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Contractile protein gene expression in primary myotubes of embryonic mouse hindlimb muscles

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SUMMARY

The time course of contractile protein [actin, myosin heavy chain (MHC) and myosin light chain (MLC)] gene expression in the hindlimb muscles of the embryonic mouse (<15 days gestation) has been correlated with the expression of genes for the myogenic regulatory factors, myogenin and MyoD, and with morphogenetic events. At 14 days gestation, secondary myotubes are not yet present in crural muscles (M. Ontell and K. Kozeka (1984) *Am. J. Anat.* 171, 133-148; M. Ontell, D. Bourke and D. Hughes (1988) *Am. J. Anat.* 181, 267-278); therefore, all transcripts for contractile proteins found in these muscles must be produced in primary myotubes. In situ hybridization, with ^{35}S-labeled antisense cRNAs, demonstrates the versatility of primary myotubes in that transcripts for (1) α-cardiac and α-skeletal actin, (2) $MHC_{embryonic}$, $MHC_{perinatal}$ and $MHC_{\beta/slow}$, and (3) MLC_{1A}, MLC_{1F} and MLC_{3F} are detectable at 14 days gestation. While the general patterns of early activation of the cardiac genes and early activation of the genes for the developmental isoforms are preserved in both myotomal and limb muscles (D. Sassoon, I. Garner and M. Buckingham (1988) *Development* 104, 155-164 and G. E. Lyons, M. Ontell, R. Cox, D. Sassoon and M. Buckingham (1990) *J. Cell Biol.* 111, 1465-1476 for myotomal muscle), there are a number of differences in contractile protein gene expression. For example, in the myotome, when myosin light chain genes are initially transcribed, hybridization signal with probe for MLC_{1A} mRNA is greater than that with probe for MLC_{1F} transcripts, whereas the relative intensity of signal with these same probes is reversed in the hindlimb. The order in which myosin heavy chain genes are activated is also different, with $MHC_{embryonic}$ and $MHC_{perinatal}$ preceding the appearance of $MHC_{\beta/slow}$ transcripts in limb muscles, while $MHC_{embryonic}$ and $MHC_{\beta/slow}$ appear simultaneously in the myotomes prior to $MHC_{perinatal}$. In the myotome, an intense hybridization signal for α-cardiac and a weak signal for α-skeletal actin transcripts are detectable prior to myosin mRNAs, whereas in the limb α-cardiac actin transcripts accumulate with myosin transcripts before α-skeletal actin mRNA is detectable. These differences indicate that there is no single coordinate pattern of expression of contractile protein genes during initial formation of the muscles of the mouse. The fact that different myogenic factors are present initially in myotomes than are present initially in the hindlimb (cf. M. E. Buckingham (1992) *Trends Genet.* 8, 144-149) suggests that there may be subtle differences in the capacity of these factors to activate different muscle genes. These results are discussed in the context of muscle cell lineages.

Key words: actin, myosin, gene expression, embryonic mouse, primary myotubes, myogenic regulatory factors

INTRODUCTION

Mammalian muscle formation is a biphasic phenomenon (Wohlfart, 1937; Ashmore et al., 1972), characterized by successive myogenic waves resulting in two discrete populations of myotubes, referred to as primary (i.e., first formed) and secondary (i.e., formed by a subsequent wave of myogenesis) myotubes. In the crural muscles of the mouse, where the time course of muscle morphogenesis has been investigated extensively, a population of primary myotubes is present by 12 days gestation. After primary myotube formation is completed, there is a delay of ~2 days before secondary myotube formation commences. Secondary myotubes form using the primary myotubes as a scaffold for their development (Ontell and Kozeka, 1984a; Ontell et al., 1988a). While it was originally suggested that

primary myotubes are the source of adult rat slow myofibers and that secondary myotubes are the source of adult rat fast myofibers (Kelly and Rubinstein, 1980), the relationship between the birthdate of a myofiber and its ultimate fiber type is more complex (Condon et al., 1990; LaFramboise et al., 1991). Recently, it has been suggested that secondary myotubes are the source of ~60% of the slow myofibers in the adult rat diaphragm (La Framboise et al., 1991).

The contractile proteins actin and myosin exist in multiple, tissue-specific and developmentally regulated isoforms (Whalen et al., 1981) encoded by families of genes (cf. Buckingham, 1989; cf. Pette and Staron, 1990). The sequential expression of myosin genes from the late fetal (15-16 days gestation) to adult stages has been analyzed at both the protein and mRNA levels in mouse and rat skeletal muscle (Rubinstein and Kelly, 1981; Whalen et al., 1981; Nadal-Ginard et al., 1982; Lyons et al., 1983; Weydert et al., 1983; Narusawa et al., 1987; Weydert et al., 1987; Barton et al., 1989). The expression of myosin (myosin heavy chain (MHC) and myosin light chain (MLC)) (Lyons et al., 1990) and actin genes (Sassoon et al., 1988) in mouse myotomal muscles during embryonic development has been studied with in situ hybridization. However, since the time course of primary and secondary myotube formation and the time course of innervation of myotomal muscles have not been determined, it has not been possible to correlate morphogenetic events with contractile protein gene expression. In the present study, specific ^{35}S-labeled cRNA probes to the 5′ and 3′ untranslated regions (UTR) of cardiac and skeletal α-actin and myosin mRNAs have been used for in situ hybridization on sections of hindlimb muscles of 10-14 day gestation mice (defined in this paper as embryonic mice). During this period, hindlimb buds first appear (Theiler, 1989) and primary myotube formation, but not secondary myotube formation, occurs in the muscles of the crus (region between the knee and the ankle) (Ontell and Kozeka, 1984a,b; Ontell et al., 1988a,b). Therefore all of the autoradiographic signal emanating from the hindlimb muscles reflects the presence of mRNAs for contractile proteins in primary myotubes. The myogenic regulatory sequences (cf. Olson, 1990; Weintraub et al., 1991; Wright, 1992) MyoD and myogenin have a different time course of expression in limb as compared with myotomal muscle (Sassoon et al., 1989). It is, therefore, of particular interest in this study to compare results obtained for the expression of actin and myosin genes in the developing limb with their expression in myotomes.

MATERIALS AND METHODS

Preparation and prehybridization of tissue sections

10 to 15 day gestation fetuses (staged using criteria described in Theiler, 1989) were removed from the uterus of anesthetized (ketamine, 0.01 mg/g body weight, I.P., and xylazine, 0.02 mg/g body weight, I.P.) Swiss Webster mice, and the dams were killed by cervical dislocation. Age-matched whole fetuses and disarticulated hindlimbs were fixed in 4% paraformaldehyde in PBS, dehydrated and infiltrated with paraffin. 6 μm thick, serial sections (2 sections per slide) were mounted on subbed slides (Gall and Pardue, 1971) and treated using a modification of the protocol of Wilkinson et al. (1987). Briefly, sections were deparaffinized in xylene, rehydrated, digested with proteinase K, postfixed, treated with dithiothreitol/iodoacetamide/N-ethylmaleimide (to reduce non-specific ^{35}S-binding; Zeller and Rogers, 1989), treated with triethanolamine/acetic anhydride, washed and dehydrated.

Probe preparation

To distinguish between transcripts within the myosin and actin multigene families, it is necessary to use probes derived from the 3′ or 5′ untranslated regions (UTR) of the mRNAs. Appropriate restriction fragments or oligonucleotides were subcloned into the vector Bluescribe$^+$ (Stratagene Cloning Systems, LaJolla, CA). Bluescribe$^+$ was grown in *E. coli* TGI. Probes (sequences described in Lyons et al., 1990 unless otherwise indicated) to the following mRNAs were used:

(a) 3′ UTR of mouse MLC_{1A} (MLC1 embryonic) mRNA (Barton et al., 1988), probe length 130 bases. This sequence is available from EMBL/GenBank/DDJB under accession numbers M20773, M19435 and J03932.

(b) 5′ UTR of mouse MLC_{1F} mRNA (Robert et al., 1984), probe length 60 bases. This sequence is available from EMBL/GenBank/DDJB under accession number K02237.

(c) 5′ UTR of mouse MLC_{3F} mRNA (Robert et al., 1984), probe length 61 bases. This sequence is available from EMBL/GenBank/DDJB under accession number K02238.

(d) 3′ UTR of the mouse MLC_{1V} mRNA (Lyons et al., 1990), probe length 82 bases. This sequence is available from EMBL under accession number X67685.

(e) 5′ UTR of rat MHCembryonic (MHC_{emb}) mRNA (Strehler et al., 1986), probe length 70 bases. Sequence data are available from EMBL/GenBank/DDJB under accession numbers X04267 and X05004.

(f) 3′ UTR of rat MHCslow/ventricular ($MHC_{\beta/slow}$) mRNA (Mahdavi et al., 1982), probe length 68 bases. Sequence data are available from EMBL/GenBank/DDJB under accession number K01463.

(g) 3′ UTR of mouse MHCperinatal (MHC_{pn}) mRNA (Weydert et al., 1985), probe length 130 bases. Sequence data are available from EMBL/GenBank/DDJB under accession number M12289.

(h) 3′ coding region of mouse MHC_{emb} (MHC_{E320}) mRNA (Weydert et al., 1985), probe length 320 bases. This probe reacts with all fast MHC transcripts, but does not hybridize to transcripts for $MHC_{\beta/slow}$ (Ontell, unpublished observation). Sequence data are available from EMBL/GenBank/DDJB under accession number M11154.

(i) 5′ UTR of mouse α-cardiac actin mRNA (Sassoon et al., 1988), probe length 50 bases, sequence as described in Sassoon et al. (1988). Sequence data are available from EMBL/GenBank/DDJB under accession number X03767.

(j) 5′ UTR of mouse α-skeletal actin mRNA (Sassoon et al., 1988), probe length 60 bases, sequence as described in Sassoon et al. (1988). Sequence data are available from EMBL under accession number X67686.

(k) Myogenin 3′ coding and noncoding region (Wright et al., 1989). This cRNA corresponds to the terminal 700 nucleotides of the rat myogenin cDNA. It was kindly provided by W. Wright.

(l) MyoD 3′ region (Davis et al., 1987). This cRNA corresponds to nucleotides 751-1785 of mouse MyoD mRNA (Sassoon et al., 1989). It was kindly provided by A. Lassar and H. Weintraub.

The cRNA transcripts were synthesized according to manufacturer's conditions (Stratagene Cloning Systems) and labeled with [^{35}S]UTP (>1,000 Ci/mmol; NEN Research Products, Wilmington, DE). cRNA transcripts larger than 100 nucleotides were subjected to alkali hydrolysis to give a mean size of 70 bases for effi-

cient hybridization. Sense (control) probes were similarly synthesized.

Hybridization and washing procedures

The hybridization procedures were as described by Wilkinson et al. (1987). Sections were hybridized overnight at 52°C in 50% deionized formamide, 0.3 M NaCl, 20 mM Tris-HCL, pH 7.4, 5 mM EDTA, 10 mM $NaPO_4$, 10% dextran sulfate, 1× Denhardt's, 50 μg/ml total yeast RNA, and 60,000 cpm/μl ^{35}S-labeled cRNA probe. They then were washed at 48°C for 30 minutes in 5× SSC containing 10 mM DTT; followed by washing at 65°C for 20 minutes in 50% formamide, 2× SSC, 10 mM DTT; and then two times at 37°C for 10 minutes in Washing Solution (400 mM NaCl, 10 mM Tris-HCl, pH 7.5, 50 mM EDTA). After treatment with 20 μg/ml RNAseA at 37°C for 30 minutes, they were washed at 37°C for 5 minutes in Washing Solution, followed by washes for 15 minutes at 37°C in 2× SSC and for 15 minutes at 37°C in 0.1× SSC. Sections were dehydrated and slides dipped in Kodak NTB-2 nuclear track emulsion and exposed for 1 week in light-tight boxes with desiccant at 4°C. After development in Kodak D-19, slides were counterstained with 0.1% toluidine blue and analyzed with both bright-field and dark-field optics of a Leitz microscope.

Evaluation of hybridization signal

In order to compare the intensity of hybridization signal with a given cRNA probe over time (i.e., from 10 days to 14 days gestation), sections at each age were hybridized with the same probe preparation, washed, dipped into emulsion, exposed and developed together. Changes in the level of hybridization signal with a given cRNA probe over time were evaluated by taking dark-field micrographs, using Polaroid film, with a constant light intensity and a constant time of exposure of the film to the light source. Since the length of the probes varied, it was not possible directly to correlate intensity of signal from different probes with the amount of transcripts for each gene unless the sizes of the probes and the number of uridines in the probes were considered. When it was desirable to compare the relative intensity of hybridization signal of two different probes at a given age, serial sections of the hindlimb muscles were reacted with the two probes, processed for autoradiography and developed at the same time. A similar protocol using Polaroid film (as described above), with a constant light intensity and a constant time of exposure of the film to the light source was used to compare the intensity of signal for these two probes at a given developmental stage. Hybridization signals over the muscles with sense (control) probes were not above background.

RESULTS

In the mouse, hindlimb buds become visible at the level of somites 23-28 at 10 days gestation (~30 somite stage; Theiler, 1989). At this stage transcripts for the myogenic regulatory factor myogenin (Wright et al., 1989), α-cardiac and α-skeletal actin (Sassoon et al., 1988), MLC_{1A} and MLC_{1F}, and MHC_{emb} and $MHC_{\beta/slow}$ (Lyons et al., 1990) were present in the somites. There was no detectable hybridization with probes for any of the mRNAs for the contractile proteins actin and myosin, nor was there any hybridization with probes for myogenin or MyoD (another myogenic regulatory factor; Davis et al., 1987) in the limbs of these mice (not shown). Despite the extensive growth of the limbs that occurred between 10 and 11 days gestation, there was still no detectable level of transcript accumulation in the hindlimbs at 11 days gestation (40-44 somites) with any of the probes used in this study (not shown).

During the next 12 hours, there was a dramatic change in expression of the genes for the myogenic factors and for the contractile proteins, actin and myosin, in the mouse hindlimb (Fig. 1). In agreement with previous studies (Sassoon et al., 1989), transcripts for both myogenin and MyoD appeared in the hindlimb bud by 11.5 days gestation (Fig. 1E,H,L), corresponding to the time of the first appearance of primary myotubes in crural muscles of the hindlimb. (The full complement of primary myotubes is present in the extensor digitorum longus muscle (EDL) by 12 days gestation; Ontell and Kozeka, 1984b.) The level of myogenin and MyoD expression remained constant from their initial appearance until 14 days gestation (results summarized in Fig. 2). Transcripts for α-cardiac actin but not α-skeletal actin also were detected in the hindlimb at this stage (compare Fig. 1D,I and K). Simultaneously with the appearance of the α-cardiac actin transcripts, MLC_{1F} transcripts (Fig. 1F) and MLC_{1A} transcripts (Fig. 1J) were present in the hindlimb muscles. However, the intensity of the hybridization signal for the MLC_{1A} transcripts was markedly less than that for the MLC_{1F} mRNAs. This occurred despite the fact that the MLC_{1A} probe is more than twice as large as the MLC_{1F} probe and has 2.5 times more uridines than the MLC_{1F} probe (Lyons et al., 1990). At 11.5 days gestation, there was no detectable hybridization with probes specific for any MHCs (with exposure to the emulsion for 7 days prior to developing the autoradiographs). However, MHC transcripts were detected with a probe (MHC_{E320}) complementary to the COOH-terminal coding region of all known fast, skeletal muscle-specific, MHC gene transcripts. In order to determine whether increased exposure time prior to development of autoradiographs would reveal signal using MHC-specific probes, serial sections were reacted with probes specific to MHC_{emb}, $MHC_{\beta/slow}$ and MHC_{pn}, and the emulsion was exposed for 12 days prior to development of the autoradiographs. A very weak signal was seen with probe for MHC_{emb} transcripts (Fig. 1B) but not with the probes for the other specific transcripts. The more intense hybridization seen with the non-specific MHC probe (Fig. 1C) was probably, in part, due to the greater length of this probe. (See Materials and Methods.) Except for the intensity of the signal resulting from hybridization with probes for myogenin, MyoD and MLC_{1F}, the signals observed with each probe in the hindlimb muscles, at this stage, were less than that observed in the somites of the same mouse (Fig. 1).

By 12 days gestation, all muscle masses in the crus of the hindlimb contained primary myotubes and a pleomorphic population of mononucleated cells. Primary myotube formation had been completed in the EDL muscle by this stage (Ontell and Kozeka, 1984b). The soleus muscle could not be identified as a discrete muscle, as its segregation from the surrounding musculature is not yet completed (Ontell et al., 1988a). At this stage of development, several additional skeletal-muscle-specific transcripts were detected in the hindlimb muscles. Message for α-skeletal actin (Fig. 3E) was detected, but the hybridization signal for this message was less than that for the transcript for the cardiac

actin isoform (Fig 4C). Transcripts for both MHC_{emb} (Fig. 3B) and MHC_{pn} (Fig. 3D) were detected after exposure of the emulsion for 7 days prior to development of the autoradiographs. In contrast, transcripts for $MHC_{\beta/slow}$ were not detected, even after a 12 day exposure (Fig. 3C). The intensity of hybridization signal from hindlimb muscles with probes for α-cardiac actin and MLC_{1A} mRNAs had increased at 12 as compared with 11.5 days gestation (compare Figs 1 and 4), but the signal for MLC_{1A} remained less intense than that for MLC_{1F} (compare Fig. 4B and D).

No new transcripts for actin or myosin were detected at 12.5 days gestation; however, the levels of signal for transcripts for MLC_{1F} and α-cardiac actin were greater at this stage than at earlier developmental ages (Fig. 2). Again at

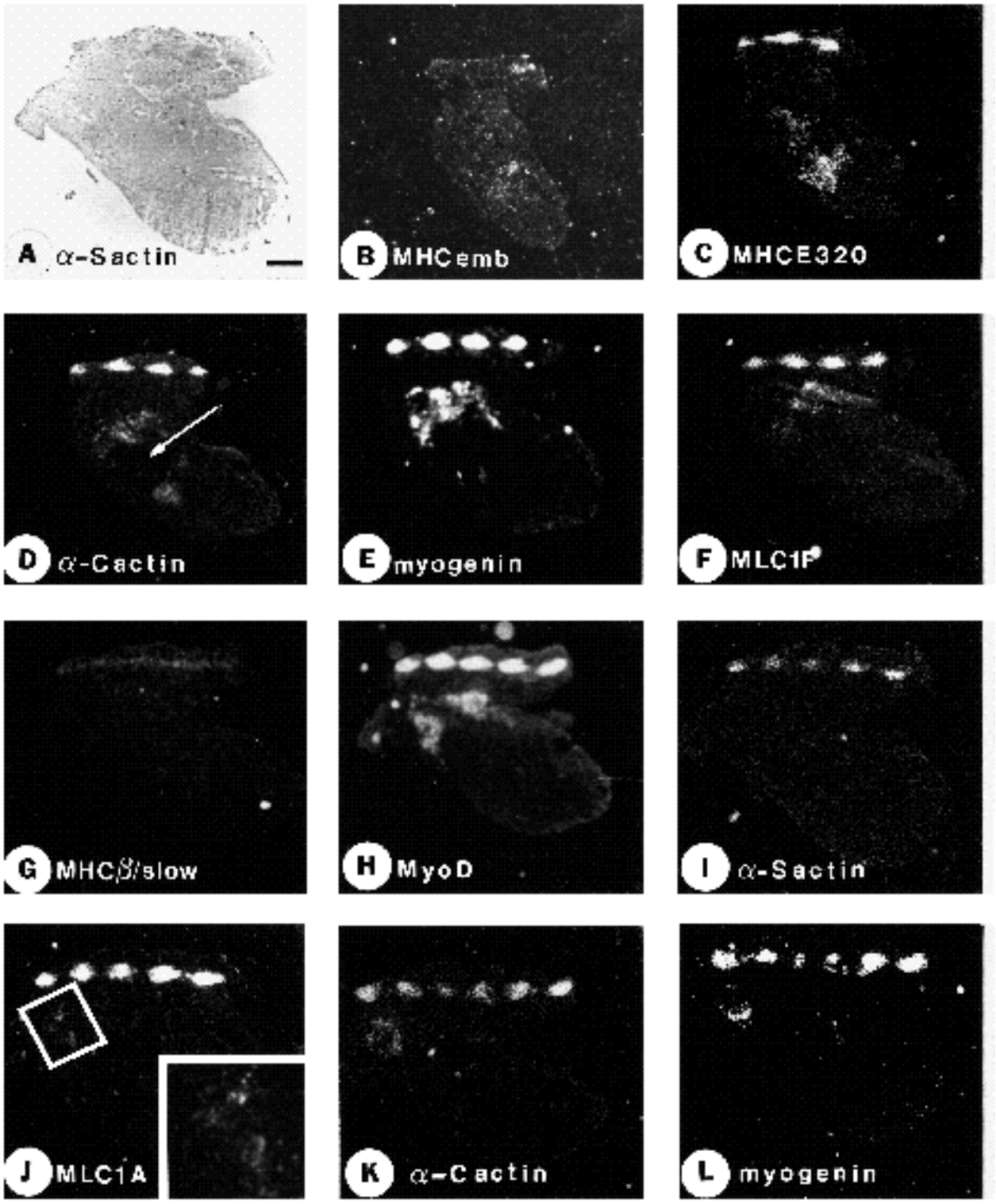

Fig. 1

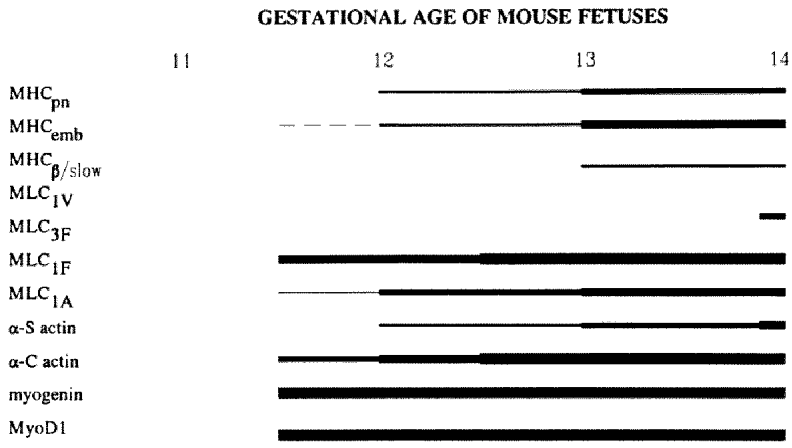

Fig. 2. The appearance and modulation of transcript accumulation for the myogenic regulatory factors (myogenin, MyoD) and the contractile protein transcripts (for actin and myosin isoforms) in the developing hindlimb muscles. The level of intensity of the signal resulting from hybridization is reflected in the thickness of the lines (see Materials and Methods). Number of specimens = 3 at 11 and 11.5 days gestation; 4 at 10 and 12. 5 days gestation; 5 at 12 days gestation; 6 at 14 days gestation; 8 at 13 days gestation.

this stage and throughout the developmental period studied, the intensity of the signal with the probe for MLC_{1A} transcripts was less than the signal for MLC_{1F} transcripts.

At 13 days gestation, transcripts for $MHC_{\beta/slow}$ were detected (Fig. 5E) and there was an increase in the intensity of hybridization signals with probes for MHC_{emb} (Fig. 2), MHC_{pn} (compare Figs 5D and 3D) and α-skeletal actin (Figs 5G, 3E) transcripts as compared with earlier stages. It was at this stage that the transcript for the developmental isoform MHC_{emb} reached its greatest level of accumulation (Fig. 2). The level of signal with the probe for α-skeletal actin transcripts (Fig. 5G) remained less than that for the cardiac isoform (Fig. 5B). At this stage, a clear proximal-distal gradient in the expression of contractile protein transcripts was evident, in that some mRNAs present in the crus were not present in the foot pad (e.g., transcripts for $MHC_{\beta/slow}$; Fig. 5E). There was a correlation between the time at which the gene was first expressed in the limb and the extent of hybridization signal along the proximal-distal gradient, with transcripts for the genes expressed earliest in the limb (e.g., MyoD, Fig. 5F; α-cardiac actin, Fig. 5B; MLC_{1F}, Fig. 5C and myogenin, not shown) exhibiting greater accumulation in the foot pad than genes expressed later in limb development (e.g., genes for $MHC_{\beta/slow}$, Fig. 5E and MHC_{pn}, Fig. 5D).

At 14 days gestation, the developmental stage at which the mouse EDL contained its full complement of primary myotubes and the soleus muscle (which now is identifiable as a discrete muscle) contained ~60% of its primary myotubes (Ontell et al., 1986b), the first primitive neuromuscular junctions were found in the EDL but not in the soleus muscle (Ontell and Kozeka, 1984a; Ontell et al., 1988a). It is at this stage that transcripts for MLC_{3F} (Fig. 6E) appeared, at a hybridization intensity just slightly above background, in all crural muscles. The intensity of hybridization was such that the signal for $MLC_{1F}>MLC_{1A}>MLC_{3F}$ (Fig. 6A,D,E, respectively; Fig. 2). The level of hybridization intensity with the probe for α-skeletal actin mRNA (Fig. 6C) increased during this period, reaching a level of intensity just slightly less than for the cardiac isoform (Fig. 6B). At 14 days gestation, it was not possible to discern differences in transcript accumulation among the crural muscles with in situ hybridization; the soleus muscle (the muscle of the crus that contains the highest percentage of slow myofibers in the adult mouse) showed no difference in muscle gene expression compared with muscle masses that eventually would have a faster phenotype (Fig. 6).

Fig. 1. Hindlimb bud and attached myotomal (upper region of each micrograph) muscles of an 11. 5 day gestation mouse reacted with cRNA probes as indicated. A is a bright-field micrograph of the same section shown in dark field in I. The area enclosed in the box in J is enlarged in the insert. Sections shown in B-L are in serial order, with B being ~210 μm away from L. All probes are specific for the transcript indicated, with the exception of the probe for MHC_{E320} (C), which hybridizes with all known skeletal, fast myosin heavy chains. Autoradiographs were exposed to emulsion for 7 days prior to development, except for the probe for MHC_{emb} (B), which was exposed for 12 days. Transcripts for the myogenic regulatory factors, myogenin (E,L) and MyoD (H), are present at this stage along with transcripts for α-cardiac actin (D,K), MLC_{1F} (F) and MLC_{1A} (which is just detectable; J). There are no reactions with any other specific probes used in this study, when the exposure time prior to development of autoradiographs was 7 days. However, there is a reaction, albeit faint, when the section treated with the specific probe for MHC_{emb} (B) is exposed to emulsion for 12 days prior to development. With the exception of B, the micrographs accurately depict the relative intensity of the reactions among the various probes. At this stage, dorsal and ventral muscle masses surround the precartilage primordia of the thigh (arrow indicates the region occupied by the precartilage; Lance-Jones, 1979). Within a few micrometers, the appearance of the precartilage mass and the muscles undergo marked changes. Compare D and K or E and L. Bar, 200 μm.

DISCUSSION

This paper provides a detailed in situ hybridization analysis of contractile protein gene expression in the hindlimb muscle of embryonic mice (i.e., 10-14 days gestation), at a time when the only myotubes present in the crural muscles are primary myotubes (Ontell and Kozeka, 1984a; Ontell et al., 1988a). The present study demonstrates the versatility of primary myotubes, which accumulate transcripts for (1) α-cardiac and α-skeletal actin, (2) MLC_{1A}, MLC_{1F} and MLC_{3F} and (3) MHC_{emb}, $MHC_{\beta/slow}$ and MHC_{pn} by 14

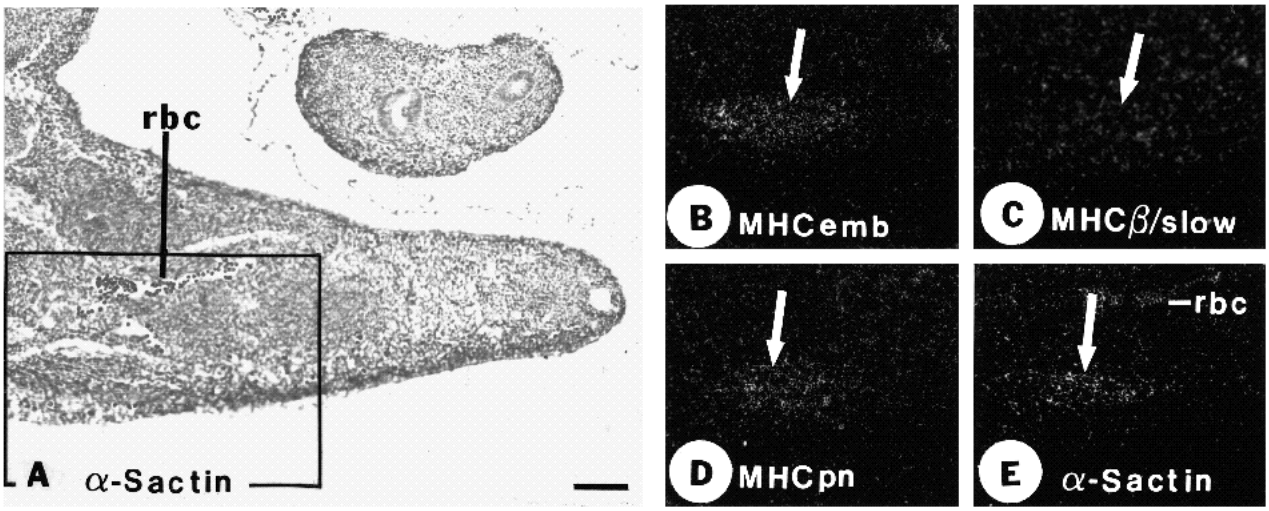

Fig. 3. Hindlimb of a 12 day gestation embryo reacted with the cRNA probes indicated. A is a bright-field micrograph of the same section shown in dark field in E. B-E are dark-field micrographs of the region of the hindlimb enclosed in the box in A, arranged in serial order. At 12 days gestation, transcripts for α-skeletal actin (E, arrow) are detectable. Transcripts for MHC_{emb} (B, arrow) and MHC_{pn} (D, arrow) are also present; however, transcripts for $MHC_{\beta/slow}$ (C, arrow) are not detected even after exposure of the autoradiograph for 12 days prior to developing. Red blood corpuscles (rbc) are present in a small blood vessel. Bar, 100 μm.

days gestation. Because the first primitive myoneural junctions appear in the EDL muscle at 14 days gestation (Ontell and Kozeka, 1984a) and in the soleus muscle at 16 days gestation (Ontell et al., 1988a) and because all of the transcripts, except those for MLC_{3F}, are present in the crural muscles by 13 days gestation, these genes can be activated in the absence of innervation. Since MLC_{3F} transcripts first appear at 14 days gestation in the soleus muscle, as well as in the EDL muscle, activation of this gene also is probably innervation-independent. This study also establishes that, while in general the same myosin and actin genes are transcribed in both myotomal (Sassoon et al., 1988; Lyons et al., 1990) and early limb muscle, the temporal pattern of gene expression (Table 1) and the relative levels of their transcripts differ, suggesting that there is no single coordinate pattern of contractile protein gene expression required as skeletal muscle differentiates. These differences may well reflect the fact that there are differences in the myogenic regulatory factors present in the developing limb muscle versus the myotomal muscle at the onset of myogenesis (Table 1).

How does contractile protein transcript accumulation differ in the myotomal versus limb musculature? (For comparison see Table 1.) Transcripts for MHC_{emb} and MHC_{pn} are detectable in the limb 1.5-2 days prior to transcripts for $MHC_{\beta/slow}$, whereas $MHC_{\beta/slow}$ and MHC_{emb} transcripts appear simultaneously in the somites, a day prior to the detection of mRNA for MHC_{pn} (Lyons et al., 1990). In myotomal muscle, the intensity of the signal for transcripts for MLC_{1A} exceeds that for MLC_{1F} from the time of the first appearance of these transcripts to 12.5 days gestation (Lyons et al., 1990). In contrast, the intensity of signal for

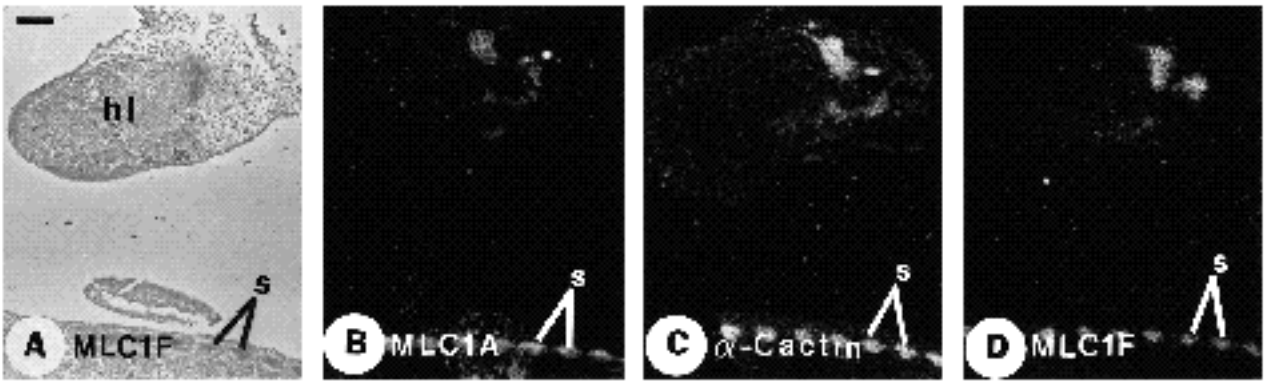

Fig. 4. 12 day gestation mouse hindlimb (hl) and caudal somites (s) of an adjacent embryo of the same age reacted with the probes indicated. A is a bright-field micrograph of the same section shown in dark field in D. B-D are displayed in serial order. In the hindlimb, the intensity of the signal with probe for MLC_{1F} (D) is greater than the signal obtained with probe for MLC_{1A} (B). The intensity of the signal for α-cardiac actin is greater at 12 days gestation than at 11. 5 days gestation (compare Fig. 4C with Fig. 1D). Bar, 200 μm.

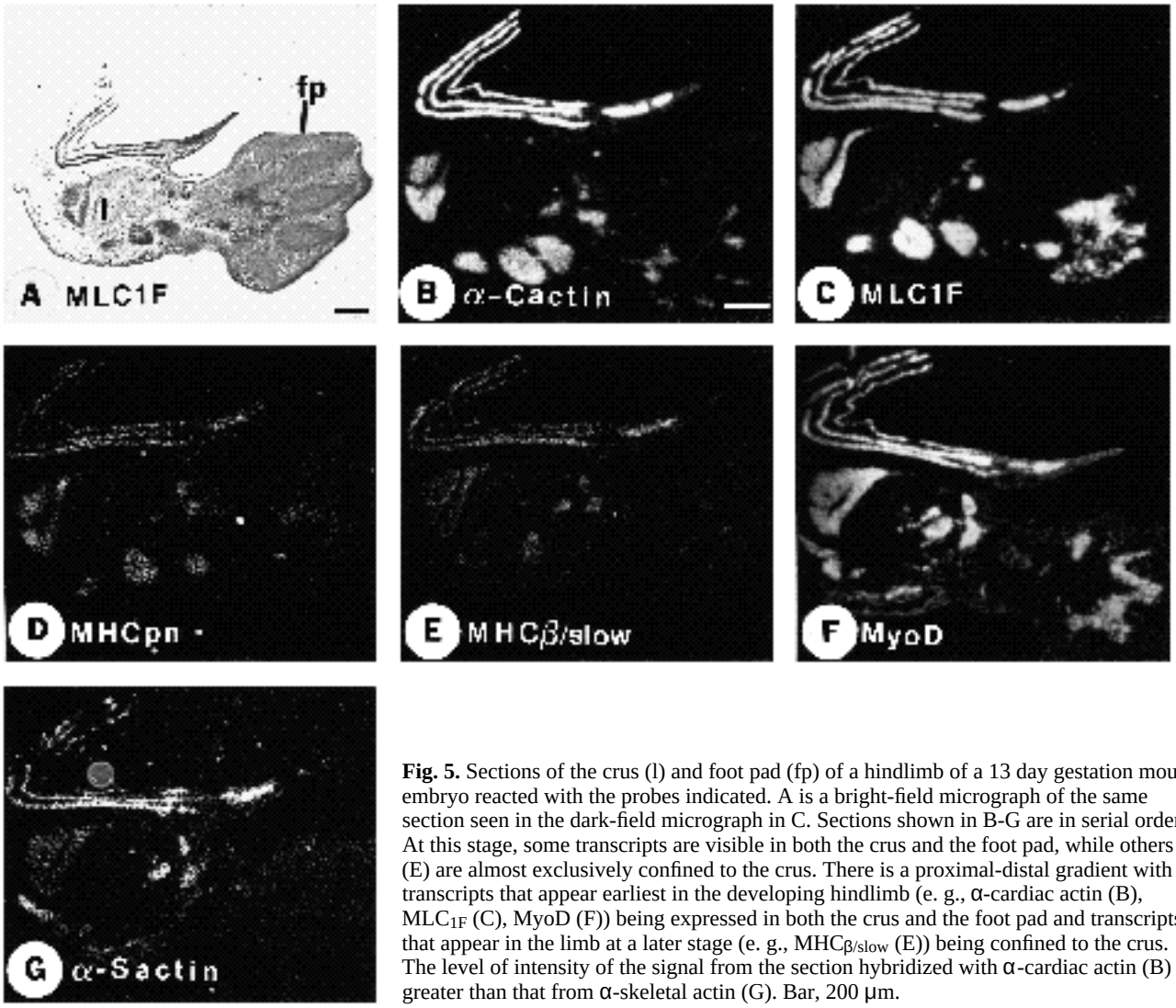

Fig. 5. Sections of the crus (l) and foot pad (fp) of a hindlimb of a 13 day gestation mouse embryo reacted with the probes indicated. A is a bright-field micrograph of the same section seen in the dark-field micrograph in C. Sections shown in B-G are in serial order. At this stage, some transcripts are visible in both the crus and the foot pad, while others (E) are almost exclusively confined to the crus. There is a proximal-distal gradient with transcripts that appear earliest in the developing hindlimb (e. g., α-cardiac actin (B), MLC_{1F} (C), MyoD (F)) being expressed in both the crus and the foot pad and transcripts that appear in the limb at a later stage (e. g., $MHC_{\beta/slow}$ (E)) being confined to the crus. The level of intensity of the signal from the section hybridized with α-cardiac actin (B) is greater than that from α-skeletal actin (G). Bar, 200 μm.

MLC_{1F} transcripts in the limb musculature exceeds that for MLC_{1A} throughout the embryonic period. In the myotomal muscle, α-skeletal actin transcripts are found as soon as the α-cardiac actin mRNA is detectable, although the intensity of hybridization signal is less for the skeletal muscle transcript (Sassoon et al., 1988). In the limbs, α-cardiac actin transcripts appear prior to those for the skeletal isoform. In myotomal muscle, α-actin transcripts precede those of any myosin genes by about one day whereas, in the limb MLC_{1F}, α-cardiac actin, MLC_{1A} and MHC_{emb} messages are detectable simultaneously.

The four members of the myogenic regulatory gene family, MyoD, myogenin, myf-5 and myf-6 (also known as MRF4 or herculin), which are present in mammals, function as skeletal muscle-specific transcription factors (cf. Olson, 1990; Weintraub et al., 1991; Wright, 1992). Co-transfection experiments have revealed a few differences in the transactivation of certain muscle genes by individual myogenic regulatory factors (e.g., Yutzey et al., 1990), but in general each member of the family appears to function similarly. Different muscle cell lines may have different combinations of myogenic factors and, in a few cases, the absence of a factor has been shown to affect the expression of a contractile protein gene (e.g., Brennan et al., 1990). In vivo, each myogenic sequence has a distinct profile of expression, suggesting that each sequence has a distinct role during skeletal muscle myogenesis (cf. Buckingham, 1992).

The pattern of expression of the myogenic regulatory factors differs between limb and myotomal muscles (Table 1). Can these differences be correlated with differences in contractile protein RNA accumulation? In the myotomal muscle, myf-5 (Ott et al., 1991), myogenin (Sassoon et al., 1989), myf-6 (Bober et al., 1991) and MyoD (Sassoon et al., 1989) transcripts are detectable from 8, 8.5, 9 and 10.5 days gestation, respectively. However, myogenin protein is not detectable until 10.5 days gestation, concurrent with the appearance of MyoD (Cusella-De Angelis et al., 1992). Therefore, although antibody studies have not yet been reported for myf-5 and myf-6, the supposition would be that at early stages in the myotome these are the two major myogenic regulatory factors present. In contrast, in the hindlimb muscles, myogenin and MyoD transcripts and proteins accumulate from 11.5 days gestation (Cusella-De Angelis et al., 1992), preceded at 11 days gestation by myf-

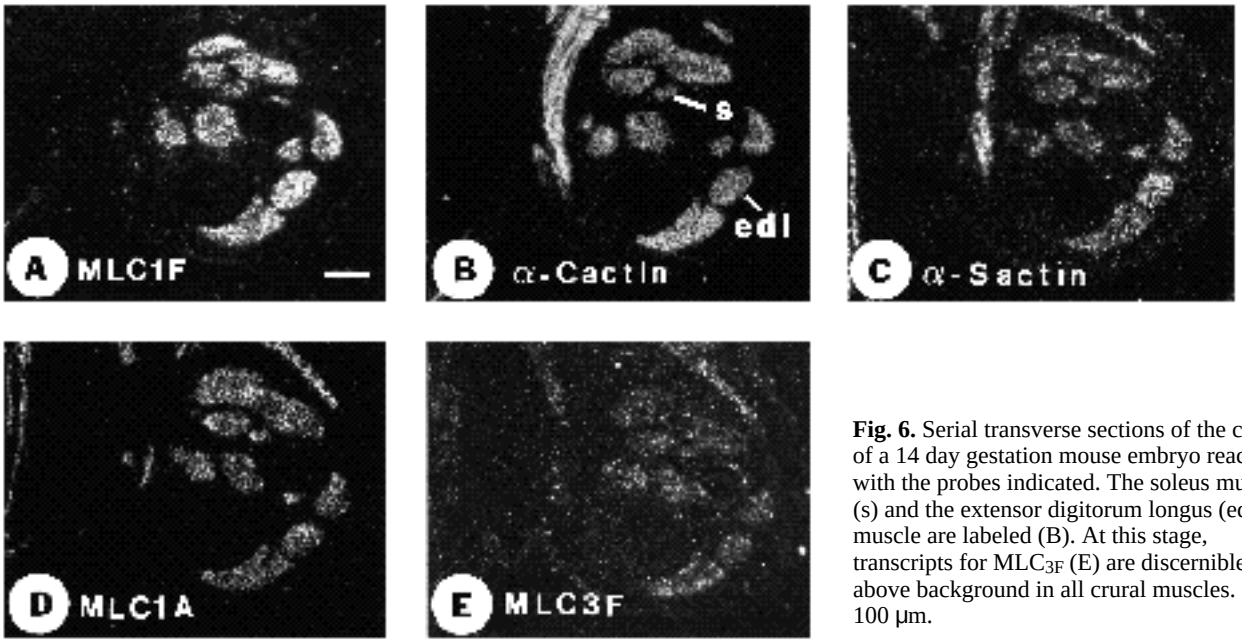

Fig. 6. Serial transverse sections of the crus of a 14 day gestation mouse embryo reacted with the probes indicated. The soleus muscle (s) and the extensor digitorum longus (edl) muscle are labeled (B). At this stage, transcripts for MLC_{3F} (E) are discernible just above background in all crural muscles. Bar, 100 μm.

5 transcripts, which are detectable for only a brief period (Ott et al., 1991). Myf-6 transcripts are not detectable in the mouse hindlimb until about 16 days gestation (Bober et al., 1991).

When the striated muscle α-actin genes are activated in the somites, myf-5 is the only known myogenic regulatory factor that could be present, while both myf-5 and myf-6 are probably present in the somites at the time when the first myosin light and heavy chain transcripts are detectable. In the limb, myogenin, MyoD and possibly some myf-5 are

Table 1. Time of appearance of hybridization signal for contractile protein mRNAs relative to myogenic regulatory factory mRNAs in developing mouse muscles

	MYOTOMES[1]							HINDLIMBS[2]			
Days Emb	8	9	10	11	12	13	14	11	12	13	14
myf 5[a]	▭	▭	▭	▭	▭	▭	▭	▭	▭	▭	
MyoD[b]			▭	▭	▭	▭	▭	▭	▭	▭	▭
myf 6[c,Δ]		▭	▭								
myogenin[b,*]	■	■	■	▭	▭	▭	▭	▭	▭	▭	▭
MHCemb[d]		+						+			
MHCpn[d]			+						+		
MHCβ/slow[d]		+								+	
MLC1A[d]		+						+			
MLC1F[d]		+						+			
MLC3F[e]							+				+
α-S actin[f]	+								+		
α-C actin[f]	+							+			

[1]In myotomes: a) Ott et al., 1991; b) Sassoon et al., 1989; c) Bober et al., 1991; d) Lyons et al., 1990; e) Ontell et al., unpublished data; f) Sessoon et al., 1988.

[2]In hindlimbs: a) Ott et al., 1991; b) Sassoon et al., 1989 and present study; c) Bober et al., 1991; d,e,f) present study.

[Δ]Myf 6 mRNA is not detectable in the hindlimb prior to 16 days gestation (Bober et al., 1991).

*While the mRNA for myogenin is present in the myotomes from 8.5 days gestation, no protein is detectable until 10.5 days gestation, concurrent with the appearance of MyoD mRNA and protein (Cusella-De Angelis et al., 1992).

present at the time of activation of the muscle actin and myosin genes. The fact that the same contractile protein genes are ultimately activated in both the myotome and the limb shows that these actin and myosin genes have no absolute requirement for a particular myogenic factor. However, the differences in the relative timing and extent of contractile protein gene activation in hindlimb versus myotomal muscle suggest that the response of some muscle genes varies with the myogenic factors that are present. The MHC_{pn} gene, for example, begins to be expressed relatively later in the myotome at the time when MyoD and myogenin begin to accumulate; whereas in the limb, in the presence of these two factors, MHC_{pn} transcripts appear more rapidly (Table 1). At the time that transcripts for MLCs begin to accumulate in the hindlimb, hybridization signal for MLC_{1F} mRNA is more intense than that for MLC_{1A} mRNA, whereas in the myotome the intensity of hybridization signals for MLC_{1A} initially exceeds those for MLC_{1F} (Lyons et al., 1990). One could argue that the MLC_{1A} gene is preferentially activated by myf-5 and/or myf-6, whereas the MLC_{1F} gene responds preferentially to MyoD and/or myogenin. In the case of $MLC_{1F,}$ it has been shown that the enhancer that modulates the activity of this gene responds preferentially to MyoD (Rosenthal et al., 1990). Furthermore, the BC_3H1 muscle cell line, which expresses myf-5 and myogenin but lacks MyoD, does not express MLC_{1F} and only does so when transfected with a MyoD expression vector (Brennan et al., 1990). It is also noteworthy that BC_3H1 cells do not fuse in the absence of MyoD, and that differentiated cells in the myotome, unlike the limb, do not form myotubes initially.

The differences between limb and myotomal muscle cells, in terms of both myogenic regulatory factors and contractile protein gene expression, may reflect differences in cell lineage and/or environmental differences. While the myotome is formed by cells from the craniomedial region of the dermamyotome adjacent to the neural tube, the precursor muscle cells that will form limb muscle migrate from the ventrolateral edge of the dermamyotome, at least in the avian model (cf. Wachtler and Christ, 1992). Thus, myotomal muscles, but not limb muscles, develop in intimate relationship with the neural tube and notochord. The migration of pre-muscle cells to the limb is influenced by the presence of the apical ectodermal ridge (e.g., Gumpel-Pinot et al., 1984), which also may have an indirect influence on subsequent muscle formation. Additionally, morphogenesis of myotomal muscle begins earlier than does development of limb musculature. Thus, the maturation of other systems (i.e., the nervous and endocrine systems) that might modify the myogenic program is more advanced at the onset of muscle formation in the limb.

In the present study, we have demonstrated a clear proximal-distal gradient in the time course of the appearance of contractile protein transcripts in the limb, similar to the rostral-caudal gradient that has been demonstrated for the mRNAs in the myotomes (Lyons et al., 1990). However, we have not found any other evidence for regionalization among or within the crural muscles, despite the fact that by 14 days gestation some of the muscles (e.g., the extensor digitorum longus muscle) display primitive neuromuscular junctions while other muscles (e.g., the soleus muscle) do not. The in situ hybridization protocols used in this study do not permit sufficient resolution to enable us to determine whether all of the primary myotubes present in the embryonic muscles simultaneously accumulate the same contractile protein transcripts. To determine this, it will be necessary to develop in situ hybridization techniques that will provide sufficient resolution to permit identification of transcripts found in individual myotubes. This would permit us to appreciate fully the versatility of primary myotubes at later gestational stages, when closely apposed primary and secondary myotubes are found in limb muscles.

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