The Muscle Regulatory Gene, Myf-6, Has a Biphasic Pattern of Expression during Early Mouse Development

Eva Bober,* Gary E. Lyons,† Thomas Braun,* Giulio Cossu,‡ Margaret Buckingham,‡ and Hans-Henning Arnold*

*Department of Toxicology, Medical School, University of Hamburg, 2000 Hamburg 13, Germany; †Department of Molecular Biology, CNRS, UA 1148, Pasteur Institute, Paris, F-75724, France; and ‡Institute of Histology and Embryology, University of Rome, 00161 Rome, Italy

Abstract. The spatial and temporal expression pattern of the muscle regulatory gene Myf-6 (MRF4/herculin) has been investigated by in situ hybridization during embryonic and fetal mouse development. Here, we report that the Myf-6 gene shows a biphasic pattern of expression. Myf-6 transcripts are first detected in the most rostral somites of the mouse embryo at 9 d of gestation and accumulate progressively in myotomal cells along the rostro-caudal axis. This expression is transient and Myf-6 mRNA can no longer be detected in myotomal cells after day 12 post coitum (p.c.). In contrast to other muscle determination genes (MyoD1, myogenin, Myf-5), Myf-6 mRNA is not detected in limb buds or visceral arches and skeletal muscle of the mouse embryo (day 8–15 p.c.). In fetal mice, Myf-6 transcripts appear at day 16 p.c. in all skeletal muscles, and the gene continues to be expressed at a high level after birth. These results suggest that early Myf-6 expression may be restricted to a population of myogenic cells that does not contribute to the embryonic muscle masses in limb buds and visceral arches. The reappearance of Myf-6 mRNA in fetal skeletal muscle coincides approximately with secondary muscle fiber formation and the onset of innervation.

The isolation of MyoD1, the first muscle regulatory protein, from the C3H mouse myogenic cell line 10T1/2 (Davis et al., 1987) was followed by the identification of three additional, related but distinct proteins from various species. Myogenin (Wright et al., 1989; Edmondson and Olson, 1989; Braun et al., 1989a), Myf-5 (Braun et al., 1989b), and MRF4 (rat) (Rhodes and Konieczny, 1989), also called Myf-6 (human) (Braun et al., 1990) and herculin (mouse) (Miner and Wold, 1990), are all members of a family of muscle-specific helix-loop-helix proteins whose forced expression leads to the muscle phenotype in 10T1/2 mouse fibroblasts (for review see Olson, 1990). In nonfibroblast cell types, regulators in addition to the helix-loop-helix proteins appear to be required to induce a phenotypic conversion to muscle (Weintraub et al., 1989). In transfection experiments using vectors that constitutively express the four myogenic factors, transcriptional activation of muscle-specific reporter genes has been demonstrated for each factor (Lin et al., 1989; Edmondson and Olson, 1989; Yutzey et al., 1990; Braun et al., 1990a). Autoactivation of the corresponding endogenous genes and cross-activation of the other members of the gene family have also been shown (Braun et al., 1989b; Thayer et al., 1989). Moreover, heterodimerization properties and DNA binding affinities in vitro have been studied and found to be similar for the human Myf proteins (Braun, T., and H. H. Arnold, manuscript submitted for publication; Rosenthal et al., 1990), suggesting that these proteins may have similar if not identical functions.

The first indication that these proteins may play distinct roles during myogenesis in vivo came from in situ hybridization experiments performed on mouse embryo sections. These studies showed that MyoD and myogenin mRNAs have different temporal patterns of accumulation in the myotome and in the developing limb bud (Sassoon et al., 1989). More recently, we have reported that Myf-5 mRNA expression is detectable in somites before myotome formation and clearly precedes the expression of myogenin and MyoD1 (Ott et al., 1991). In contrast to the expression of the latter two factors which, once initiated, continue to accumulate in all developing skeletal muscles, Myf-5 is transiently activated in an anterior posterior gradient but is down-regulated in limb and body muscle from 11.5 d p.c. (Ott et al., 1991). The early expression of Myf-5 in dermamyotomal cells may indicate its involvement in the determination of myoblasts rather than in the establishment of terminally differentiated muscle cells.

The expression pattern of the fourth myogenic factor of this family, Myf-6 (MRF4, herculin) during mouse development has not been described. Rhodes and Konieczny (1989) reported the first detection of MRF4 mRNA by Northern blot analysis in the skeletal muscle of a rat fetus at 18 d p.c. Here, we describe the detailed analysis of Myf-6 expression during mouse embryogenesis using in situ hybridization. We compare the time course of Myf-6 mRNA synthesis with that of other Myf genes. It is particularly interesting to compare the expression of Myf-5 and Myf-6 transcripts since both genes are closely linked on human chromosome 12 (Braun et al.,...
Figure 1. Nucleotide sequence of the mouse Myf-6 cDNA. The mouse sequence is compared to the homologous cDNAs from rat (MRF4) and human (hMyf-6). Identical nucleotide residues are indicated by dashes. Dots indicate gaps which have been introduced for optimal alignment. Start and stop codons are marked by asterisks. The fragment used as hybridization probe is underlined. These sequence data are available from EMBL/GenBank/DDBJ under accession numbers X51591, X51592, and X51593.
1990a) and in the mouse genome (Miner and Wold, 1990). We show that Myf-6 has a biphasic pattern of expression in embryonic and fetal mouse skeletal muscle.

Materials and Methods

Isolation and Nucleotide Sequence Determination of the Mouse Myf-6 cDNA

The Myf-6 cDNA was isolated by screening 1 x 10^6 p.f.u, of a phage lambda gt-11 library prepared from skeletal muscle of a newborn mouse (kind gift of J. P. Merlie, Washington University, St. Louis, MO). The probe was derived from the first exon of the human Myf-6 gene and is represented by a 160-bp PstI fragment that lacks homology to other myf cDNAs (Braun et al., 1990a). The largest of three independent cDNA isolates was sequenced by the dideoxy chain termination method (Sanger et al., 1977) on both strands using subcloned restriction fragments and nested deletions (Henikoff, 1984).

Northern Blot Analysis

Total RNA was prepared from limbs and trunks of mouse embryos and fetuses staged between days 12 to 19 p.c., and from newborn and adult mouse limb skeletal muscle by the LiCl/urea method (Auffray and Rougeon, 1980). Gel electrophoresis, RNA transfer, and hybridization conditions have been described (Braun et al., 1989a). The probes for Northern blot analysis were DNA fragments corresponding to the 3'-specific regions of myogenin (Sassoon et al., 1989), and Myf-6 cDNAs as described for cRNA probes used for in situ hybridizations. DNA was labeled to a specific activity of 1-3 × 10^6 cpm/μg using the multiprime labeling kit from Amersham International (Amersham, UK).

Preparation and Prehybridization of Tissue Sections

C3H and BALB/c mouse embryos and fetuses were fixed and embedded as described previously (Lyons et al., 1990). Two sections were mounted per slide.

Preparation of Probes for In Situ Hybridization

Restriction fragments, which specifically detect Myf-5, Myf-6, and myogenin transcripts were subcloned into the Bluescribe transcription vector (Stratagene Cloning Systems, La Jolla, CA) and grown in E. coli TG1. For the Myf-6 probe, the 680-bp PstI fragment indicated in Fig. 1 was used. The Myf-5 probe is a 310-bp Bal-ApaI fragment of the first exon of the mouse Myf-5 gene as described in Ott et al. (1991). The myogenin probe corresponds to the 3'-terminal 700-bp of the rat myogenin cDNA (Wright et al., 1989; Sassoon et al., 1989). The cRNA transcripts were synthesized according to manufacturer's conditions (Stratagene Cloning Systems), and labeled with 35S-UTP (>1,000 Ci/mmol; Amersham International). cRNA transcripts were partially hydrolyzed to a mean size of 70 nucleotides for efficient hybridization.

Hybridization and Washing Procedures

Hybridization and washing procedures were performed as described in Lyons et al. (1990). Slides were dipped in undiluted Kodak NTB-2 nuclear track emulsion and exposed for 1 wk at 4°C. Slides were developed in Kodak D-19 and analyzed using light- and dark-field optics of a Zeiss Axioplan or Axiopt microscope.

Results

Using the human Myf-6 cDNA (Braun et al., 1990a) as hybridization probe, we have isolated the almost full-length mouse Myf-6 homologue which corresponds to the previously described herculin gene (Miner and Wold, 1990). As shown in Fig. 1, the nucleotide sequence of the mouse cDNA is highly conserved and has diverged ~10 and 20% from MRF4 rat and Myf-6 (human) sequences, respectively (Braun et al., 1990a; Rhodes and Konieczny, 1989). A high degree of sequence conservation exists even in the untranslated regions of the cDNA, which was used to generate the Myf-6-specific complementary RNA probe, encoded by a 680-bp PstI restriction fragment. It was cloned behind the T7 promoter in the pBS vector (Stratagene Cloning Systems). The specificity of the probe was determined on a Northern blot (data not shown). In embryonic (day 8–15 p.c.) and fetal (day 15–19 p.c.) limb and trunk RNA preparations from mice, Myf-6 mRNA was first detected on Northern blots at day 16 p.c. with continued expression into the postnatal period. In contrast, myogenin mRNA was already weakly present in 12-d embryos in both limb and trunk, and was found to be reduced.
in its level in the adult limb muscle (Fig. 2). This first observation confirmed the result obtained for the rat MRF4 expression which also showed late onset in development (Rhodes and Konieczny, 1989).

To investigate Myf-6 expression in more detail, mouse embryos between 9 and 11.5 d p.c. were sectioned and hybridized in situ with various Myf probes. As shown in Fig. 3, in transverse sections of two embryos of 9 (18 somites; Rugh, 1990) and 9.5 d (24 somites) p.c., we detected the accumulation of both Myf-5 and Myf-6 transcripts on serial slides of rostral somites. Myf-5 transcripts can first be detected in the newly formed somites at day 8 (Ott et al., 1991), whereas Myf-6 transcripts are first seen at day 9 p.c. These transcripts gradually accumulate as shown in a transverse section of a 9.5-d p.c. embryo (Fig. 3).

To extend the analysis to later stages of development, parasagittal and transverse sections were performed on two 11-d-old littermates (39–44 somites). Serial sections were hybridized to Myf-5, Myf-6, and myogenin probes. To ascertain that somites were cut in the appropriate plane, slides chosen for hybridization with the Myf-6 probe were sandwiched between slides which were hybridized to the other probes (Myf-5 and myogenin). At least two sections were probed on each slide. As shown in Fig. 4, a–c, in parasagittal sections of a region slightly anterior to the hind limb bud, somites expressed approximately equal levels of Myf-6, Myf-5, and myogenin transcripts. In the limb bud itself, Myf-5 mRNA was abundantly expressed (Fig. 4 a), but myogenin and Myf-6 mRNAs were not detected. In parasagittal sections of more posterior somites, again, similar levels of all three transcripts, Myf-5, Myf-6, and myogenin were observed (Fig. 4, d–f). In adjacent somites, we detected coexpression of Myf-5 and myogenin at relatively high levels, but no Myf-6 mRNA. The most caudal and therefore least developed somites immediately adjacent to the neural tube (Fig. 4 d), however, hybridized exclusively to the Myf-5 probe, confirming that Myf-5 expression precedes that of the other members of the MyoD gene family. This series of hybridizations shows that the activation of the Myf-6 gene, like that of other myogenic factor genes, follows a rostro-caudal gradient. Each myogenic regulatory factor gene follows a distinct temporal sequence of activation in the somites during embryonic development.

The onset of Myf-6 expression lags slightly behind the initial detection of myogenin transcripts, but the relative levels of both mRNAs are approximately equal at a later stage (Fig. 4). As myotomes develop, the level of Myf-6 mRNA starts to decline in comparison to that of myogenin mRNA. The rostro-caudal gradient of the decrease in Myf-6 mRNA concentration is demonstrated in serial transverse sections of an 11.5-d-old embryo in the forelimb bud region. At this time of development, the more rostral somites contain less Myf-6 mRNA compared to that of myogenin, while the more caudal somites still express high levels of Myf-6 mRNA (Fig. 5).

We did not detect Myf-6 transcripts between day 12 and 15 p.c. (data not shown) by in situ hybridization, which is consistent with the results obtained by Northern blot analysis (see Fig. 2). In conclusion, these results together with those shown in Fig. 4 indicate that the Myf-6 gene is transiently activated in myotomes during somitogenesis, and that the inactivation of Myf-6 proceeds in the same anterior-posterior gradient as activation.

Although Myf-6 mRNA is detected between 9 and 12 d p.c. in developing myotomes, in situ hybridization performed on limb buds and visceral arches in embryos of the same age shows that Myf-6 mRNA is never detected in these structures. For example, Fig. 6, a–c shows the limb bud of an 11.5-d-old embryo, demonstrating hybridization of myogenin and Myf-5 mRNA, but not detectable hybridization with the Myf-6 probe. Fig. 6, d–f shows the mandibular arch of an 11-d p.c. embryo. There again Myf-6 transcripts are not detected, whereas Myf-5 is expressed at high levels and myogenin at low but detectable levels. Similar results were obtained at different stages of embryogenesis (data not shown).

The time of transient Myf-6 and Myf-5 expression in somites is presumably followed by a period when only myogenin and MyoD1 mRNA are detected in myotomal cells as previously shown by Ott et al. (1991) and our results (Fig. 7). To examine Myf-6 mRNA accumulation at later stages, we extended the analysis up to fetal stages (15–19 d p.c.). As shown in Fig. 7, body wall muscles of a 17.5-d fetus contain high levels of Myf-6 transcripts as well as myogenin mRNA. At this time, Myf-5 transcripts can no longer be detected (data not shown; Ott et al., 1991). From these observations we conclude that the Myf-6 gene becomes reactivated in fetal skeletal muscle where it is coexpressed with MyoD1 (data not shown) and myogenin. Tables I and II summarize the results for in situ hybridization with each of the myogenic factor probes in developing embryonic myotomes, limb buds, and visceral arches.

**Discussion**

The detailed analysis of Myf-6 transcripts in mouse embryos performed by in situ hybridization completes a series of investigations on the temporal and spatial expression pattern of those muscle regulatory genes which have been identified so far. The results confirm that the expression of all members of the MyoD family is restricted to skeletal muscle cells and their precursors. Synthesis is not detected in the heart at any time of development. The tissue and cell types expressing the four genes appear to be identical or at least extensively overlapping, although the temporal pattern of these gene activities are quite distinct. Myf-5 is the first gene that is activated in dermamyotomal cells of newly formed somites before the differentiation of dermatocele and myotome (Ott et al., 1991). Transcription of myogenin and Myf-6 genes is initiated, in this order, in more mature somites 12–24 h later when myotomal cells have formed. The onset of MyoD1 expression follows ∼24 h later (Sassoon et al., 1989). The activation of the genes encoding the muscle regulatory factors occurs

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**Figure 3.** Myf-6 mRNA first appears in 9 d p.c. embryos and accumulates in developing myotomes. Transverse sections of an 18 somite embryo hybridized to the Myf-5 probe (a and c) and the Myf-6 probe (b). Transverse sections of a 24 somite embryo (9.5 d p.c.) hybridized with the Myf-6 probe (d and e) and the Myf-5 probe (f); a and d are phase contrast micrographs, b, c, e, and f are dark field micrographs. Myotomes are indicated by arrowheads. NT, neural tube. Bars: (a–c) 120 μm; (d–f) 240 μm.
Figure 4. Myogenic factors follow a distinct pattern of gene activation during embryonic development. Serial sections at the level of the hindlimb bud of an 11 d p.c. (39–44 somites) embryo were hybridized to the probe for Myf-5 (a), Myf-6 (b), and myogenin (c). More caudal sections of the same embryo as in a–c were hybridized with the probes for Myf-5 (d), Myf-6 (e), and myogenin (f). NT, neural tube; L, limb bud. Bars: (a–c) 200 μm; (d–f) 250 μm.
Figure 5. Myf-6 expression in embryonic myotomes is down regulated in a rostro-caudal gradient. Parallel transverse sections of an 11 d.p.c. embryo (39-44 somites) were hybridized to probes in the following order: Myf-5 (a), Myf-6 (b), and myogenin (c). FL, fore-limb bud. Some myotomes are indicated by arrowheads. Bar: (a-c) 120 μm.

along the same rostro-caudal gradient as somitogenesis. Therefore, the critical parameter for gene activation appears to be the developmental stage of the somites.

There is no indication in vivo of the rapid reciprocal activation phenomenon seen between myogenic factors in cell transfection experiments. Any such phenomenon acting in the somites is more subtle. The initial kinetics of expression might suggest that Myf-5 activates myogenin which activates Myf-6, and that activation of MyoD1 has a negative effect on the initial wave of Myf-6 expression and subsequently on Myf-5. However, what happens in the limb and what happens in fetal muscle when Myf-6 expression reappears suggests that any such regulatory circuits between myogenic factors, if they exist in vivo, are subject to extrinsic modifications.

It was recently shown by Lyons et al. (1990) that mRNAs encoding different isoforms of both cardiac and skeletal muscle myosin heavy chains and myosin light chains are first detected between 9 and 10 d.p.c. in the myotomes of the most rostral somites and appear in more caudal somites at later stages. If, as suggested for the other myogenic factors (see Olson, 1990 for review) and shown in terms of a transcriptional activation domain for Myf-5 (Braun et al. 1990b), these proteins act as transcription factors for muscle-specific genes, Myf-6 could be involved in the activation of these myosin genes, since its transcription starts approximately at the same time of development. However, in tissue culture cells there appears to be no simple pattern of coordinated activation between muscle regulatory genes and structural protein genes (Miller, 1990). There rather may be cooperation between one or more of the myogenic factors and positive or negative general transcription factors, such as El2 (Murre et al., 1989) and Id (Benezra et al., 1990), and this may determine the expression patterns of muscle structural genes.

In addition to their expression in the myotome, initially Myf-5, and slightly later myogenin and MyoD are transcribed in myogenic cells which have migrated into the limb buds and visceral arches (Sassoon et al., 1989; Ott et al., 1991). We have also observed the expression of these three factors in the developing extraocular muscles (Lyons, G. E., and E. Bober, unpublished observations). Within the limits of detection of the in situ hybridization technique, Myf-6 is not expressed in these embryonic structures. These results are particularly interesting because they suggest either that different populations of myogenic precursor cells exist in somites or that extrinsic factors which are involved in the initiation of the myogenic program are different for myotomal cells when compared to myogenic cells which have migrated away from the somite. Myotomal cells are thought to arise from a different portion of the differentiating somite than the cells that migrate out to form muscle in axial structures. Cells from the dorsomedial lip (Ede and El-Gadi, 1986) or from the cranial edge (Kaehn et al., 1988) of the dermamyotome migrate underneath the dermatome to form the myotome. However, cells from the ventrolateral edge of the somite are thought to migrate out to developing limb buds before myotome formation (Jacob et al., 1979; Milaire, 1976).

Little is known about the physiological signals that influence skeletal muscle differentiation in embryos. As myotomes form and muscle gene transcripts begin to accumulate, processes from the neural tube have been observed to contact the myotomal cells (Filogamo and Gabella, 1967). Vivarelli and Cossu (1986) have shown that the presence of the neural
Figure 6. Myf-6 mRNAs are not detected in embryonic limb buds and visceral arches. Serial sections of the forelimb bud of an 11.5 d p.c. embryo were hybridized with probes for Myf-5 (a), Myf-6 (b), and myogenin (c). Sections of the mandibular arch of an 11.5 d p.c. embryo were hybridized to the probes for Myf-5 (d), Myf-6 (e), and myogenin (f). D, dorsal premuscle mass; V, ventral premuscle mass. Bars: (a–c) 240 μm; (d–f) 120 μm.

In contrast, premyogenic cells that migrate to the limb buds and visceral arches are not in close proximity to the neural tube in cultures of 8.5 d p.c. somitic cells is essential for the appearance of myosin-positive cells, but in cultures of 10.5 d p.c. somites, the number of myosin-positive cells appearing is largely independent of the neural tube. Since the four myogenic regulatory factor genes are activated between 8 and 10.5 d p.c., the influence of the neural tube may be essential at least for the initiation of the myogenic program in myotomal cells.

In contrast, premyogenic cells that migrate to the limb buds and visceral arches are not in close proximity to the neural tube.
Figure 7. Myf-6 is reexpressed in fetal skeletal muscles. Transverse sections of the body wall muscles of a 17.5 d.p.c. fetus were hybridized with the probes for Myf-6 (a) and myogenin (b). Bar: (a and b) 200 μm.
neural tube except for a short time before migration. These cells do not express detectable levels of myogenic factor transcripts during their migration and for at least 24 h in the developing limb bud (Sassoon et al., 1989; Lyons, G. E., and M. Buckingham, unpublished observations). For these cells, other extrinsic factors may influence the onset of the myogenic program and the pattern of myogenic factor gene expression. For example, the accumulation of extracellular matrix components involved in the condensation of chondrogenic cells and the redistribution of premyogenic cells into the regions of dorsal and ventral premuscle mass formation may be one process which influences the onset of the myogenic program in somite-derived cells. In contrast, mitogens that are enriched in developing limb buds may delay muscle differentiation.

Considering the developmental time span during which the muscle regulatory genes are actively transcribed, two general patterns can be distinguished. One, exhibited by Myf-5 and Myf-6, is characterized by transient embryonic expression in somites, and in the case of Myf-5, in limb buds and visceral arches. The other, exhibited by myogenin and MyoD, is a pattern of continued expression in embryonic and in fetal muscle. The different timing of the onset of Myf-5 and MyoD gene activation in somites and also their slightly different pattern of down regulation argues against a common regulatory control of both genes despite their close linkage in the genome. This is further supported by the differential regulation of both genes in extrasomatic structures. Finally, in contrast to the Myf-5 gene which is expressed in one wave from day 8 to day 13 p.c. of embryonic development, Myf-6 exhibits a biphasic pattern of expression. The first phase lasts from 9 to 11.5 p.c. in myotomes, and the second phase begins around 16 d.p.c. in fetal skeletal muscle with expression continuing into adult muscle.

The reappearance of Myf-6 transcripts in fetal skeletal muscle occurs at approximately the same time as the beginning of the formation of secondary muscle fibers and the establishment of neuromuscular junctions (Ott et al., 1989). Thus at least the initiation of Myf-6 expression in fetal skeletal muscle may be controlled by innervation. Experiments on mouse embryos denervated in utero should provide further information.

In conclusion, the analysis of the patterns of expression of the four related myogenic factor genes Myf-5, myogenin, Myf-6, and MyoD during embryonic and fetal development in the mouse indicates that each of these genes follows a distinct time course of activation, accumulation and inactivation. This pattern is different in two populations of myogenic cells, those in myotomes and those in other structures in the embryo. These results provide a basis for further investigation of which signals and which genes may be involved in the control of the muscle-specific regulators.

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