Introduction

Little is known regarding the mechanisms for establishing and maintaining pattern in the tissues of mammals. Two general types of developmental strategy have been described in invertebrates. In some cases, the differentiated state of a cell is controlled by its environment through local cell–cell interactions. In Drosophila, a clear example of this type of environmental control is provided by cells of the ommatidia (Banerjee and Zipursky, 1990). These cells specialize late, remaining plastic until interaction with their immediate neighbors dictates their expression of a particular phenotype. In other cases, the differentiated state of a cell is controlled by its lineage, and pattern results from directed cell migration or selective adhesion. In Caenorhabditis elegans, the differentiation of sex myoblasts provides an example of lineage control (Thomas et al., 1990). These cells specialize early, before reaching their final position, and once relocated express their phenotype irrespective of their neighbors. Until recently, it has been difficult to address questions regarding pattern development in mammals. However, a novel approach involves genetically marking individual cells in situ using replication incompetent retroviral vectors and monitoring the fate of their progeny (Price, 1989). This method has revealed that both strategies are used to establish pattern in the central nervous system. Environmental cues control terminal differentiation of retinal neuroblasts (Turner et al., 1990), whereas lineage determines the fate of the precursors of neurons and glia (Luakin et al., 1988; Price and Thurlow, 1988).

Here we use retroviral lineage marking to examine the cellular basis for the fiber type pattern observed in the muscles of the mammalian limb. Muscles are composed of fibers, multinucleated cells that grow by the fusion of mononucleated myoblasts. In postnatal development, this growth continues so that muscle mass increases 10-fold even after the pattern of diverse fiber types has been established. Fiber types differ in their rate of contraction, a property that is, in part, determined by the complement of myosin heavy chain (MyHC) isoforms the fiber contains; fast and slow MyHCs have high and low ATPase activities, respectively (Barany, 1967). In general, slow fiber types are responsible for holding posture, whereas fast fiber types perform movements. Although both fast and slow fiber types occur in all muscles of the rat lower hindlimb, the ratio of the two classes differs dramatically among muscles and even among regions of a single muscle. Whereas superficial muscle regions typically have more fast fibers than deep regions near bone, within any given region fibers are mixed in a pepper and salt fashion. Moreover, a gradient of fast to slow muscle fibers in one muscle can be entirely opposite to that observed in a neighboring muscle (Condon et al., 1990). How spatial and temporal cues operate to generate and maintain this complex pattern in developing muscles is unclear.

It has long been known that environmental factors, such as innervation (Buller et al., 1960; Pette and Vrbova, 1985), can affect fiber type. However, innervation is not sufficient to account for fiber type diversity for two reasons. First, in the denervated embryo, pattern develops in the complete absence of neurons (Butler et al., 1982; Condon et al., 1990, Harris et al., 1989). Second, in the adult, surgical alterations, such as the innervation of slow muscles by fast nerves, cannot interconvert all fiber types (Gundersen et al., 1988; Westgaard and Lomo, 1988). This restriction of plasticity in response to innervation has led to the concept of "adaptive range" (Westgaard and Lomo, 1988), which proposes that fibers fall into several intrinsically different classes, and that innervation can influence the fiber type within a class only over a limited range. Thus, although innervation and other extrinsic influences, such as hormones (Izumo et al., 1996), can modulate muscle fiber type, their effects appear to be limited by intrinsic differences among fibers.

A mechanism based on lineage could lead to intrinsic differences and fiber type diversity. According to this hypothesis (Schafer et al., 1987), fiber diversity derives from the distribution of distinct populations of myoblasts that are committed to the generation of fast or slow fiber types. Studies of myoblasts in tissue culture lend support to this "lineage" hypothesis. Myoblasts isolated from muscle tissue differ with respect to their expression of fast or slow MyHC isoforms, and this property is inherited by their progeny (Feldman and Stockdale, 1991; Miller et al., 1985; Miller and Stockdale, 1986a). Moreover, when myoblasts from cat jaw muscle are implanted into ablated regions of leg muscles, the newly formed fibers express MyHCs

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characteristic of the jaw muscle of origin, not their new location (Hoh and Hughes, 1988). As a result, it has been suggested that distinct fiber types derive from committed myoblasts that selectively adhere to and fuse with like myoblasts. The distribution of these precursor cells could determine and maintain the complex pattern of diverse interspersed fiber types.

In this report, we examine the cellular basis for muscle fiber heterogeneity and test the hypothesis that selective fusion of committed myoblast lineages plays a role in maintaining muscle fiber phenotype during postnatal growth and development. Toward this end we have genetically marked myoblasts in vivo with retroviral vectors and followed the fate of their progeny (Hughes and Blau, 1990). Our results show that myoblast clones in developing postnatal rat muscle are not committed to fuse into particular fiber types, but contribute progeny to the entire spectrum of fast and slow fiber types in their vicinity. Furthermore, retrovirally marked myogenic clones that appear specialized for slow contraction in vitro, following implantation into diverse muscles in vivo, contribute to all fiber types. These results demonstrate that although postnatal myoblasts appear to be committed, possibly due to their lineage, they are reprogrammed upon fusion into muscle fibers.

Results

A Single Myoblast Clone Contributes to Both Fast and Slow Fibers

We have previously shown (Hughes and Blau, 1990) that single myoblast clones, genetically marked in vivo by infection with a retroviral vector that expresses β-galactosidase (β-gal), frequently contribute cells to a cluster of several muscle fibers during the postnatal development of rat lower hindlimbs. Such clusters were shown to represent true clones, the progeny of a single retrovirally infected myoblast, by the use of a dual retroviral technique (Hughes and Blau, 1990; Galileo et al., 1990). Upon coinjection of a mixture of two retroviral vectors that express β-gal in the nucleus (nls-β-gal) or cytoplasm (cyt-β-gal), we observed well separated clusters of fibers that in 97% of cases contained either nuclear or cytoplasmic label, but not both. These results showed that “satellite” myoblasts could cross basal lamina.

Here we use the retrovirus MMuLVSVnlsLacZ (Bonnett et al., 1987) under identical conditions to those described previously (Hughes and Blau, 1990). The fibers that receive a cell from a clone marked with this retroviral vector express nls-β-gal, a modified form of β-gal that is targeted to nuclei because of the addition of the SV40 T antigen nuclear localization signal to the N terminus. Because nuclei targeted β-gal in syncytial muscle fibers can be taken up by nuclei other than those that produce it (Ralston and Hall, 1989), fibers containing a nucleus derived from a retrovirally marked myoblast can be identified in serial transverse cryostat sections after X-Gal staining for β-gal activity. In adult fibers, nuclei are typically located at the fiber periphery (Schmalbruch, 1985), and those containing nls-β-gal are readily apparent (Figure 1A) in serial sections. There are two lines of evidence that confirm that such series of labeled nuclei are within muscle fibers, not adjacent cells. First, we frequently observed minute crystals of blue X-Gal reaction product in the cytoplasm of fibers with labeled nuclei (Figure 1A, small arrows). This β-gal activity typically occupied a relatively small area and was presumably caused by nls-β-gal that had not yet reached its nuclear target. Such crystals were not seen in the surrounding cells. In addition, our previous study (Hughes and Blau, 1990) showed that clones marked with nls-β-gal did not differ significantly in fiber number and morphology from clones identified with a retrovirus that expressed cyt-
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β-gal, which could clearly be seen to label the whole sarco-
plasmic space.

To test whether myoblasts are specialized to fuse to
either fast or slow fibers, we determined the phenotype of
fibers to which the cells of a clone contributed. MMuLVSV-
nisLaCZ was injected into postnatal day 15 (P15) rat legs,
where it infected single myoblasts in situ that proliferated,
migrated, and contributed cells to between one and ten
fibers by the time of analysis three weeks later (P37). La-
beled fibers were identified by staining alternate sections
for β-gal. Figure 1A shows four fibers (large arrows) in the
lateral gastrocnemius to which a single myoblast clone
contributed (illustrated schematically in Figure 1C). Figure
1B shows serial adjacent sections in which muscles are
stained to reveal fibers with slow MyHC isoforms using
monoclonal antibody 4A.840 (Webster et al., 1988b).
Clearly, the fibers to which this clone contributed were
heterogeneous in their MyHC expression: three contained
no detectable slow MyHC, whereas the fourth expressed
this isoform at a high level. Of 40 individual clones ana-
yzed in this manner, 5 (12.5%) appeared heterogeneous,
containing fibers that did and did not express the slow
MyHC recognized by 4A.840.

Myosin Heavy Chain Antibodies Distinguish
Numerous Fiber Types

Although the presence of slow MyHC distinguishes slow
fibers from fast fibers, the diversity of muscle fiber types
is more complex than this simple dichotomy would suggest
(Schiaffino et al., 1989; Harris et al., 1989; Condon et al.,
1990). Multiple different fiber types can be distinguished
both physiologically and by isoform-specific monoclonal
antibodies (Pette and Staron, 1985; Laframboise et al.,
1991). It seemed possible that myoblasts were restricted to
a subset of slow or fast fiber types. Before addressing this
possibility, it was necessary to determine the complex-
ity of fiber types within the muscles of the rat limb. For
this purpose we used four monoclonal antibodies to MyHC
produced in our laboratory (Silberstein et al., 1986; Web-
ster et al., 1988b). This new antibody-based classification
reveals a greater complexity of fiber types than previous
ATPase-based classifications. Since additional fiber type
diversity is likely to be revealed upon the development of
new reagents, this classification is used here to dis-
tinguish, not to define, diverse fiber types. The antibodies
were raised against purified preparations of human
myosin and have been shown to detect different epitopes
on MyHC isoforms by Western blotting, competition ELISA,
and patterns of staining in human and rodent
muscle sections (Webster et al., 1988b, Condon et al.,
1990); further molecular characterization of the specific
MyHC isoforms they recognize and the genes encoding
them will be described elsewhere (M. Cho, S. M. H.,

In the rat posterior lower hindlimb, the four monoclonal
antibodies distinguish eight different muscle fiber types,
designated A–H based on the combinations of MyHC epi-
topes they contain (Table 1). For example, in Figure 2,
adjacent regions of the soleus, plantaris, and lateral gas-
trocnemius muscles can be seen to contain diverse fiber
types that differ in MyHC composition. Some fiber types,
such as B fibers, are present in several muscles, whereas
others, such as E fibers, are restricted in their distribution.
E fibers are only present in the deep region of lateral
гastrocnemius. Some of the fiber types characterized by
MyHC content correspond to fiber types that have been
shown physiologically to contract in a particular manner.
For example, antibody 4A.74 reacts with a MyHC epitope
present in fibers in rat plantaris that also react with antibod-
ies N3.36 and 4A.1519 but not 4A.840 (fibers) and are likely
to correspond to classical type IIb fibers, a subset of fast
fibers that are intermediate in contraction speed and
fatigue resistance. Other fiber types, characterized by the
combination of MyHC epitopes they contain, do not appear
to correspond to previously described fiber types, for ex-
ample the 4A.840, 4A.74, 4A.1519, N3.36 (type D) fi-
bers of the deep lateral gastrocnemius. In some cases, fibers
containing mixtures of MyHC epitopes may repre-
sent transient developmental stages (Laframboise et al.,
1991). However, the fiber types in Table 1 can all be de-
tected in the adult, and therefore presumably are required
to perform distinct physiological functions. Complex com-
binations of MyHC expression have been detected pre-
viously in muscle spindle fibers (Pedrosa et al., 1989), but
the extent of the diversity we detect in regions of some
muscles has not previously been reported. Thus, our
MyHC antibodies permit an analysis of myoblast fate be-
yond the simple fast/slow dichotomy, allowing us to deter-
mine whether myoblasts are restricted to subsets of fiber
types within these broad classes.

| Table 1. Summary of MyHC Epitope Combinations Found in
Muscle Fibers of the Postnatal Rat Lower Hindlimb |
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<td>Fiber Type</td>
<td>Monoclonal Antibody</td>
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<td>B</td>
<td>C</td>
<td>D</td>
<td>E</td>
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<tr>
<td>4A.74</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4A.1519</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4A.840</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>N3.36</td>
<td>+</td>
<td>+</td>
<td>+</td>
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Serial adjacent sections of adult rat lower hindlimbs were analyzed
immunohistochemically by reaction with each of four monoclonal anti-
bodies that recognize different epitopes on MyHC isoforms. The same
fibers were identified in each section and their MyHC epitope profile
determined by visual inspection of whole fascicles of 10 to 30 fibers.
Fascicles selected for analysis represented all muscles of the lateral
and posterior regions of the rat lower hindlimb. Other fiber types may
exist at low frequencies in other muscles or developmental stages.
Some fiber types shown here appear to correspond to the classical
fiber types designated by ATPase activity: A and G correspond to
classical type I fibers, B corresponds to type IIb fibers, and C to type I.
Other fiber types have not previously been described.

Clones Are Not Restricted to Contribute to Subsets
of Fiber Types

We observed that single clones of myoblasts contributed to
fibers with many different combinations of MyHC (Figure
3). Serial transverse sections were analyzed for the pre-

ence of β-gal-labeled fibers and were reacted with each of
the four antibodies to MyHC. As shown in Figure 3A, β-gal
Figure 2. Eight Fiber Types Are Distinguished by Monoclonal Antibodies to Myosin Heavy Chain Isoforms.

Fiber type diversity of rat lower hindlimb is shown in four serial 30 µm sections at postnatal day 37 (P37) analyzed following reaction with monoclonal antibodies 4A.74 (A), 4A.1519 (B), 4A.840 (C), and N3.36 (D), as described in Figure 1. Inset illustrates the borders of the three muscles included in each section: deep red strip of lateral gastrocnemius (lg), soleus (sol), and plantaris (pln) at the mid level of the rat lower hindlimb. Note that fiber distribution not only differs among muscles but among regions of a single muscle. Examples of MyHC fiber types A, B, C, D, E, G, and H are indicated by arrows and have MyHC compositions as described in Table 1. Fiber type F was not present in this leg region. Bar, 100 µm.
A clone generated by infection of a single myoblast with MMuLV/Sin5LacZ at P15 and analyzed at P37. Three fibers in the deep lateral gastrocnemius (arrows X, Y, and Z) have received cells from the clone and contain β-gal-positive nuclei (A). Nearby unfixed frozen sections in the same region, schematized in (C), were histochemically stained for both β-gal activity and reactivity with antibodies 4A.840 (B), 4A.1519 (D), N3.36 (E), or 4A.74 (F). Each fiber has a distinct MyHC phenotype. Fiber X is a type A fast fiber (only N3.36' in Table I), fiber Y is a type B fiber (N3.36-, 4A.74', and 4A.1519' in Table I), and fiber Z is a type C fiber (4A.840' in Table I). Bar, 25 µm.

Activity is best revealed in fixed sections: three fibers in the deep region of the lateral gastrocnemius muscle are shown, into which the retrovirally labeled progeny of a clone had fused. These fibers are shown schematically in Figure 3C to facilitate identification of the same fibers in serial sections. Figures 3B, 3D, 3E, and 3F show four nearby sections that were not fixed to permit optimal visualization of MyHCs by immunohistochemistry; the same clone can still be identified in these unfixed sections by weak nuclear β-gal staining. Figure 3B shows that, like the clone in Figure 1, this clone contributed to fibers that do (fiber Z) and do not (fibers X and Y) contain slow MyHC. Figures 3D, 3E, and 3F show that fiber Z contains only slow MyHC, since it does not react with any of the other antibodies, all of which recognize epitopes on fast MyHC isoforms. Fibers X and Y both contain fast class MyHC, as they react with antibody N3.36 (Figure 3E); they are not identical, however, as fiber Y contains epitopes detected by both 4A.74 and 4A.1519 that are not present in fiber X. Because 4A.74 reacts specifically with type Ila fibers defined previously by ATPase histochemistry (Webster et al., 1988b), fiber Y is likely to correspond to a classical type Ila fast fiber. Thus, the clone in Figure 3 has contributed myoblasts to three distinct fiber types: A, B, and C in Table
1, also known as fast type IIb or IIx, fast type IIa, and slow type I, respectively. We conclude that at least some myoblasts give rise to progeny that can fuse with numerous fiber types in the course of normal postnatal muscle development.

These data do not rule out the possibility that individual myoblast clones are restricted, or specialized to fuse with subsets of the full spectrum of fiber types. To address this possibility, we analyzed the frequency with which all possible pairs of MyHC fiber types, classified A–H based on the combination of the four MyHC epitopes they contain (Table 1), were found together in a duetor of β-gal-labeled fibers. Table 2 shows that almost all pairwise combinations of fiber types were detected within clonal clusters, making it highly unlikely that the fusion of myoblasts is restricted to particular subsets of fiber types. Indeed, the frequency with which each pairing was observed generally correlated with the frequency expected if fiber types were randomly paired. Thus, although several pairs of fiber types were not observed within a single clone, most of these "missing" pairs would not have been expected, given the low frequency of the fiber types in question. However we also failed to observe pairwise combinations of some common fiber types, such as C and G, in the same cluster. Although at first sight this observation suggests a restriction of clones either to "C-type" or "G-type" fiber clusters, it can be accounted for by the nonrandom distribution of MyHC fiber types within the leg (see Figure 2). C fibers, which are the slowest contracting fibers, occur mainly in the soleus, whereas G fibers, which are among the fastest contracting fibers, occur only in other muscles; only in the small "red strip" of the lateral gastrocnemius do both fiber types C and G occur in the same muscle region (see Figure 2). Thus, the chance of a clone contributing to both of these two fiber types is extremely low. Similarly, the apparent excess of identical pairs of fibers can be accounted for by the frequent presence of clones in regions containing only a single fiber type. Overall, our data suggest that myoblasts are not restricted to fuse with any particular subset of fiber types in their environment.

**Clones Show No Detectable Preference for Fusion to Particular Fiber Types**

Although the studies described above suggested that clones were not restricted, they did not determine whether clones exhibited a preference for particular fiber types. To examine this possibility, we compared the MyHC profile of the fibers to which the clone had contributed with the fibers in the area around each clone. Sections were reacted with each MyHC antibody, and 100–150 fibers located symmetrically around the clone were evaluated (Figure 4). In most cases, clones present in an area of mixed fiber types contributed to a mixture of fiber types (e.g., Figure 4A, clone I). For each MyHC epitope, clones fell on a diagonal, indicating that the percentage of fibers in a β-gal-positive cluster that expressed a particular MyHC was typical of the environment in which the clone was found. Occasionally, a clone contributed to a set of similarly staining fibers in a region that contained a mixture of fiber types. For example, in Figure 4A, 100% of clone II fibers were 4A.840-positive in a region in which 80% of surrounding fibers were also 4A.840-positive. Since there were only five β-gal '4A.840' fibers within the clone, 5 out of 5 (100%) 4A.840-positive is not statistically different from the expected 4 out of 5 (80%) value based on surrounding fiber phenotypes. Such homogeneous clones could either reflect the labeling of a myoblast clone committed to fusion with a particular set of fibers, or a chance event resulting from the fusion of the progeny of the clone with a small number of fibers that happened to be similar, as predicted by binomial probability theory. Three lines of evidence suggest that the latter explanation is correct.

First, homogeneous clones in regions containing a mixture of fiber types were generally small (Figure 4). Moreover, most large clones that were homogeneous for expression of one MyHC epitope were heterogeneous for expression

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**Table 2. Clones Are Not Restricted and Can Contain Most Pairwise Combinations of Fiber Types**

<table>
<thead>
<tr>
<th>Fiber Types</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
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<tr>
<td>A</td>
<td>18 (3)</td>
<td>10 (7)</td>
<td>1 (11)</td>
<td>4 (2)</td>
<td>0 (1)</td>
<td>4 (2)</td>
<td>5 (21)</td>
</tr>
<tr>
<td>B</td>
<td>8 (4)</td>
<td>5 (11)</td>
<td>2 (2)</td>
<td>2 (1)</td>
<td>3 (3)</td>
<td>13 (22)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>27 (8)</td>
<td>10 (3)</td>
<td>4 (2)</td>
<td>0 (1)</td>
<td>0 (1)</td>
<td>0 (5)</td>
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<tr>
<td>D</td>
<td>0 (0)</td>
<td>2 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (1)</td>
<td>4 (7)</td>
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<td>E</td>
<td></td>
<td></td>
<td>1 (0)</td>
<td>0 (0)</td>
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<td>F</td>
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<td></td>
<td>1 (1)</td>
<td>4 (7)</td>
<td>62 (31)</td>
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<td>G</td>
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Total number of fibers of each type 14 15 22 4 2 5 43

Summary of frequency of pairs of fibers in clusters to which a single myoblast clone contributed. The number of times each fiber type pair occurred in each clone was determined by including all possible pairings. Thus, in clones containing two, three, and four fibers, one, three, and six pairs were counted, respectively. Therefore, due to the variation in clone sizes, the total number of pairs analyzed was 188. The observed frequency was determined by summing the number of pairs of fibers of each type from all clones. For example, the 14 A fibers were distributed among seven clones with 2, 2, 4, 5, 5, 6, and 6 total fibers of which 2, 2, 1, 1, 4, and 5 were A type, respectively (14 total). These contained 1, 1, 0, 0, 6, and 10 AA pairs, respectively (18 total). The expected frequency (parentheses) of each pairing was calculated based on the binomial probability of selecting each pair when 188 pairs were randomly selected from the pool of 105 total fibers of the types indicated below the table. For example, if 188 pairs were selected at random from a pool of 105 fibers of which 14 are type A and 15 type B, the expected number of times one A and one B would be selected in the same pair would be seven (7). The antibody profile of each fiber type (A-G) is shown in Table 1.
Figure 4. Myoblast Clones Labeled In Situ Contribute to All Fiber Types in Their Environment
The proportion of fibers expressing each MyHC epitope in a clone reflects the proportion in its neighboring fibers, or muscle environment. Myoblast clones labeled in vivo with MMuLVV513LacZ at P15 were analyzed at P37. The total of 56 clones analyzed is shown here. Clone locations and sizes were determined within legs by staining alternate 30 μm sections for β-gal after fixation. In the remaining sections, the fibers to which a clone had contributed were stained for both β-gal and for MyHC composition by peroxidase immunohistochemistry with monoclonal antibodies 4A.640 (A), N3.36 (B), 4A.1519 (C), and 4A.74 (D). Each column represents a single clone. The height (y) of each column represents the total number of fibers in a single clone with a percentage (x) of MyHC positive fibers in an area of a muscle in which a percentage (y) of the surrounding fibers were MyHC-positive. Six clones, labeled I–VI, are marked in each panel to facilitate comparison.

of other epitopes (e.g., Figures 4B, 4C, and 4D, clone II).
The third piece of evidence comes from a statistical analysis. We modeled our data assuming that all clones had a certain preference to fuse with fibers containing a particular MyHC epitope. We then determined what the limits on that preference could be, given the data for the four antibodies in each panel (A–D) of Figure 4. Although a small preference of all myoblasts or a small subpopulation of restricted myoblast clones could not be ruled out, the probability was low (P < 0.05). In summary, this analysis suggests that the progeny of a clonal founder myoblast contribute randomly to fibers in the environment of the clone.

Implanted Myoblasts Have Restricted Potential In Vitro but Not In Vivo
Although the results described above suggested that myoblast clones do not select fibers with which to fuse from their immediate environment, the possibility remained that because of the apparently limited extent of their migration, they were never exposed to fiber types that occurred in other muscles and regions of the leg. It therefore seemed possible that the marked differences between one muscle region and another could arise by the segregation into each region of myoblasts committed to generate precisely the range of fiber types present in that region. To test the hypothesis that myoblasts are homogeneous within a region but differ among regions in their intrinsic differentiative capacity, a clone of primary myogenic cells was derived from adult mouse leg. This clone was genetically marked in vitro by infection with the cytoplasmic β-gal-expressing BAG retrovirus, selected in G418, and then returned to various muscles in the legs of day P28-P35 mice by injection. The injected cells contributed to fibers in many muscles, including lateral gastrocnemius (Figure 5A), soleus (Figure 5D), plantaris, peroneus longus, extensor digitorum longus, and tibialis anterior. Three to ten days after injection, labeled myoblasts had clearly fused into fibers as demonstrated by β-gal staining in the fiber cytoplasm in serial transverse sections. No difference in the rate, efficiency or extent of fusion was detectable in the different muscles. Although some cells fused with one
another to form aberrant small myotubes at early times after injection (data not shown), many of the β-gal-labeled fibers were of a mature fiber diameter. It is therefore likely that these large labeled fibers were endogenous preexisting muscle fibers into which injected labeled myogenic cells had fused. Several weeks after injection, the small aberrant myotubes were generally not present, possibly due to death of these uninnervated cells, and the tissue had resumed a normal morphology with many β-gal-labeled fibers near the site of injection (Figures 5A and 5D). Thus a single clone of myoblasts is able to contribute to fibers in muscles with radically different physiology and fiber type composition.

We determined that the primary cells, an adult leg myoblast clone, had restricted potential in tissue culture. When allowed to differentiate into myotubes, they expressed MyHCs reactive with N3.36, 4A.1519, and 4A.040, but not 4A.74 (data not shown). Yet, when injected into either lateral gastrocnemius (Figures 5A, 5B, and 5C) or soleus (Figures 5D, 5E, and 5F) muscles, these myoblasts contributed to fiber types that both did and did not express 4A.74 (Figures 5C and 5F). Similarly, the injected cells fused with fibers that expressed slow MyHC in the soleus (Figure 5E) as well as with fibers that did not express this type of MyHC in the lateral gastrocnemius (Figure 5B). The C2C12 established clonal myoblast cell line, like the primary cells, expresses a subset of both fast and slow MyHCs upon differentiation in vitro. An extensive analysis using all four antibodies to MyHC revealed that when either C2C12 cells or the primary mouse myoblasts were implanted, they contributed to all fiber types present in the leg (Table 3). For each MyHC isoform we could detect no significant preference for fusion to specific fibers. Thus, a clone of myoblasts derived from one site in the leg of an adult mouse can contribute to diverse fiber types in muscles and regions of contrasting MyHC fiber type profile and adaptive range. As with myoblast clones labeled in vivo, injected cells do not appear to have a preference for, or select, certain muscle fiber types with which to fuse.

To determine whether the injected cells were able in vivo
to continue to express the MyHCs typical of their "intrinsic program" of differentiation revealed in tissue culture, we examined gene expression in retrovirally marked cells after injection. In muscles such as the superficial lateral gastrocnemius, in which only fast MyHCs were expressed, we were unable to detect any slow MyHC expression in the large endogenous fibers into which the slow MyHC-expressing myoblasts fused (e.g., Figure 5B). Slow MyHC was not detected in any of the 350 β-gal-positive fibers analyzed in Table 3. Furthermore, when serial sections through the entire β-gal-positive region of the fibers were analyzed, mosaic fibers with slow MyHC were never observed in muscles that normally contained exclusively fast fibers. This contrasted sharply with the small aberrant fibers formed by the same injected cells shortly after injection, in which slow MyHC was abundantly expressed (data not shown). Similar results were obtained when we assayed for the expression of fast MyHCs in the slow soleus muscle. These results suggest that myoblasts in postnatal mice can express their intrinsic program if they differentiate alone, but this program is overridden upon fusion with a fiber that expresses a different phenotype.

Discussion

Muscle Fiber Pattern in Postnatal Development Is Independent of Cell Lineage

Cells can differentiate according to their lineage or in response to their environment. Here we examined the role of these two mechanisms in the course of muscle development in the postnatal rodent. Specifically, we asked whether the maintenance of a complex pattern of eight interspersed fiber types results from selective fusion of committed myoblast lineages to subsets of fiber types or involves nonselective fusion to all fiber types. We selected the postnatal period for study because the initial pattern of fiber types in limb musculature has been laid down at this point, yet growth by the addition of new myoblasts to preexisting fibers is occurring at a rapid pace, and muscle mass is increasing in excess of 10-fold. Moreover, our previous studies indicated that at this developmental stage, lineage analysis was possible, because satellite myoblasts could cross the basal lamina sheath that surrounds each fiber and contribute to multiple muscle fibers (Hughes and Blau, 1990). The results presented here show that myoblasts in situ or injected into muscle fuse nonselectively with all fiber types in their environment.

The random fusion of myoblasts with diverse fibers has profound implications for the generation and maintenance of muscle pattern in postnatal development. Myoblasts exhibit intrinsic commitment to the synthesis of particular MyHCs when they differentiate in vitro (Feldman and Stockdale, 1991, Miller and Stockdale, 1996a, Keyton, S. Webster, and H. M. B., unpublished data). However, this does not appear to be the case in vivo. Muscle fibers formed in vivo are mosaics with respect to the nuclei they
contain; the nuclei are of diverse origin. If myoblasts retained their intrinsic commitment after fusion with fibers, such myofibers would contain a patchwork of MyHC isoforms encoded by different nuclei. However, in hundreds of serially sectioned β-gal-positive fibers, no mosaic fibers were found (Figure 5; Table 3). This was not because mosaic fibers cannot be readily assayed. As we have shown previously, mosaic fibers arise upon muscle damage when fetal MyHC is reexpressed (Webster et al., 1988b). Mosaic fibers also arise when patterns of innervation are altered (Salviati et al., 1986). Therefore, when it occurs, mosaicism is readily detectable, because MyHCs do not diffuse but are localized in nuclear domains (Pavlath et al., 1989).

Thus, the data presented here show that during postnatal development, the expression of the intrinsic program of the myoblast is overridden upon fusion with preexisting fibers. It appears that environment, not lineage, specifies how myoblasts differentiate during postnatal growth and development.

**Myoblasts Are Committed but Still Plastic**

There is ample evidence that undifferentiated myoblasts are committed to express a subset of the myogenic repertoire of contractile protein genes. Clonal analyses of the progeny of single cells in vitro reveals that myoblasts differ in their expression of MyHC isoforms upon differentiation and that those differences are heritable (Feldman and Stockdale, 1991; Miller and Stockdale, 1986a; M. Cho, S. Webster, and H. M. B., unpublished data). Moreover, myoblasts transplanted to new muscle sites in vivo continue to express a program characteristic of their muscle of origin (Hoh and Hughes, 1988). However, in both of these types of experiment, the environment has been destroyed and fibers are formed de novo. These experiments suggest that myoblast heterogeneity in the postnatal animal may serve to regenerate fiber type diversity in the event of total fiber loss. Evidence that myoblasts are committed was also provided by our own studies, but this commitment could be overridden. When implanted myoblasts fused to one another to form new small diameter fibers, they expressed MyHCs typical of their differentiation in vitro (data not shown). By contrast, myoblasts that fused into large diameter preexisting multinucleated muscle fibers, in a manner typical of normal growth and development, expressed myosins characteristic of the host fiber. This was clear from the finding that myoblasts that expressed slow MyHCs upon differentiation in tissue culture did not express detectable levels of these isoforms following fusion with fast fibers in a region of muscle containing no slow fibers in vivo. Moreover, the proportion of each fiber type in regions of retrovirally labeled fibers was similar to that in adjacent regions of unlabeled fibers. This was true either when myoblast clones were labeled in situ by direct retroviral infection or when clones were labeled in vitro and then injected en masse into diverse muscles. Thus, in their normal environment, committed myoblasts in postnatal limbs are plastic and do not express their intrinsic MyHC program.

**Genetically Engineered Myoblasts Stably Contribute to Fibers: Clinical Implications**

The plasticity of postnatal myoblasts may prove useful therapeutically. The results reported here suggest that myoblasts could serve as a vehicle for recombinant gene delivery for the treatment of inherited and acquired diseases. A gene encoding β-gal was introduced into both the C2C12 myoblast line and a clone of primary mouse myoblasts, and the cells were returned to postnatal mouse muscle tissue by injection. The genetically engineered myoblasts fused with endogenous muscle fibers within 5 days (data not shown) and assumed the pattern of MyHC gene expression of the host fiber while maintaining transgene expression for several weeks. In other experiments, we have observed stable, high level retroviral β-gal expression in muscle fibers for more than 6 months (data not shown). Such long-term gene expression has not been seen with most other genetically engineered cell types following injection in vivo (for review, see Miller, 1990).

Our experiments suggest several advantages of muscle over other tissues as a vehicle for gene therapy. Large quantities of myoblasts can be grown from a small biopsy (Webster et al., 1988a). Myoblasts, in contrast with most cell types, can cross basal lamina and readily disseminate within the tissue (Hughes and Blau, 1990). Myoblasts can contribute to all types of preexisting fibers, and after fusion, myoblasts become irreversibly postmitotic, yet remain viable within fibers for years, minimizing the potential for tumorigenesis. Moreover, myoblast implantation into nonvital muscles can be safely reversed. Finally, muscle is well vascularized, allowing access of secreted gene products to the blood (Dhawan et al., 1991). These considerations suggest that genetically modified myoblasts may provide a convenient, stable vehicle for delivery of nonmuscle as well as muscle products.

**Fiber Environment Controls Myoblast Gene Expression**

Myoblasts appear to be instructed in their gene expression either by the extracellular microenvironment surrounding, or by intracellular trans-acting factors within the fiber to which they fuse. The latter possibility seems more likely. Previous studies showed that gene expression could be changed in heterokaryons formed by fusing nonmuscle cells with muscle in tissue culture (Blau et al., 1985). The present studies suggest that trans-acting factors may also change the phenotype of a committed myoblast when a heterokaryon forms naturally in vivo. Candidate trans-acting factors include the myogenic regulators MyoD, myogenin, myf5, and MRF4 (for reviews see Olson, 1990; Weintraub et al., 1991), which regulate their own expression. In addition to their presumed function in the commitment of somitic cells to myogenesis (Sassoon et al., 1989), these regulators may play another role in later development. Indeed, their continued low level expression in the adult (Eftimie et al., 1991), the presence of their consensus binding sites in most muscle structural genes (Olson, 1990), and their differential expression in muscles with
different fiber composition (Rhodes and Konieczny, 1989) make them ideal candidates for instructing fibers in the set of MyHCs they should express.

How is fiber type maintained? Clearly, extrinsic influences such as innervation are involved. Changes in innervation or electrical activity can modulate fiber type by inducing the expression of MyHC isoforms with different ATPase activity (Buller et al., 1960; Pette and Rovbva, 1995). However, nerves cannot induce complete interconversion of all fiber types. Fibers intrinsically fall into one of several classes, or "adaptive ranges," and each class limits the range of influence of neurons (Westgaard and Lomo, 1988; Gundersen et al., 1988; Hoh and Hughes, 1988). MyOD and myogenin levels are also modulated by innervation (Ettline et al., 1991), which might suggest that these proteins provide a molecular focus for the interaction between intrinsic and extrinsic influences on fiber type. Thus, fiber type appears to be maintained by a program intrinsic to fibers, some aspects of which can be modulated by extrinsic factors, such as neuronal stimulation and hormones.

Muscle Fiber Pattern in Early Development May Be Specified by Lineage

Although our data argue strongly that myoblast lineages in the postnatal rodent are not responsible for the maintenance of fiber type pattern, they do not rule out the possibility suggested by Schaefer et al. (1987) that lineages play a role in the establishment of that pattern. The distribution of distinct myoblast populations could specify the position of diverse fiber types. This distribution could be achieved by targeted migration of myoblast lineages from the somites to particular regions within the limb. On the other hand, local influences could herald a previously homogeneous population of myoblasts. If lineage does provide the basis for muscle patterning, the first myoblast to find a new fiber could determine its phenotype and then instruct other myoblasts as they fuse, as occurs in postnatal development. Alternatively, myoblasts in embryonic development could fuse selectively with myoblasts committed to a similar fiber type.

Spatial cues within the limb, perhaps in the form of gradients of homeobox proteins, retinooids, or polypeptide growth factors, must be responsible for generating the pattern (De Robertis et al., 1991), but how these early signals are translated into the complex fiber type array is unclear. That temporal cues also play a role is suggested by the finding that primary and secondary muscle fibers with distinct MyHC isoforms arise at different times in development, when distinct myoblast precursors are present (Bonner and Hauschka, 1974; Miller and Stockdale, 1986; Kelly and Rubinstein, 1986; Consoli et al., 1988).

Neurons are unlikely to be involved in establishing the initial pattern, since this pattern can form in their absence (Butler et al., 1982; Harris et al., 1989; Condon et al., 1990). Indeed, it is probable that either the muscle instructs the nerve with respect to its fast/slow phenotype or that specific fiber types are matched with specific neuronal types in the early phases of muscle development. To reveal the relative roles of lineage and environment in establishing muscle fiber pattern, retrovirial marking experiments, like those described here in postnatal life, must be performed in embryonic development.

Experimental Procedures

Clonal Analysis

MMuLV/SV tNLS LacZ retrovirus was obtained from Du Bucce's modified Eagle's medium plus 10% defined supplemented calf serum conditioned overnight by confluent ω-12-c3 cells, concentrated by 6 hr of centrifugation at 16,000 rpm in an SW27 rotor at 4°C, and resuspended by repeated titration in 400 μl of phosphate buffered saline plus 25 μg/ml polymyxin. Finely ground charcoal was added to mark the injection site and 60 μl of suspension containing about 5 x 10^6 infective particles was immediately injected using a 27 gauge needle into the legs of anesthetized postnatal day 14 or 15 rats. On postnatal day 37 or 38, injected hindlimbs and un.injected controls were dissected from the animal, the tibia removed, and frozen in freezing isopentane for subsequent cryostat sectioning at 30 μm. Alternate serial sections were collected onto two sets of gelatin-coated slides. Charcoal was generally observed between soleus, lateral gastrocnemius, and peroneus longus muscles, suggesting that most of the retroviral particles were injected between muscles and that muscle tissue was not extensively damaged by the injection procedure. No signs of regeneration, such as small, fetal MyHC-positive or centrally nucleated fibers, were observed. Retroviruses do not infect postmitotic cells (Varmuza et al., 1977). Therefore, labeled muscle fibers, which expressed β-gal in nuclei in several serial sections, must have arisen by the fusion of a retrovirally marked myoblast. Clones were identified as regions containing one or more labeled fibers separated from all other labeled fibers by at least 10 fiber diameters. At the injection levels used (around 30 labeled regions per leg), our previous studies have shown that few, if any, clusters of labeled fibers represent infections by more than one retroviral vector (Hughes and Blau, 1990).

Histology

Clones were initially visualized by staining alternate serial sections for β-gal. One set of sections was fixed for 4 hr at 4°C in 4% paraformaldehyde, 0.25% glutaraldehyde, 100 mM NaH₂PO₄, (pH 7.4), to prevent loss of β-gal, and then washed in phosphate-buffered saline (PBS) and incubated overnight at 37°C in 1 mM 5-bromo-4-chloro-3-indolyl β-p-galactosidase (X-Gal), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl₂ in PBS. Sections were washed in PBS, mounted in gelvatol, and photographed using Nomarski optics on a Zeiss Axioptot microscope. The number, fiber composition, and location of clones was determined by inspection of all serial sections and a "clone map" was created for each leg. No differences in the size of clones were detected based on position in the limb.

Immunocytochemistry

Unfixed 10–30 μm cryostat sections were stained to permit antibody reaction with native MyHC. Sections were incubated with unlabeled hybridoma tissue culture supernatant for 1 hr at room temperature, followed by biotin-conjugated class-specific anti-mouse IgG (Vector) diluted in PBS with 0.2% Tween 20, followed by avidin–biotin complex horseradish peroxidase (Vectorstain ABC kit) and visualized using 1 mg/ml diaminobenzidine, 0.035% H₂O₂ (enhanced with 0.02% CoCl₂) on occasion (Hsu and Soban, 1982) in 50 mM Tris-HCl (pH 7.2). Double staining for MyHC and β-gal was performed by first X-Gal stained unfixed sections overnight at 37°C, washing in PBS, and then staining for MyHC as described above. The unfixed tissue showed significantly weaker X-Gal staining than sections prefixed with paraformaldehyde, but sufficient to identify the location of the clone. Four MyHC epitope-specific monoclonal antibodies were used in this study, all of which recognize the 220 kd myosin band on Western blots. 4A 840 (Webster et al., 1988b) recognizes slow fibers and the in vitro expression product of the cloned slow MyHC gene and slow MyHC from tissue in immu-
Implantation of Retrovirally Marked Myoblasts
C2neoBAC myoblasts were produced by double labeling C2C12 cells with both cyt-β-gal and nls-β-gal-expressing retroviruses. For analysis in vivo, the cells were injected into synergistic Nestin C3H mouse thymi.

Acknowledgments
We are grateful to Marilyn Travis for help in the production of C2neoBAC clones. Lydia Pan for cloned mouse primary myoblasts, to Drs. Richard Mulligan for VcmCAGneo and Jean-François Nicolas for Vcm-12 retroviral producer cells, and to Brad Efron for help with statistics. Drs. Frank Stockdale, Patricia Salinas, Phillipa Webster, and the members of our laboratory made helpful comments on the manuscript. This work was supported by a Lucille P. Markey Visiting Fellowship to S. M. H. and grants from the National Institutes of Health (HD-18179 and AG-09521), the Muscular Dystrophy Association, and the March of Dimes Birth Defects Foundation to H. M. B.

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Received September 10, 1991; revised December 9, 1991.

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