Unprocessed Myogenin Transcripts Accumulate during Mouse Embryogenesis*

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Alejandro Sánchez and Jeffrey Robbins

From the Department of Pediatrics, Division of Molecular Cardiovascular Biology, Children's Hospital Research Foundation, Cincinnati, Ohio 45229-3039

The gene myogenin encodes a helix-loop-helix protein whose function is critical to the integrity of the mammalian myogenic cascade. In vitro, the expression of this gene immediately precedes terminal differentiation, as measured by the synthesis of those proteins that make up the contractile apparatus. However, during mammalian development, expression of myogenin and the appearance of sarcomeres are separated by at least 1 week. The observation that early embryos (10.5 days post coitum) do not possess myogenin protein despite the fact that transcripts are detected (Cusella-De Angelis et al. (1992) J. Cell Biol. 116, 1243-1255), suggests the possibility of post-transcriptional regulation. Using polymerase chain reaction and in situ analyses, we report here that the mouse embryo accumulates a significant pool of unprocessed myogenin RNA in the developing somites (10.5 days post coitum). These results indicate that post-transcriptional regulation of myogenin may occur at the RNA processing level.

Members of the myc family of helix-loop-helix (HLH)
proteins play fundamental roles in myogenesis. For example, MyoD encodes a protein whose expression is able to convert fibroblasts to skeletal muscle in vitro (1), and other members of this class, including myogenin, myf-5, and myf-6, also encode peptides that are critical for establishing the terminal myogenic program. Surprisingly, recent gene ablations of MyoD (2), and myf-5 (3) are not capable in of themselves of arresting myogenesis in mice, although the double mutants do show marked deficits in skeletal muscle.2 These results indicate that a possible redundancy in the whole animal context may exist for at least parts of the myogenic cascade.

In contrast with these results, ablation of myogenin results in a total inhibition of terminal myogenic differentiation (4). Thus, while the possibility of functional redundancy may exist at the beginning of the myogenic cascade (where MyoD is thought to play a critical role), this may be lost toward the end of the cascade, where the expression of myogenin appears obligatory for the progression of terminal differentiation. In vivo, myogenin displays a transcriptional pattern that differs from the other myogenic effectors in that it is expressed in all of the apparent embryonic skeletal muscle lineages (5, 6). Evidence suggests that myogenin can be modulated by other HLH proteins (7) as well as by MEF-2, a member of the MADS box family of transcription factors (8, 9). Transgenic analyses are consistent with the hypothesis that myogenin expression during myogenic lineage specification is controlled by an interplay between HLH factors and MEF-2 (6). Mutation of the E1 box in the myogenin promoter, for example, greatly diminishes its expression in 11.5-day post coitum (p.c.) limb buds, without significantly affecting its expression in somites (6). However, these results differ from those obtained with experimenters carried out in cultured cells, where E1 mutations failed to affect myogenin expression (10).

A second, intriguing discrepancy between the in vitro and in vivo profiles of myogenin expression/function is that in vitro, expression of myogenin immediately precedes terminal differentiation (11, 12), while in the embryo this is not the case. During murine embryogenesis, the myogenin transcript is detected using in situ hybridization techniques as early as day 8.5 p.c., that is, during early somitogenesis (13, 14). However, the formation of striated muscle occurs significantly later during development, at approximately day 14 p.c. (15). It can be argued that the apparent lag between the expression of myogenin and the appearance of the terminal phenotype in vivo is the result of myogenin’s participation in complex regulatory circuits not mimicked by cell cultures. However, the observation that the expression of myogenin during somite proliferation is not accompanied by the protein’s synthesis (16) weakens this hypothesis and suggests that the protein’s synthesis might be regulated at the post-transcriptional level. Thus, current data indicate that myogenin is transcriptionally active during somitogenesis, but the transcripts are not immediately utilized by the translational apparatus during myogenic lineage specification.

Because of these observations, we wished to examine more closely the transcriptional patterns of myogenin and the nature of the transcripts during late embryogenesis and early fetal development. Here we report that a significant pool of heterogeneous nuclear (hn) myogenin transcripts exists during the stages of somite formation and proliferation, and that its appearance correlates temporally with the formation of skeletal muscle at day 14 p.c.

EXPERIMENTAL PROCEDURES

Reverse Transcription and PCR Analyses—Specific amplimer sets for the detection of MyoD and myogenin transcripts were designed. The sequences are as follows: MyoD, 5'-AGTGGTCCTGAGACCAGGG-GAAAGG-3'; MyoD, 5'-GGGAAGAGCAGAAGTCGGCTG-TCTAG-3'; Myog, 5'-GGAGGTGGAGGGTGGAATTCGAGG-3'; Myog, 5'-CTGGAAGTCGGCTGAGACCAGGG-3'. The oligonucleotides complementary to the MyoD and myogenin 3'-untranslated regions (UTRs) (MyoD, and Myog,) were used to direct first strand synthesis of the cDNA. Total RNA from FVB/N mouse embryos was prepared using RNAzol (Cinna Biotec, Friendswood, TX). The cDNA synthesis and the PCR reactions were carried out as described (17). The cycling sequence was as follows: an initial denaturation for 3 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 30 s at 65 °C, and 30 s at 72 °C. A final 10-min extension at 72 °C was performed.

For the detection of contaminating DNA templates in the RNA samples analyzed, two oligonucleotides specific for a promoter region of
Experimental Procedures). One to two pg of total RNA was used for each RT-PCR reaction. The expected positions and sizes of the myogenin and MyoD PCR products are indicated. Using RNA from day 7.5 p.c., the myogenin amplimers generate a 1007-bp PCR product (hnMg) as well as the expected 480-bp fragment (Mg). Lane 1, myogenin amplimers were used; lane 2, MyoD amplimers were used. M, HaeIII-digested dX174.

the myogenin gene were designed: Myogenin1 (5'-CTTCCTGCCTGTC- CACCAGCTGC-3'), and Myogenin2 (5'-CCCTCCTGCTGGCATGAACCAG-3'). The PCR cycling sequence for these amplimers consisted of 30 cycles of 1 min at 94°C, 30 s at 60°C, and 30 s at 72°C. A final 10-min extension at 72°C was performed. All PCR samples were analyzed by electrophoresis of 10-µl aliquots in 2% agarose gels.

Riboprobe Construction and Synthesis—The PCR products obtained with the MyoD- and myogenin-specific amplimers were subcloned into pIBI31 (Kodak-IBI, New Haven, CT). The clones were sequenced. In order to generate the riboprobes, 1 pg of each recombinant plasmid DNA was linearized with either Sty I (myogenin) or BamH I (MyoD and hn-myogenin). The riboprobes were then synthesized using [α-32P]UTP (1500 Ci/mmol; DuPont NEN) and 10–15 units of T7 RNA polymerase (Kodak-IBI) as described (17).

In Situ Hybridization—FVB/N mouse embryos were dissected, fixed, and cryoprotected using standard protocols (18, 19). Two 8-µm sagittal sections were mounted per slide. All sections were hybridized overnight with 1 x 106 cpm/µl of the [α-32P]UTP-labeled cRNA probe at 55°C as previously described (17). The sections were washed twice at 55°C for 30 min in 50% formamide, 2 x SSC, and 10 min dithiothreitol, followed by two 15-min washes in 2 x SSC, and a final wash in 0.1 x SSC at 50°C. The slides were dehydrated, dipped in Ilford K5 emulsion (Electron Microscopy Sciences, Fort Washington, PA), and exposed for 10 days in light-tight boxes with desiccant at 4°C. Kodak D19 developer was used to process the slides, and the sections were analyzed using the phase-contrast and dark-field optics of an Olympus BHTU microscope.

RESULTS

Detection of HLH Transcripts in Mouse Embryos: MyoD and Myogenin—The pattern of myogenin transcription was compared with that of another myogenic gene, MyoD. RNA was isolated from early embryos, and transcripts originating from MyoD and myogenin were detected using reverse transcription and PCR (RT-PCR) (17). Oligonucleotide pairs specific for the detection of these transcripts were designed (see “Experimental Procedures”) such that the resulting PCR products amplified from the myogenin and MyoD transcripts are 480 and 369 bp, respectively. Representative RT-PCR reactions are shown in Fig. 1.

At 7.5 days p.c. when somites are first formed, the MyoD transcript is readily apparent using these techniques (Fig. 1, 7.5 days p.c., lane 2). The 480-bp fragment diagnostic for myogenin transcripts is also present (Fig. 1, 7.5 days p.c., lane 1).

Surprisingly, a 1007-bp fragment was also generated using the myogenin amplimers. This DNA product appears to be re-

Fig. 1. Temporal expression of the myogenin and MyoD genes during in vivo development. RNA was isolated from whole mouse embryos (7.5 and 14 p.c.) and the adult gastrocnemius (Ad) (see “Experimental Procedures”). One to two pg of total RNA was used for each RT-PCR reaction. The expected positions and sizes of the myogenin and MyoD PCR products are indicated. Using RNA from day 7.5 p.c., the myogenin amplimers generate a 1007-bp PCR product (hnMg) as well as the expected 480-bp fragment (Mg). Lane 1, myogenin amplimers were used; lane 2, MyoD amplimers were used. M, HaeIII-digested dX174.

A

DNA

315 bp

introns

hnRNA

mRNA

UTRs

B

FIG. 2. Ruling out DNA contamination in the RT-PCR analyses.

Panel A, the intron/exon organization of myogenin is shown (the sequence was generously provided by Dr. Eric Olson), as well as the primary and processed transcription products (hnRNA and mRNA). The locations of the amplimer sets used to detect DNA (rectangles 1 and 2) and RNA (rectangles 3 and 4) are indicated. The products expected from the amplification reactions are also shown. A 361-bp PCR fragment with amplimers 1 and 2 is diagnostic for DNA contamination in RNA samples. Panel B, DNA contamination assays of embryonic RNA. The expected sizes of PCR products are shown on the right. Lanes 1 and 2 are DNA markers (HaeIII-digested dX174 and EcoRI-HindIII-digested λ phage). Lane 3 shows the control, 361-bp diagnostic band generated by amplimers 1 and 2 (panel A) from mouse genomic DNA. Lane 4 shows the fragments generated when 7.5-day p.c. total RNA was amplified with the promoter-nested amplimers. Lane 5: the 1007-bp fragment was generated as expected when the oligonucleotides nested in exons 2 and 3 (panel A) were used with a different aliquot of the same RNA sample after reverse transcription.
MyoD and myogenin Gene Expression in Embryos: In Situ Hybridization Analyses—The detection of unprocessed myogenin RNA in the form of a 1007-bp amplified fragment (Figs. 1 and 2) was intriguing. The identity of the DNA was confirmed by subcloning and sequencing the 1007-bp PCR fragment (data not shown). The fragment contains the 3' end of the second exon, the second intron (530 bp), and part of the third exon (Fig. 2, panel A). To confirm and extend these data, we undertook an analysis of the processed and unprocessed myogenin transcripts in the developing embryo using in situ analysis. In order to design a riboprobe that differentiated between the processed and unprocessed transcripts in situ, a RsaIRsAI restriction fragment within myogenin's second intron was subcloned such that antisense riboprobes specific for the unprocessed RNA could be generated (see “Experimental Procedures”). A probe corresponding to the 3'-end of the myogenin transcript was also used. The sequence in this region has been used to detect myo-D and myogenin, and indicate that the myogenin hybridization signal detected in 10.5-day somites results, in part, from the accumulation of unprocessed myogenin RNA.

MyoD and myogenin probes to sagittal sections of 14-day p.c. fetuses further corroborate the RT-PCR data (Fig. 3). Both the MyoD and 3'-UTR myogenin riboprobes hybridize to regions of skeletal muscle (Fig. 3, 14 days p.c., panels D and E, arrows). However, the intron-derived myogenin riboprobe did not yield a signal above background in an adjacent section of the same embryo (Fig. 3, panel F). It is interesting to note that the unprocessed myogenin transcripts cannot be detected at the developmental stage (14 days p.c.) at which myotube formation occurs.

**DISCUSSION**

These data are consistent with the possibility that post-transcriptional regulation of the myogenin transcript occurs during embryogenesis. The myogenic gene myogenin, implicated in the terminal differentiation of myoblasts in vitro (5, 20), is transcriptionally active during the early stages of somitogenesis. However, instead of properly spliced transcripts, a large pool of unprocessed RNA (hn-myogenin) is present and is localized in the somites. This observation may explain previous data, which showed that, in 10.5-day p.c. embryos, somites expressing myogenin did not cross-react with a monoclonal antibody against myogenin (16). The probes used by these and other investigators (14) hybridize to both the processed and unprocessed myogenin transcripts. During the early stages of somite formation, hn-myogenin is present in relatively significant amounts (Figs. 1 and 3). As embryogenesis progresses, the PCR fragment diagnostic for properly spliced myogenin (480 bp) becomes more prevalent, until it is the only PCR product detectable in the fetus (Fig. 1, 14 days p.c.) and in adult skeletal muscle (Fig. 1, Ad). The accumulation of unprocessed transcripts, as detected by our hn-myogenin probe, and the apparent lack of myogenin protein is consistent with post-transcriptional regulation of myogenin occurring during the early stages of the myogenic program.

Post-transcriptional regulation of genes during mammalian embryogenesis has been reported for other transcription factors as well as for two contractile proteins. For example, growth hormone factor 1 mRNA has been detected during the early stages of murine pituitary organogenesis, while the corresponding protein is not detected until 2 days later, after adenohypophysis differentiation is complete (21). During rodent development the neonatal myosin heavy chain (18) and the cardiac troponin I (22) mRNAs are detected several days before the cognate proteins. Post-transcriptional regulation of at least one HLH gene, Id, has been postulated. During myoblast proliferation Id is expressed and steady state transcript levels increase. When the proliferating myoblasts begin to differentiate, Id transcripts become unstable and are rapidly degraded (23).

In several organisms, splicing is used to control gene expression and complex developmental pathways (24, 25). In *Drosophila*, sex determination in somatic cells is regulated by splicing. The generation of female transcripts from transformer results not through the activation of alternative splicing, but rather by blockage of one of the splicing sites (26). This is accomplished by the direct binding of the Sex-lethal (Sxl) gene product to a poly(U) sequence run in the polypyrimidine tract of the 3' end of the splicing site (27, 28). Interestingly, the transcriptional activation of Sxl is modulated by the proteins acheta-scute (29) and daughterless (30), both of which are members of the basic HLH family of transcriptional regulators. Whether or not an HLH-regulated mammalian homologue of Sxl exists is not known, but it is intriguing that a poly(U) sequence homologous to that of the transformer gene in *Drosophila* exists in the polypyrimidine tract of the 3' splice site of myogenin's second intron.

Another provocative possibility is that the unspliced myo-
genin transcript itself serves as a regulatory factor. In certain cases, 3'-UTRs are able to act as trans-acting regulators capable of modulating cell division and differentiation, probably by binding, and thus sequestering, proteins involved in these processes (31). The large abundance of unprocessed myogenin transcripts, as well as their absence in isolated polysomal fractions (16), is consistent with this possibility.

The in situ hybridization data (Fig. 3, 10.5 days p.c.) show that when an intronic sequence is used for a riboprobes, some of the myogenin transcripts present in the somites are unspliced and, therefore, presumably untranslated. The restricted ability of early myoblasts to splice hn-myogenin and subsequently generate detectable protein might explain why this factor's activity is relegated to the final stages of myogenic differentiation (Figs. 1 and 3, day 14). This mechanism provides a possible explanation for the functional position of myogenin in the later events of the myogenic cascade.

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REFERENCES


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