Myogenin Is Required for Late but Not Early Aspects of Myogenesis during Mouse Development

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Abstract. Mice with a targeted mutation in the myogenic basic helix-loop-helix regulatory protein myogenin have severe muscle defects resulting in perinatal death. In this report, the effect of myogenin's absence on embryonic and fetal development is investigated. The initial events of somite differentiation occurred normally in the myogenin-mutant embryos. During primary myogenesis, muscle masses in mutant embryos developed simultaneously with control siblings, although muscle differentiation within the mutant muscle masses was delayed. More dramatic effects were observed when secondary myofibers form. During this time, very little muscle formation took place in the mutants, suggesting that the absence of myogenin affected secondary myogenesis more severely than primary myogenesis. Monitoring mutant neonates with fiber type–specific myosin isoforms indicated that different fiber types were present in the residual muscle. No evidence was found to indicate that myogenin was required for the formation of muscle in one region of the embryo and not another. The expression patterns of a MyoD-*lacZ* transgene in myogenin-mutant embryos demonstrated that myogenin was not essential for the activation of the MyoD gene. Together, these results indicate that late stages of embryogenesis are more dependent on myogenin than early stages, and that myogenin is not required for the initial aspects of myogenesis, including myotome formation and the appearance of myoblasts.

SKELETAL muscle in vertebrates originates from somitic mesoderm as pluripotent mesodermal cells become committed to a myogenic fate. Committed myoblasts populate areas throughout the developing embryo, ultimately differentiating into bundles of multinucleate myofibers. Four key players in myogenic events are the basic helix-loop-helix (bHLH)[1] regulatory proteins: MyoD, Myf5, myogenin, and MRF4 (for recent reviews see Emerson, 1993; Weintraub, 1993; Olson and Klein, 1994). These muscle-specific transcription factors are individually able to initiate the entire muscle differentiation program when introduced into tissue culture cells of nonmuscle origin. Using gene-knockout technology, several laboratories have created mice lacking functional myogenic bHLH factors and are now providing useful models for studying skeletal muscle development (Braun et al., 1992; Rudnicki et al., 1992; 1993; Hasty et al., 1993; Nabeshima et al., 1993). To date, null mutations in three of the four myogenic bHLH factors (MyoD, Myf5, and myogenin) have been analyzed.

Null mutations in MyoD or Myf5 result in no observable muscle abnormalities, though unexpectedly, Myf5 mutants are missing the distal portions of their ribs (Braun et al., 1992; Rudnicki et al., 1992). Because studies with tissue culture cells strongly implicate MyoD and Myf5 as important regulators of muscle development, the results suggest functional overlap among the myogenic factors. Indeed, cross-breeding MyoD- and Myf5-mutant mice to create double mutants has demonstrated that MyoD and Myf5 can compensate for each other (Rudnicki et al., 1993). Mutations in both MyoD and Myf5 prove to be lethal at birth and produce a strong muscle phenotype. These mice lack skeletal muscle and do not have discernable myoblasts. Thus, the MyoD/Myf5-mutant mice are unable to initiate myogenesis.

Myogenin-mutant mice are born with severe muscle deficiency, although less severe than MyoD/Myf5-mutant mice, and they die within minutes of birth (Hasty et al., 1993; Nabeshima et al., 1993). All regions that are normally populated by multinucleate muscle fibers instead contain large numbers of mononucleate cells and a reduced number of myofibers. The residual myofibers have an apparent normal morphology, indicating that some myogenic differentiation occurs. Although myogenin is normally expressed be-

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1. *Abbreviations used in this paper*: bHLH, basic helix-loop-helix; MHC, myosin heavy chain; PBS-BSA, 0.2% BSA in PBS; RT-PCR, reverse transcription PCR.

fore MyoD in the embryo, myogenin-mutant neonates have normal levels of MyoD transcripts, suggesting that myogenin is not required for MyoD expression. Surprisingly, while the presence of MyoD in mutant mice is not sufficient for myoblast differentiation, myogenin-mutant myoblasts differentiate into seemingly normal myotubes when cultured in vitro (Nabeshima et al., 1993; Rawls, A., D. Edmondson, and E. N. Olson, unpublished results). The myogenin-mutant mice provide direct evidence for myogenin's essential role in skeletal muscle development in vivo but not in vitro.

Comparisons of myogenin-mutant mice with MyoD/Myf5 mutants suggest a genetic hierarchy whereby MyoD and Myf5 act upstream of myogenin and are likely to have redundant functions associated with early events of myoblast formation (Weintraub, 1993; Rudnicki et al., 1993; Olson and Klein, 1994). Myogenin is probably involved in the subsequent downstream events required for differentiation of myoblasts into myotubes. MRF4 may function similarly to myogenin or may lie further downstream in the pathway to control later events associated with myofiber formation.

Our initial characterization of the myogenin-mutant mice was based almost entirely on the phenotype of mutant neonates, and it did not directly address the muscle-forming ability of these mice during embryogenesis. In wild-type mice, myogenin transcripts are expressed in the myotome on day 8.5, ~0.5 d after Myf5 and 2 d before MyoD (Sassoon, 1993; Smith et al., 1993). It is not known if the absence of myogenin affects the formation of the myotome, the somitic compartment responsible for producing myoblasts, or if the initial expression of the bHLH factors is altered.

Myogenesis occurs as two sequential waves, a primary wave beginning on about day 12.5 and a secondary wave beginning on about day 15.5 (Kelly, 1983). It is possible that the bHLH myogenic factors have different roles in these processes. Similarly, these factors may be associated with fiber-type specialization, which occurs during fetal development (Buckingham, 1992). In an earlier report, we speculated that the residual myofibers observed in myogenin-mutant mice may correspond to a particular fiber type, and that myogenin is essential for certain fiber types but not others.

Here, we describe the embryonic phenotype of the myogenin-mutant mice. We show that the initial events of somitic differentiation are normal in the mutants, and that while muscle masses in developing mutant and wild-type embryos develop simultaneously, cells in the masses do not express muscle-specific markers at the same time and to the same extent. Moreover, the organization of tissue in the developing muscle masses is distinct in mutant and wild-type embryos with fewer differentiated myocytes in the mutants. The major differences between mutant and wild-type embryos becomes apparent during the time when secondary myofibers are forming, suggesting that the absence of myogenin may affect secondary myogenesis more severely than primary myogenesis. We find no evidence that the residual myofibers formed in the mutants correspond to a particular myofiber type or that myogenin is required for the formation of muscle in a particular region of the embryo. Finally, we show that a MyoD-*lacZ* transgene present in a myogenin-mutant genetic background is activated normally in myotomes on embryonic day 10.5, and that MyoD is expressed normally throughout development.

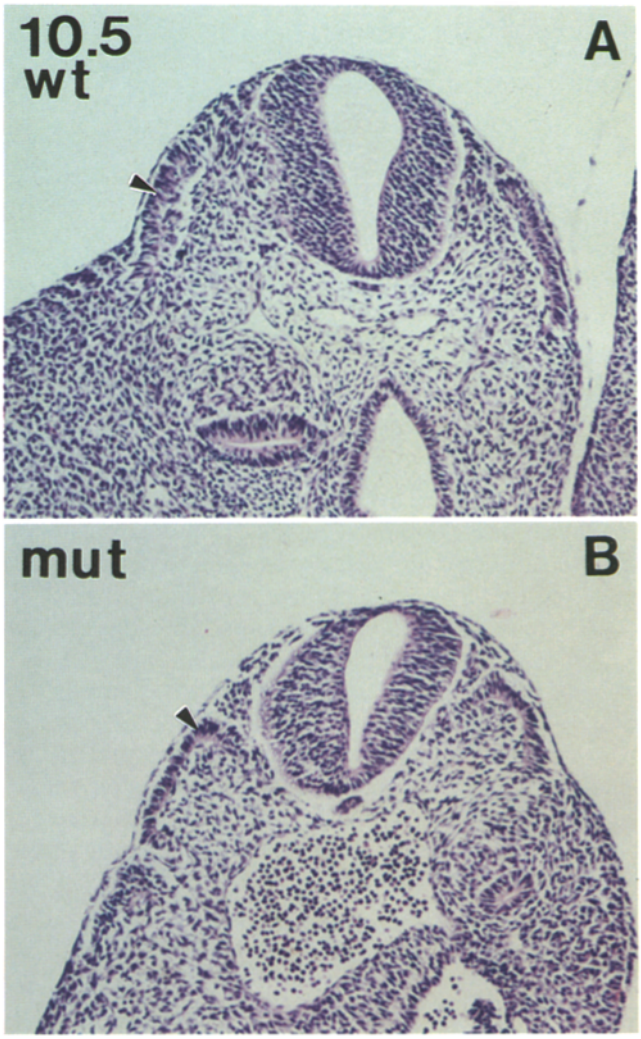


Figure 1. Somite and early muscle development in myogenin-mutant embryos. The differentiation of somites into the dermatome, myotome, and sclerotome occurred on schedule and with normal morphology in both wild-type (*A*) and myogenin-mutant embryos (*B*) at 10.5 d postcoitum. The arrowhead marks the myotome compartment; above and below the myotome are the dermatome and sclerotome compartments, respectively. At 12.5 (*C* and *D*) and 14.5 d (*E* and *F*), embryos show comparable morphology over muscle forming regions lateral to the neural tube (*arrow*). At 18.5 d (*G* and *H*), differences are observed in corresponding muscle regions between wild-type and mutant embryos (*arrow*).

Materials and Methods

Animals and Genotyping of Embryos

Mice heterozygous for the myogenin-null mutation were generated as described in Hasty et al. (1993). Mice were genotyped by Southern blot hybridization of genomic DNA prepared from tails of neonates and yolk sacs of embryos using a 900-bp EcoRI/SstI fragment of mouse myogenin cDNA as probe and EcoRI-digested genomic DNA (Hasty et al., 1993). MyoD-*lacZ* transgenic mice, generated as described by Goldhamer et al. (1992), were obtained from Moshe Shani (The Volcani Center, Bet Dagan, Israel) and crossed with myogenin-mutant mice to generate mice that were doubly heterozygous for both the MyoD-*lacZ* transgene and myogenin. The double heterozygotes were then crossed to generate mice that were homozygous for the myogenin mutation and also carried the MyoD-*lacZ* transgene.

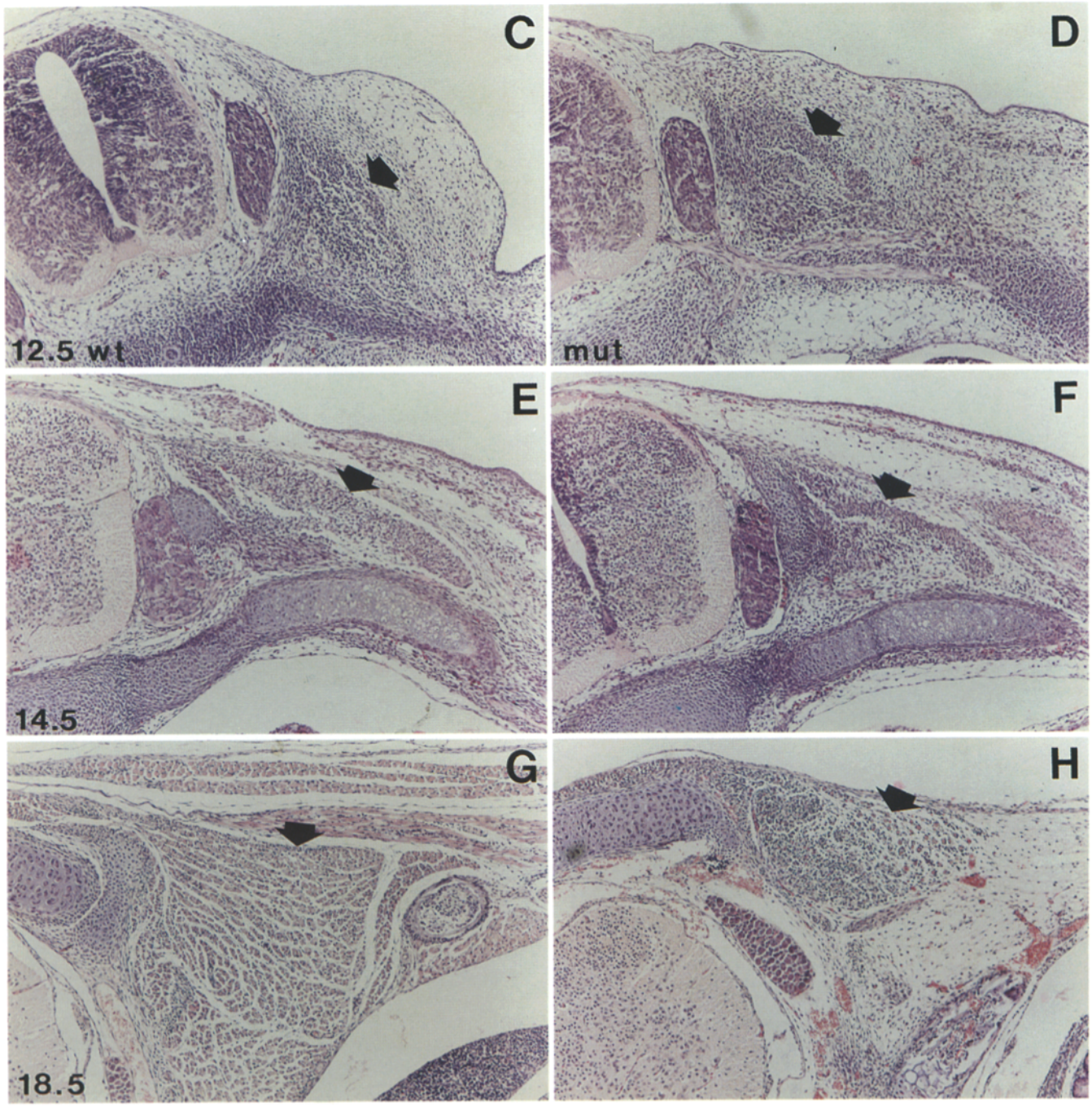


Figure 1.

Tissue Preparation

Embryos were fixed in 4% paraformaldehyde and embedded in paraffin for both indirect immunofluorescence and nucleic acid in situ hybridization analyses on sections through whole embryos (Lyons et al., 1990). Embryos older than 16 d of development and neonates were skinned to facilitate penetration and infiltration. Transverse sections were cut at 6 μm, and for each time point, parallel sections of mutant and control siblings from the same litter were placed on the same slide.

Frozen sections through hindlimbs were used to identify different myosin isoforms in neonates by indirect immunofluorescence. Hindlimbs from neonates were frozen rapidly in isopentane cooled to −60°C in liquid nitrogen and mounted for sectioning. Frozen sections were cut at 10 μm and stored at −10°C. Sections through approximately the same level in mutant and nonmutant hindlimbs were examined by indirect immunofluorescence for the expression of myosin heavy chain (MHC) isoforms.

Immunohistochemistry

For the analysis of MHC and desmin protein expression, sections through embryos were deparaffinized in two changes of 100% xylene for 10 min each. The xylene was then removed and sections gradually introduced to PBS by sequential 10-min incubation through 1:1 xylene/methanol, 100% methanol, 1:1 methanol/PBS, and finally 100% PBS. The sections were then blocked for a minimum of 30 min in 3% normal goat serum diluted in PBS. Incubations in both primary and secondary antibodies were for a minimum of 1 h, and the antibodies were washed from the sections after each antibody incubation by three 10-min washes with PBS. A monoclonal

antiskeletal fast MHC (MY-32) was diluted 1:600 in PBS, and a polyclonal antidesmin antibody was diluted 1:20 in PBS. Antibodies were purchased from Sigma Immunochemicals (St. Louis, MO). Secondary antibodies were FITC-conjugated goat anti-mouse (Sigma Immunochemicals) diluted 1:100 in PBS and tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit (Cappel-Organon-Teknika, West Chester, PA) diluted 1:200 in PBS. After the final washes, slides were mounted in FITC-Guard™ (Testog, Inc., Chicago, IL).

Indirect immunofluorescence of frozen sections with monoclonal antibodies to different MHC isoforms was used to compare fiber types in mutant and nonmutant neonate limbs (Condon et al., 1990). Essentially, slides of frozen sections were incubated in 0.2% BSA in PBS (PBS-BSA) for 30 min and then in primary antibody diluted in PBS-BSA overnight at 4°C in a humid chamber. The slides were then rinsed five times with ice-cold PBS, then washed three times for 5 min each in ice-cold PBS-BSA. Secondary incubations were for 1 h at 4°C in a humid chamber in the goat anti-mouse, FITC-conjugated antibody described above. The sections were rinsed and washed again (as described above) and mounted in FITC-Guard™. The primary antibodies used were monoclonal antibodies to slow (4A.840) and embryonic MHC (F1.652), as detailed by Condon et al. (1990), and antiskeletal fast MHC (MY-32).

In Situ Hybridization

In situ hybridization using muscle-specific markers was performed as described in Lyons et al. (1990). The MHCemb and MHCpn riboprobes were described in Lyons et al. (1990). MyoD sense and antisense riboprobes were transcribed from a full-length cDNA as described in Edmondson et al. (1994). 6-μm transverse sections through embryos at approximately the same anatomical level were positioned on slides, such that mutant and nonmutant siblings were exposed to identical conditions. Parallel slides labeled with sense riboprobes corresponding to each antisense probe showed no specific labeling above background.

Detection of Vascular Cell Adhesion Molecule-1 (VCAM-1) Transcripts by Reverse Transcription PCR (RT-PCR)

Total RNA was isolated from either fore- and hindlimb or carcass (internal organs and head removed) at different developmental stages from myogenin-mutant and heterozygous embryos as previously described (Hasty et al., 1993). 1 μg of RNA per reaction was used to synthesize cDNA using random primers and reverse transcriptase (Boehringer Mannheim Biochemicals, Indianapolis, IN) according to the manufacturer's specifications. One tenth of this reaction was then used for PCR using 100 ng of each of the primers described below, 1 μCi of [α^{32}P] dCTP, and 1.5 mM $MgCl_2$. PCR amplification conditions were 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for 23 cycles. This cycle number was chosen to keep the yield of the PCR product proportional to the mRNA abundance. For VCAM-1 amplification, the primers were ACACTTTTCCCAGACACTTTTCACG (nucleotides 1374–1398) and AGTGGGCTGTCTATCTGGGTTCTCC (nucleotides 2093–2117) (Cybulsky et al., 1993), giving a predicted band of 235 bp. One tenth of this reaction was then fractionated on a 6% polyacrylamide gel and visualized by autoradiography. To control for variation of RNA into each reaction, ribosomal protein L7 mRNA levels were analyzed using identical PCR conditions and previously described primers (Hollenberg et al., 1993).

X-Gal Staining

To observe expression pattern of the MyoD-*lacZ* transgene in the myogenin-mutant background, litters of embryos from matings between mice doubly heterozygous for the myogenin-null mutation and the MyoD-*lacZ* transgene were killed at different embryonic stages and stained as whole mounts for β-galactosidase activity (Cheng et al., 1993).

Results

Somite Development and Development of Muscle Masses in Myogenin-Mutant Embryos

Between days 8.5 and 10.5 in mouse development, somites compartmentalize into the dermatome, myotome, and sclerotome. We compared identical somitic regions from wild-type and myogenin-mutant embryos at 9.5 and 10.5 days, a time when the myogenin gene is first activated, and found no significant morphological differences. For example, the three regions of a somite comprising the dermatome, myotome, and sclerotome appeared normal in a mutant embryo (Fig. 1 *B*) when compared to a wild-type embryo at 10.5 d (Fig. 1 *A*). These results suggest that somites can form at the proper time and with normal morphological features in the absence of myogenin activity.

From 10.5 to 14.5 d of embryonic development, hematoxylin-eosin-stained sections of embryos revealed little difference between the muscle-forming regions of wild-type and mutant embryos (Fig. 1, *C–F*). After 15.5 d, however, the muscle-forming regions in mutant embryos was greatly reduced in mass compared to those of wild-type siblings (Fig. 1, *G* and *H*). These results suggest that at least initially, the muscle-forming regions contained cells with myogenic potential, and that this potential is lost in the myogenin mutants as development proceeds.

Appearance of Muscle-specific Gene Products in Myogenin-mutant Embryos

We collected embryos from the time muscle masses are first morphologically distinguishable (day 12.5) until birth, and we analyzed them for the appearance of MHC. On day 12.5, sections through wild-type embryos labeled with the MHC antibody showed evidence of developing myofibers throughout muscle-forming masses (Fig. 2, *12.5 d*, +/+). Identical sections of 12.5-d mutant embryos also stained for MHC indicated that muscle masses were present to the same extent, but the staining was significantly weaker, and the cells that stained appeared less organized (Fig. 2, *12.5 d*, −/−). Similar results were observed on day 15.5. At this time, well-differentiated myofibers were detected with the MHC antibody in wild-type embryos, whereas in mutant embryos, the same areas labeled, but less intensely, and myofibers were more diffuse (Fig. 2, *15.5 d*, +/+ vs −/−). Between 15.5 d and birth, there was a dramatic increase in myofiber number and intensity of MHC labeling in the wild-type embryos (Fig. 2, *16.5 d* and *Neonate*). It is during this time interval that major differences between wild-type and mutant embryos became most apparent. Mutant 16.5-d-old embryos or neonates showed the presence of myofibers, but their numbers and their levels of MHC expression were greatly reduced with respect to their wild-type counterparts.

To extend these results, we made use of an antibody against desmin, an intermediate filament protein that is expressed early in the myogenic pathway (George-Weinstein et al., 1993). At 12.5 d, muscle masses from the dorsal regions of mutant embryos contained intensely labeled desmin-positive cells, while the same section showed only weak MHC labeling when compared with controls (Fig. 3, *12.5 d*, *back*, +/− vs −/−). Wild-type muscle masses showed both desmin and MHC-labeled cells in limbs at 12.5 d, but the mutant showed only weak desmin staining and no detectable MHC (Fig. 3, *12.5 d*, *limb*, +/− vs −/−). These results suggested that the mutant embryos lagged behind their wild-type siblings in the differentiation of myoblasts in premuscle masses.

As development proceeded, cells exhibiting intense desmin staining were consistently observed at the periphery of wild-type muscle masses in both the back and hindlimbs and elsewhere in the body (Fig. 3, *14.5 d*, *back*, *limb*, +/−).

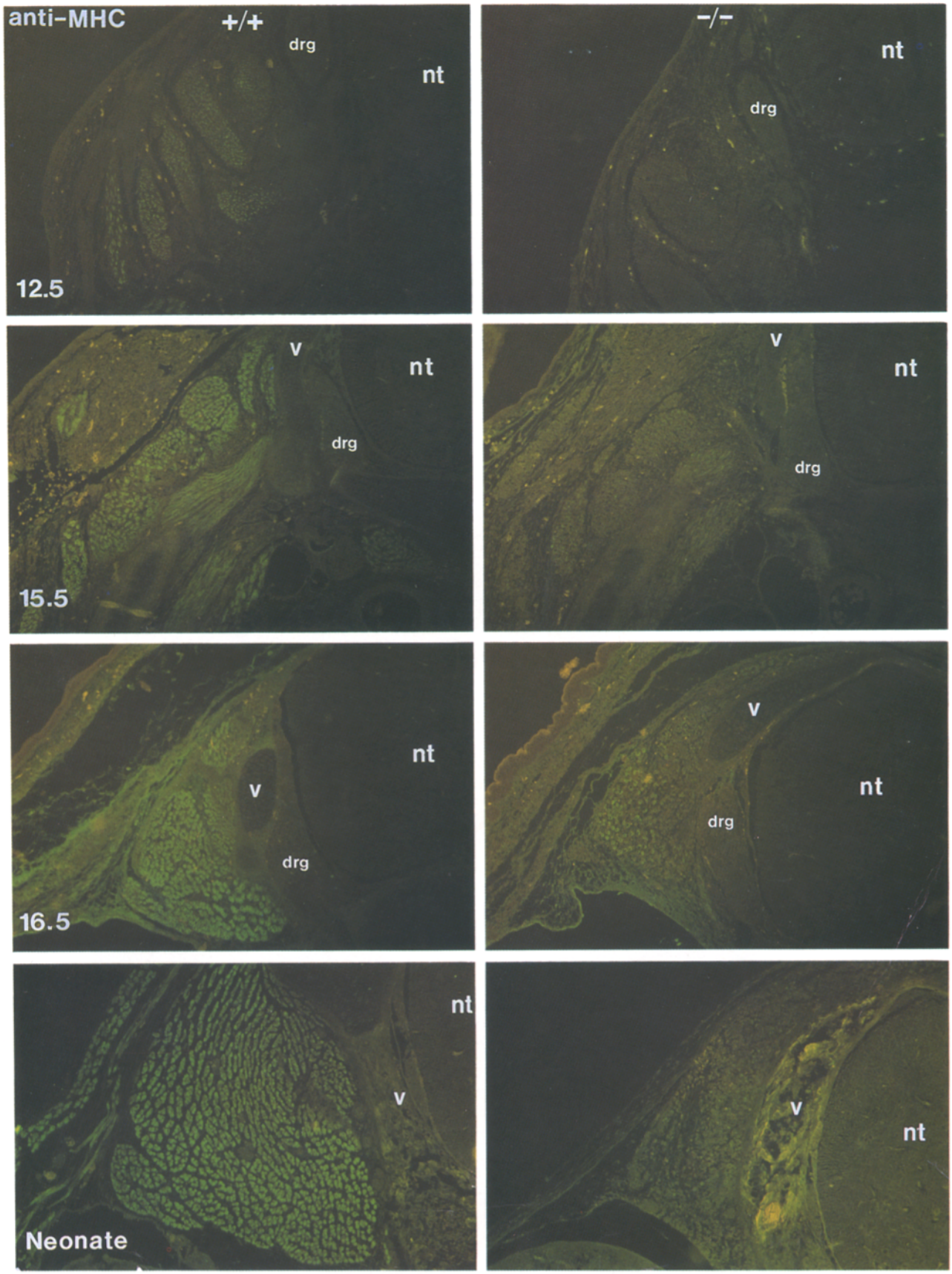
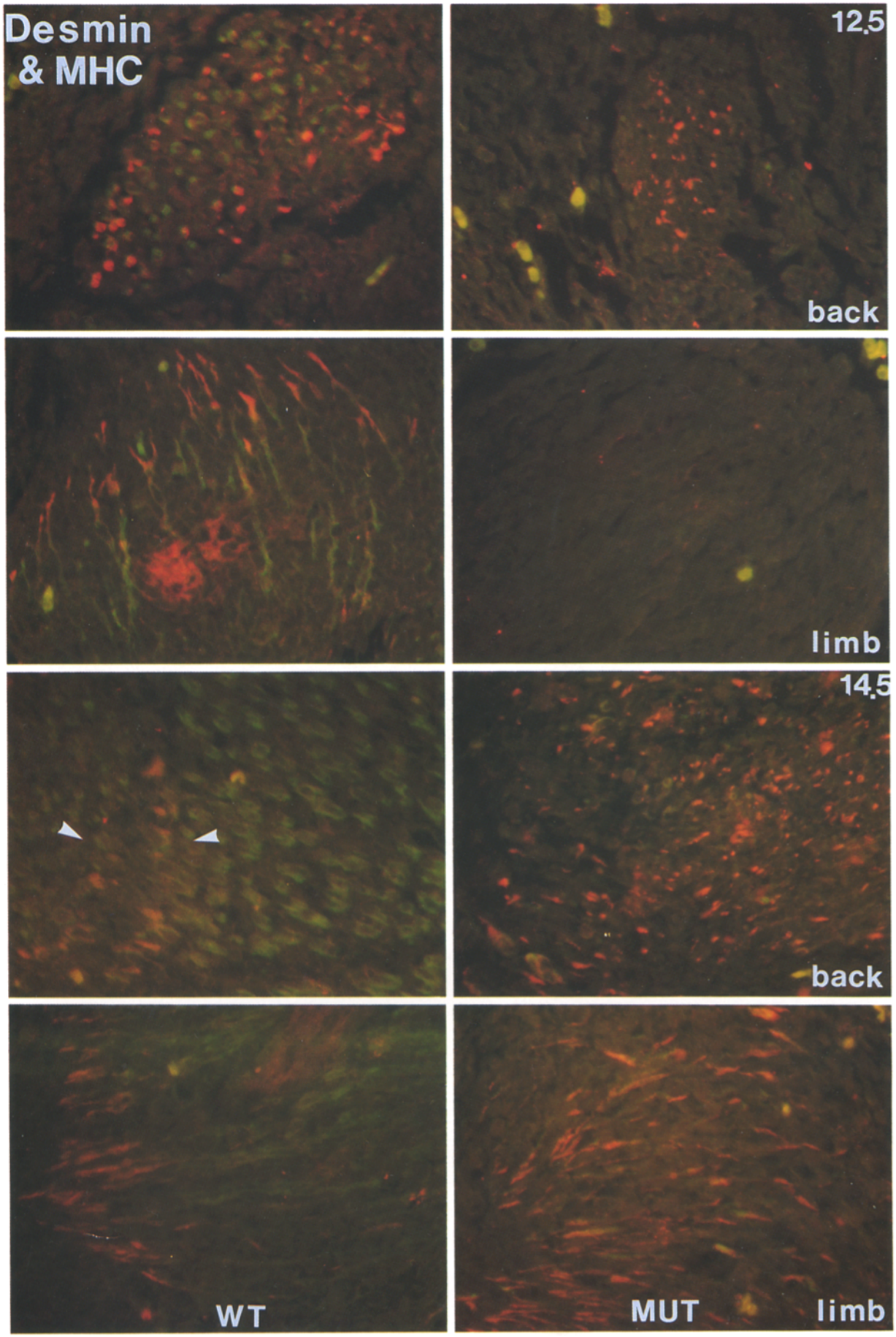


Figure 2. Comparison of MHC expression in myogenin-mutant and wild-type embryos. Discrete MHC-positive cells were detectable in the back region of nonmutant embryos as early as day 12.5. In mutant embryos, the same muscle-forming regions could be discerned, but the level of MHC expression was reduced and MHC staining was seen in fewer cells. As development continued, increased MHC expression was observed in nonmutants, but comparable levels of MHC expression were never achieved in the myogenin mutants. While the most comparable levels of MHC protein expression were seen on day 15.5; thereafter, nonmutant 16.5-d-old embryos and neonates showed a dramatic increase in the number of cells expressing MHC, whereas 16.5-d-old mutant embryos and neonates failed to exhibit a similar increase. *nt*, neural tube; *drg*, dorsal root ganglia; *V*, vertebral body. *Left column*, +/+; *right column*, −/−.


Desmin
& MHC
12.5
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WT
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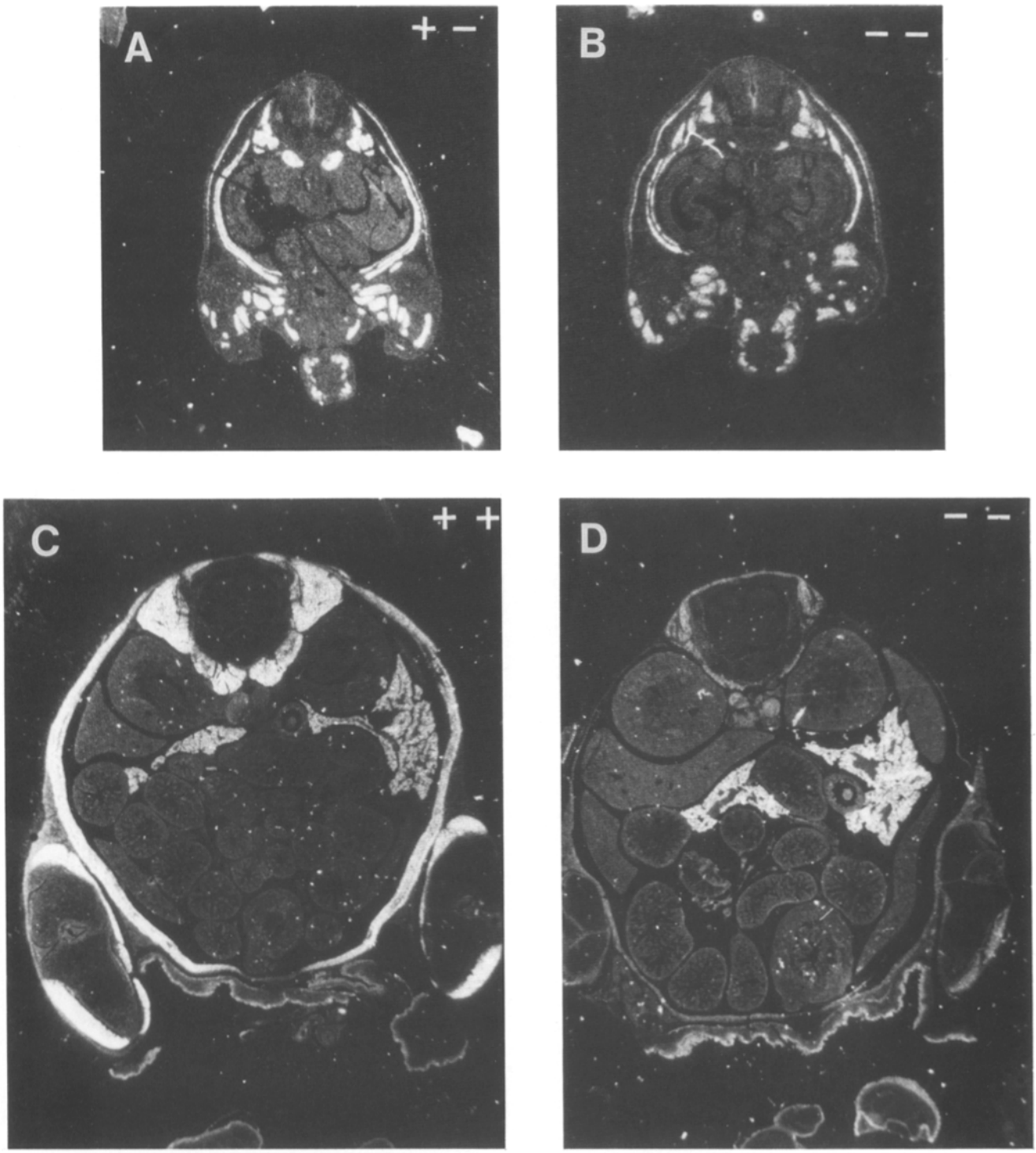

Figure 4. Embryonic and perinatal MHC transcript accumulation during embryogenesis in myogenin-mutant and wild-type embryos. Detectable levels of transcripts for embryonic MHC are observed in nonmutant (*A*) and mutant (*B*) embryos during early embryonic stages as shown here for day 14.5. On day 18.5, the perinatal isoform of MHC showed that the level of MHC expression in the mutants (*D*) was greatly reduced compared to that seen in nonmutant siblings (*C*). The intense labeling seen in *C* and *D* over the liver and heart are refraction artifacts.

Figure 3. Desmin expression in myogenin-mutant and wild-type embryos. Antibodies to MHC (*green*) and desmin (*red*). On day 12.5, the back and limb muscle-forming regions of the nonmutants showed distinct labeling of cells by both muscle markers, whereas comparable regions in mutant embryos showed only weak desmin staining in the back and even weaker staining in the limbs. MHC labeling in mutant embryos at this stage was extremely weak. On day 14.5, the level of expression of both antigens increased dramatically in back and limb muscle-forming regions of both mutant and nonmutant embryos. The organization of desmin-positive cells in the mutant back and hindlimb, however, more closely resembled that of the immature back and limb seen in nonmutants on day 12.5. Desmin-positive cells in the back and limbs in the nonmutants were found in the periphery of the muscle groups (*arrows*). *N*, neural elements that are also recognized by the desmin antibody). *Left column*, +/−; *right column*, −/−.

These cells appeared to be differentiating myocytes that were aligning within the developing muscle. In contrast, mutant embryos showed a greater number of intensely stained desmin-positive cells that were scattered throughout the muscle masses (Fig. 3, *14.5 d, back, limb, −/−*) and appeared as myoblasts that persisted in the mutant muscle-forming regions. These results provided further evidence that the myogenic process was initiated in mutant embryos, but complete muscle differentiation did not occur. The organization of desmin-positive cells in the mutant muscle masses of later embryos resembled that seen in the muscle masses of younger mutant embryos, suggesting that myoblasts are blocked in their ability to differentiate.

In early embryogenesis, MHC is first expressed as an embryonic isoform, which then switches to a perinatal form on day 16.5 (Lyons et al., 1990). We used riboprobes specific to untranslated regions of embryonic or perinatal MHC mRNAs to determine if the MHC embryonic-to-perinatal switch took place normally in the myogenin mutants. Up to day 14.5, wild-type and mutant embryos displayed detectable signals in muscle-forming regions using the embryonic probe (Fig. 4, *A* and *B*). As observed with the MHC antibody, labeling of muscle masses in mutant embryos was generally less intense, suggesting that muscle differentiation was perturbed. By day 18.5, the embryonic probe yielded only very low signals with either wild-type or mutant embryos, suggesting that appropriate downregulation of the embryonic MHC variant had occurred (not shown). When day 18.5 embryos were probed with the perinatal form of MHC, dramatic differences were observed between wild-type and mutant embryos (Fig. 4, *C* and *D*). While the wild-type embryos produced an intense signal over muscle-forming regions, mutant embryos showed very weak signals. The relative differences in signal intensity were largely attributable to a deficiency of muscle in the mutants at this developmental stage. These results further supported the notion that up to 15.5 d, muscle masses were capable of forming in the myogenin-mutant mice, but after that time, while wild-type embryos underwent extensive muscle formation, mutant embryos were severely impaired.

Expression of VCAM-1 in Myogenin-mutant Embryos

A possible explanation for the defects observed in the myogenin-mutant mice at later stages is the inability of secondary myoblasts to fuse. The integrin VLA-4 and its counterreceptor, VCAM-1, have been implicated in the process of alignment and fusion of secondary myoblasts (Rosen et al., 1992). In particular, VCAM-1 is expressed at sites of secondary myogenesis (Rosen et al., 1992). A lack of VCAM-1 expression in mutant embryos would suggest that secondary myoblasts would be deficient in fusion. We used RT-PCR to monitor the expression of VCAM-1 in wild-type and mutant embryos. VCAM-1 transcripts were expressed at approximately equal levels in limbs of wild-type and mutant embryos at 13.5 d (Fig. 5, lanes *1* and *2*). Levels increased slightly at 14.5 d in both wild-type and mutant limb muscle (Fig. 5, lanes *3* and *4*). RNA isolated from carcasses, which includes all muscle regions, again showed equal VCAM-1 expression in wild-type and mutant embryos (Fig. 5, lanes *5* and *6*). By 16.5 d, VCAM-1 expression decreased somewhat, but no significant differences were found between the wild-type and mutants (lanes *7* and *8*). These results demonstrate that VCAM-1 expression is not affected by the absence of myogenin. Moreover, the presence of normal levels of VCAM-1 are not sufficient for secondary myoblast fusion.

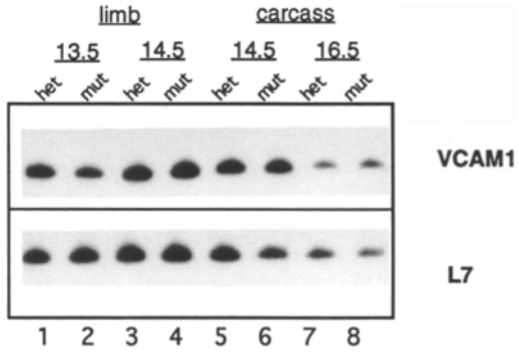

Figure 5. Expression of VCAM-1 in myogenin-mutant embryos. The level of VCAM-1 mRNA in limb muscle from 13.5- and 14.5-d-old embryos, and carcass from 14.5- and 16.5-d-old embryos was examined by RT-PCR analysis (*top row*). Omission of the reverse transcriptase from the reaction resulted in no amplification (not shown). RNA loading was assessed by ribosomal L7 transcript levels (*bottom row*). *het* and *mut*, heterozygous and homozygous myogenin-mutant embryos, respectively.

Lack of Regional Differences in Muscle Formation in the Myogenin-mutant Mice

Nabeshima et al. (1993) reported that in myogenin-mutant embryos there is a difference in the degree of muscle differentiation in the myotome vs the limb and body wall. The myotomal cells are derived from differentiation of myoblasts in situ, whereas the myoblasts that populate the limbs and elsewhere migrate from the dermamyotome and colonize the muscle-forming regions. It has been proposed that different myogenic lineages arise from the somite, and that the different bHLH myogenic factors play a role in defining these lineages (Ordahl and Le Douarin, 1992). Although some differences in the extent of muscle differentiation were evident in different regions of the mutant embryos during early stages of development (not shown), by 18.5 d, this difference was no longer apparent, and the extent of muscle deficiency seen in the back, body wall, and limbs was comparable. Thus, as monitored by MHC antibodies, muscles in the back, ventral-lateral body wall, and forelimb of 18.5-d-old mutant embryos all showed far fewer muscle fibers than seen in comparable regions of control littermates (Fig. 6; *A*, *C*, and *E* vs *B*, *D*, and *F*). These results argue against the hypothesis that myogenin function is more essential for terminal differentiation of nonmyotomal-derived muscles. A likely explanation for the difference between these results and those of Nabeshima et al. (1993) is that the latter study only analyzed 14.5 d-old embryos, a time when delays in the differentiation of limb and ventral body wall muscle in myogenin mutants are apparent.

Myogenin-mutant Neonates Express Both Fast and Slow MHC Isoforms

To assess the properties of the residual myofibers in the myogenin-mutant neonates, we made use of antibodies that distinguished between embryonic, fast, and slow MHC iso-

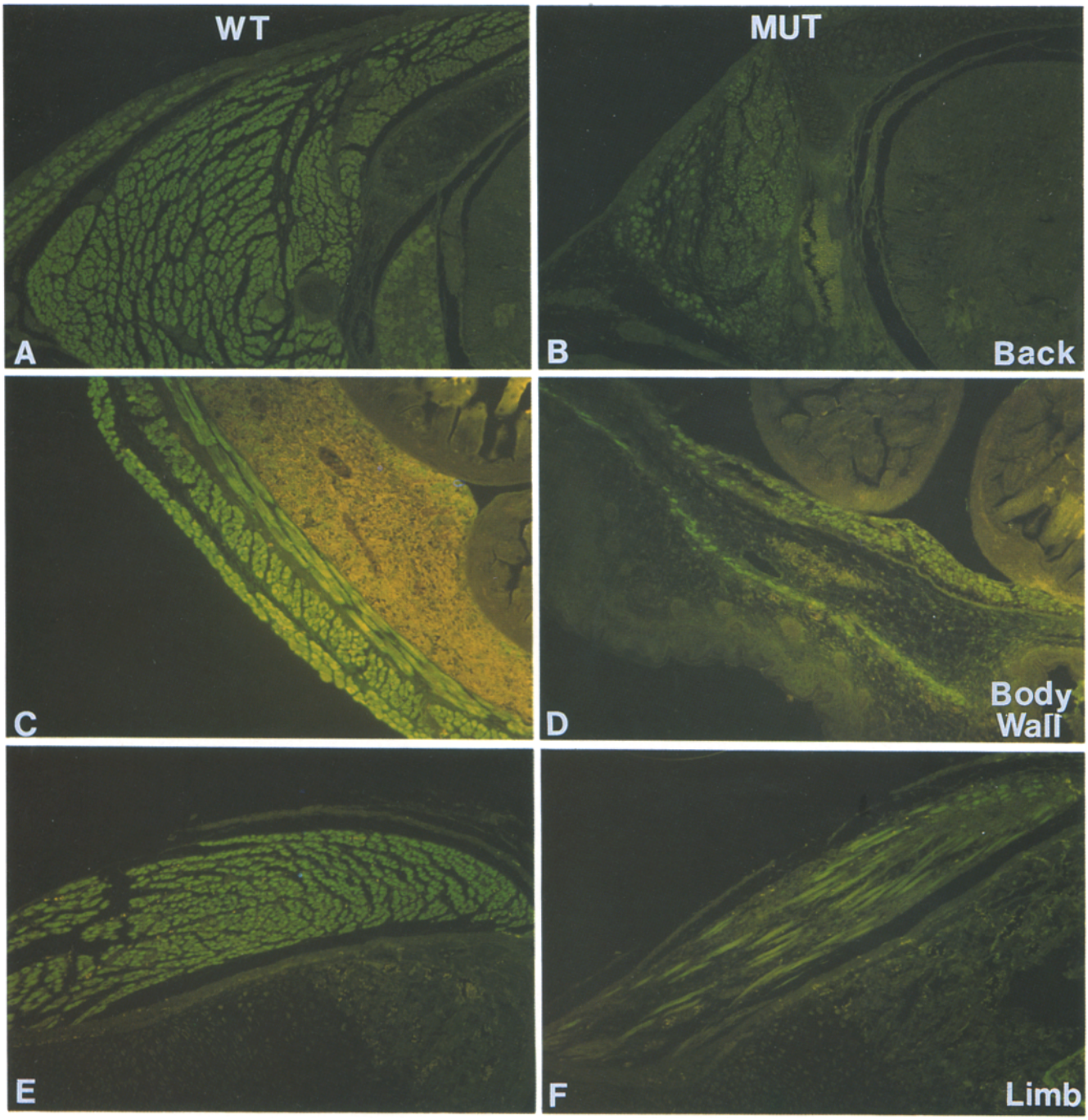

Figure 6. Lack of regional differences in the density of residual myofibers in the myogenin-mutant embryos. Antibodies to MHC demonstrate that the muscle deficiency was as severe in the back region (*A* and *B*) as it was in the ventral body wall (*C* and *D*) and limbs (*E* and *F*) of mutant embryos compared with similar regions in nonmutants.

forms (Condon et al., 1990). As expected, sections through hindlimbs of wild-type neonates demonstrated a higher proportion of fibers labeled with antibody to fast MHC than to slow MHC (Fig. 7, +/+). When the same antibodies were used to label mutant hindlimbs, a similar ratio of fast vs slow anti-MHC–labeled fibers was observed (Fig. 7, −/−). Thus, both fiber types could still form, despite the absence of myogenin.

Surprisingly, all the different muscle groups that compose the crural region were evident in the mutant hindlimb, albeit with reduced fiber number (Fig. 7, *A–C*). The overall number of muscle fibers in the mutant limbs, however, was considerably reduced compared to the number seen in wild-type limbs. These results, together with those obtained from MHC labeling through sections of whole embryos, suggested that the formation of myogenic precursors in the myogenin-

+/+
−/−
Fast
Slow
A
B
C
t
f

mutant mice was normal, but that the subsequent proliferation and differentiation of these precursors was abnormal.

Expression of the MyoD Gene in Myogenin-mutant Embryos

In an earlier study, we showed that MyoD transcripts in myogenin-mutant neonates were present at similar levels as in wild-type, and that on day 14.5, MyoD protein was present at normal levels (Hasty et al., 1993). These results did not address whether the MyoD gene was activated at the appropriate time and place in the absence of myogenin, and if its mRNA accumulated in a normal fashion. To answer the latter question, we followed MyoD transcript accumulation through embryogenesis using in situ hybridization with a MyoD-specific antisense riboprobe.

MyoD transcript levels were similar in mutant and wild-type embryos on embryonic days 12.5 and 14.5 (Fig. 8). MyoD expression appeared normal in all muscle-forming regions of the mutant embryos where, presumably, transcripts were accumulating in myoblasts and differentiating myofibers (+/+ vs −/− on day 12.5 and +/− vs −/− on day 14.5). Similarly, at later stages of development (18.5 days and neonates), no obvious difference in the levels of MyoD expression were observed between mutant and wild-type embryos (not shown).

Since myogenin transcripts are detected 2 d before those of MyoD (Smith et al., 1993), myogenin's absence could affect the initial activation of the MyoD gene. To address this question, we cross-bred the myogenin-mutant mice with a transgenic line harboring a MyoD-*lacZ* fusion gene capable of expressing *lacZ* with the same temporal and spatial patterns as the endogenous MyoD gene (Goldhamer et al., 1992). Mice heterozygous for the myogenin mutation and hemizygous or homozygous for the MyoD-*lacZ* transgene were mated with one another to produce embryos deficient in myogenin, and hemizygous or homozygous for the transgene.

On day 10.5, cells in the myotomal compartments of all somites labeled with approximately equal intensity in both mutant and control embryos (Fig. 9, *10.5 d*, −/− vs +/−). As development proceeded, MyoD-*lacZ* expression extended from the somitic region into all major muscle-forming regions of the embryo so that at 11.5 d, expression was seen in trunk, body wall, limb, and facial muscles of both mutant and wild-type embryos (Fig. 9, *11.5 d*, −/− vs +/−). By 13.5 d, labeling was as intense in the mutant embryos as in the wild-type controls, although the muscle masses throughout the mutants were more disorganized, indicating the presence of large numbers of undifferentiated myoblasts (Fig. 9, *13.5 d*, −/− vs +/−). From these results, it is clear that myogenin is not required for either the activation or maintenance of MyoD gene expression.

Discussion

Our analysis of the myogenin-mutant phenotype during embryogenesis extends our initial characterization of these mice, and it demonstrates that myogenin plays an essential role in skeletal muscle development from the time muscle masses first appear until birth. An intriguing feature of the myogenin-mutant mice is that they are capable of generating semblances of differentiated muscle during embryogenesis. Our results clearly show that "myogenic" cells are present in myogenin-mutant embryos. An unexpected finding showed that mutant and wild-type embryos appear more similar in their myogenic regions between 9.5 and 15.5 d than between 15.5 d and birth. The initial wave of myogenesis occurs between 10.5 and 14.5 d, and it results in the initial appearance of myofibers in most regions of the embryo (Kelly, 1983). In the myogenin-mutant embryos, muscle masses, which correspond to areas where mature muscle will ultimately differentiate, are capable of some differentiation, but they are clearly abnormal in both their morphological features, as well as patterns of MHC and desmin expression. Nevertheless, the absence of myogenin did not prevent at least some remnants of muscle differentiation from occurring at these early times, arguing that myoblasts can align, fuse, and differentiate normally. These findings indicate that some myogenic cells may be capable of differentiating along the myogenic pathway through a myogenin-independent mechanism.

It is only after day 14.5 that dramatic differences appear between the myogenin-mutant and wild-type embryos. This is the time when secondary myogenesis occurs (Kelly, 1983). The increase in the number of myofibers in wild-type embryos is the result of the differentiation of myoblasts that are associated with the primary myofibers and subsequent fusion of these cells to produce extensive myofiber bundles. Our data demonstrate that this process is severely retarded in the myogenin-mutant mice, and may not take place at all. The particular defect that prevents the differentiation of myoblasts to form secondary myotubes is not well understood. We suggest from these results that myogenin affects both primary and secondary myogenesis, but that its role in the two processes may not be identical. Regulatory factors other than myogenin may compensate somewhat for the loss of myogenin during primary myogenesis. In contrast, secondary myofibers may require myogenin-dependent activation of a specific set of genes that cannot be activated by other myogenic regulators.

It has been proposed that secondary fiber formation is dependent on the number of primary myofibers that form, and alternatively, that secondary myogenesis is dependent on innervation of primary myotubes (Condon et al., 1990; Wilson and Harris, 1993). One theory argues that denervation reduces the number of primary fibers that are formed, which leads to reduced secondary fiber formation (Condon et al., 1990). The opposing theory argues that primary myotubes form autonomously, and denervation specifically affects secondary myotube formation (Wilson and Harris, 1993). Both theories argue that innervation plays a role in muscle development. Relevant to this, we have shown that myogenin-mutant mice do not make detectable levels of the α and γ-subunits of the acetylcholine receptor (Hasty et al., 1993),

Figure 7. Detection of fast and slow isoforms of MHC in the limbs of myogenin-mutant and wild-type neonates. Sections through hindlimbs of neonates with antibodies specific to different isoforms of MHC revealed that mutant hindlimbs contained myofibers that expressed both fast and slow isoforms of MHC in a ratio comparable to that seen in wild-type embryo hindlimbs. In addition, the mutant hindlimbs showed many of the same muscle groups that were present in wild-type hindlimbs (labeled *A*, *B*, and *C* for comparison). *t*, tibia; *f*, femur.

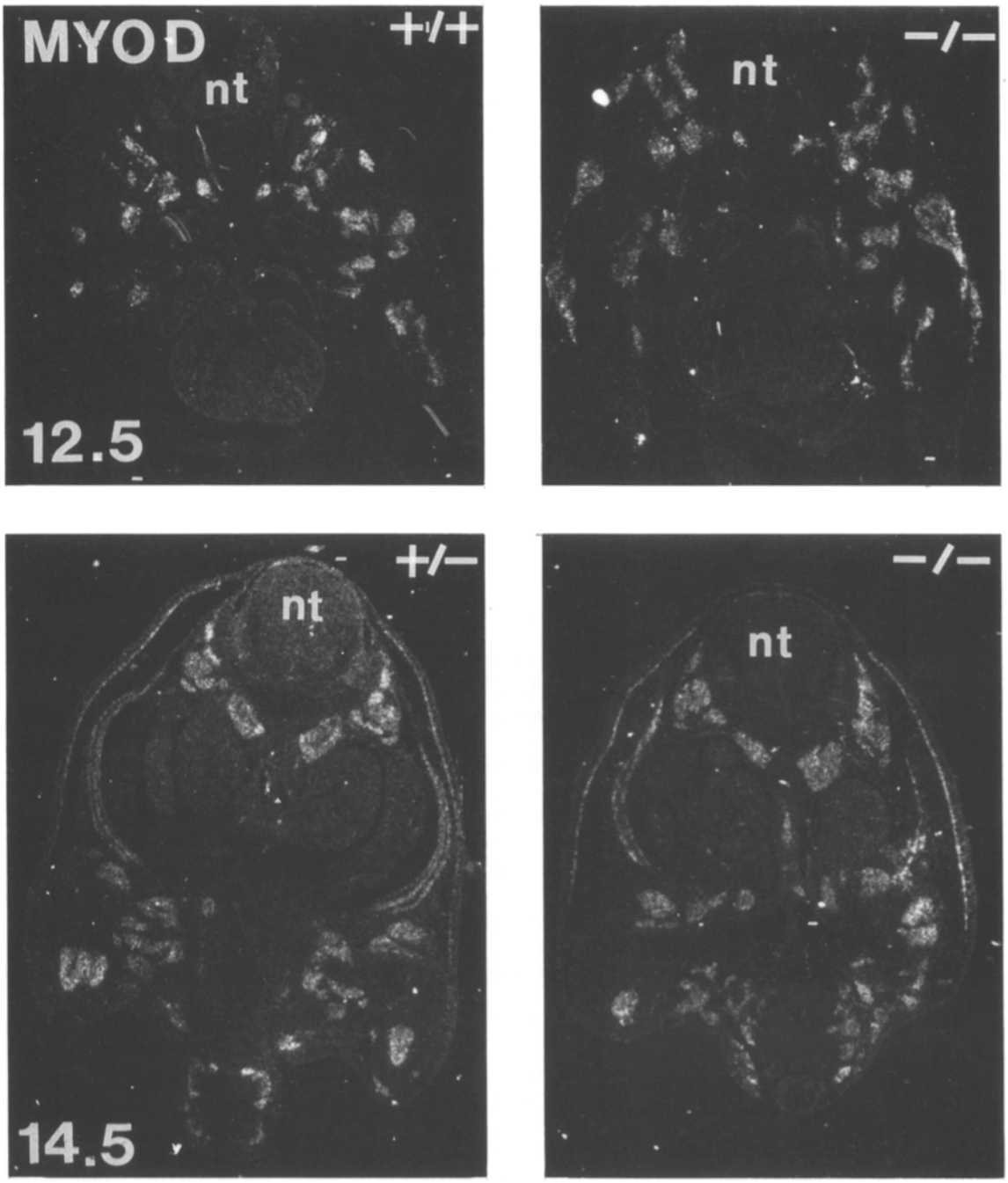

Figure 8. Accumulation of MyoD transcripts in myogenin-mutant and wild-type embryos. Comparable levels of expression of transcripts were seen in both wild-type and mutants at early embryonic stages (day 12.5 and 14.5). As development progressed, the levels of expression decreased in both the wild-type and mutant with slightly lower levels observed in mutants after 16.5 d. *nt*, neural tube.

which would lead to an absence of receptors on the cell surface and an inability to make proper nerve connections.

An additional feature of the myogenin-mutant phenotype is the presence of residual myofibers with seemingly normal morphology in late-stage embryos and neonates. These residual fibers reflect a normal ratio of physiologically distinct fiber types. A recent report presents evidence that MyoD expression is associated with slow-twitch fiber types and myogenin with fast-twitch fiber types in adults (Hughes et al., 1993), but if so, such differential expression is not manifested in the myogenin-mutant embryos.

From the results presented here and from our previous analysis (Hasty et al., 1993), it is apparent that MyoD expression is unaltered in myogenin's absence. Of course, this does not prove that myogenin does not regulate the MyoD gene in wild-type mice, since other myogenic factors, particularly Myf5, may compensate for the loss of myogenin. Recent evidence shows that Myf5 expression also appears to be unaffected in the myogenin mutants (Rawls, A., and E. Olson, unpublished results). Furthermore, the embryonic expression of a myogenin-*lacZ* transgene containing the appropriate control region of the myogenin gene is identical in

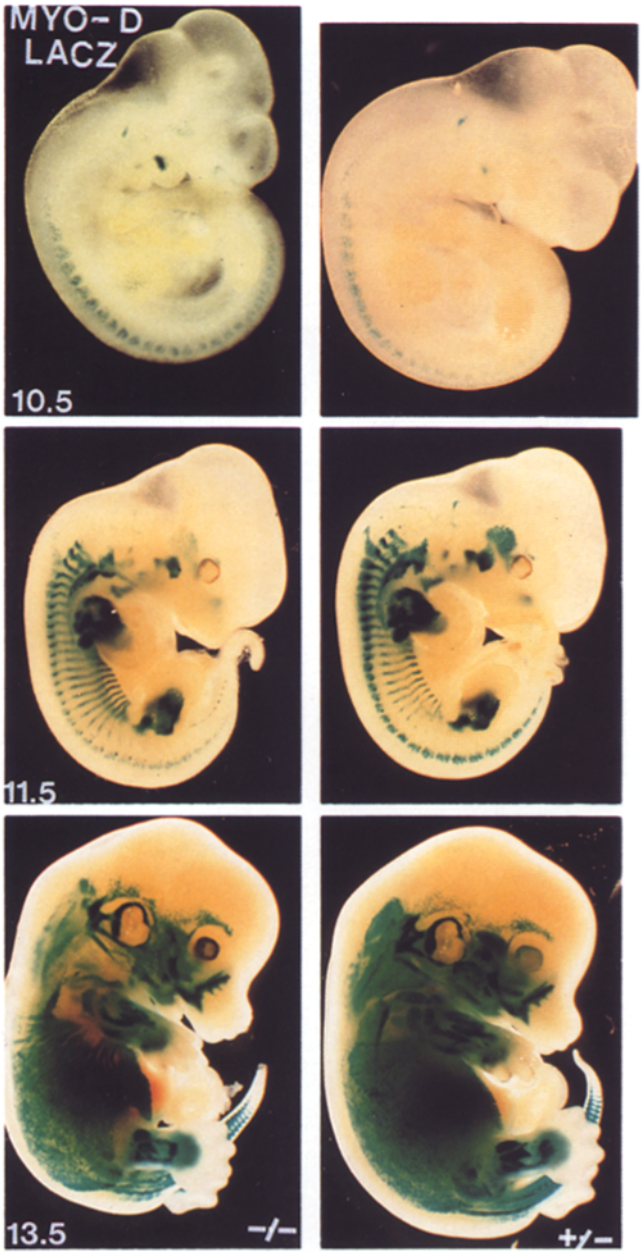

Figure 9. Expression of a MyoD-*lacZ* transgene in myogenin-mutant embryos. Comparable levels of staining were seen in the somites of 10.5-d-old nonmutant and mutant embryos. (The slight difference between the wild-type and mutant embryos seen in the figure is variable and not significant). Similar levels of expression were also seen as development continued such that nonmutant and mutant embryos were almost indistinguishable at 11.5 d. At later stages, differences in expression became apparent as the mutant embryos showed a lesser degree of organization of the β-galactosidase–positive cells into organized myofibers than did their nonmutant siblings. This can be seen as more diffuse staining throughout the body of the mutant embryo when compared with the wild-type embryo, particularly along the posterior-dorsal regions.

wild-type and myogenin-mutant backgrounds (Cheng et al., 1995). Hence, myogenin is not essential for regulating its own gene, nor the genes for Myf5 or MyoD. Interestingly, the only bHLH myogenic factor that may be affected is MRF4, whose transcripts are dramatically reduced in myogenin-mutant neonates (Hasty et al., 1993).

MyoD and Myf5 appear to have redundant functions, and they lie upstream of myogenin in the myogenic pathway (Weintraub, 1993; Rudnicki et al., 1993; Olson and Klein, 1994). It has also been hypothesized that MRF4 lies downstream of myogenin. Thus, MyoD and Myf5 may act directly to control the expression of myogenin, as evidenced by the lack of myogenin in MyoD/Myf5-mutant mice (Rudnicki et al., 1993). In contrast, MyoD and Myf5 expression is unaltered in the myogenin-mutant mice, whereas the MRF4 gene appears not to be expressed. This implies that MyoD and Myf5 are responsible for early events of myoblast formation, including the activation of myogenin. Myogenin, in turn, is responsible for later events associated with myotube differentiation, including the activation of MRF4. Finally, MRF4 may be responsible for the final myogenic events of the fully differentiated myofiber. A testable prediction from this model is that the myogenic bHLH factors activate different sets of target genes during muscle development, and that the activation of these sets follows a precise temporal sequence as myoblasts begin their differentiation program.

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