

## Early expression of the myogenic regulatory gene, *myf-5*, in precursor cells of skeletal muscle in the mouse embryo

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### Summary

We have analysed by *in situ* hybridization the expression of *myf-5*, the murine homologue of the human myogenic regulatory sequence *myf5*, during embryogenesis in the mouse. *myf-5* sequences were first detected in the earliest somites (from about 8 days *p.c.*) in the dermomyotome, before formation of the dermatome, myotome and sclerotome. The dermomyotome is classically considered to give rise to the precursor muscle cells of body and limb skeletal muscle. *myf-5*-positive cells were also detected early in the visceral arches and limb buds. In this case, as in somites, *myf-5* expression precedes that of the two related myogenic regulatory sequences, myogenin and MyoD1, and indeed any other skeletal muscle marker examined to date. *myf-5* is not detected at any

stage in developing cardiac muscle. From 11.5 days *p.c.*, the level of *myf-5* transcripts begins to decrease to become undetectable (by *in situ* hybridization) from 14 days *p.c.* Both the appearance and disappearance of *myf-5* follow the anteroposterior gradient of somite formation and maturation in the embryo. The time and place of *myf-5* expression are consistent with a role in the early events of myogenic differentiation, possibly during determination of the myogenic lineage.

Key words: mouse embryo, myogenesis, dermomyotome, *myf-5*, *in situ* hybridization.

### Introduction

During embryonic development, multipotential cells become progressively committed to follow a defined differentiation pathway. The differentiated state is characterized by the expression of a specific subset of genes. Prior to the acquisition of a differentiated phenotype, the process of cell commitment or determination is probably characterized by the expression of 'master' regulatory genes involved in the activation of specific structural genes.

Where myogenesis is concerned such candidate regulatory genes have been identified. Recently, two cDNA sequences from established muscle cell lines, MyoD1 (Davis *et al.* 1987) and myogenin (Wright *et al.* 1989), have been cloned, and shown to induce myogenic conversion when transfected into the embryonic fibroblastic cell line C3H 10T1/2, which otherwise gives rise to skeletal muscle cells only after 5-azacytidine treatment (Taylor and Jones, 1979). A genomic sequence, called 'myd', also has this property and may in fact lead to MyoD1 activation in transfected cells; however, it remains to be fully characterized (Pinney *et al.* 1988). Two additional cDNAs encoding

myogenic factors, namely *myf5* (Braun *et al.* 1989a) and *myf6* (Braun *et al.* 1990a), have been isolated from human muscle tissue by cross hybridization to the MyoD1 sequence. *Myf6* cDNAs have also been isolated from rat and mouse where they were termed MRF-4 (Rhodes and Konieczny, 1990) and herculin (Miner and Wold, 1990). All of these factors have been demonstrated to convert 10T1/2 cells to the muscle phenotype. On the basis of their homology to MyoD1, members of this family have also been isolated in other species: CMD1 in chicken (Lin *et al.* 1989), XMyoD in *Xenopus* (Hopwood *et al.* 1989; Harvey, 1990; Scales *et al.* 1990) and *qmf1* in quail (Charles de la Brousse and Emerson, 1990).

MyoD1, myogenin, *myf5* and *myf6* are distinct but related proteins. All these myogenic factors belong to the 'helix-loop-helix' super family of DNA-binding proteins (Murre *et al.* 1989), which includes several *Drosophila* genes involved in the control of cell determination and development (Villares and Cabrera, 1987; Caudy *et al.* 1988; Thisse *et al.* 1988; Klaembt *et al.* 1989). They are thus potential transcriptional factors. In fact, MyoD1 and myogenin have been shown to bind directly to a consensus recognition sequence in the

muscle-specific enhancer of the phosphocreatine kinase gene and are involved in its transcriptional activation (Lassar *et al.* 1989; Brennan and Olson, 1990). The human myf factors also bind to the same consensus sequence, present in regulatory elements of a number of muscle genes including myosin light chains (Braun *et al.* 1990a,b). A further important observation is that myogenic conversion induced by transfection of MyoD1 or myogenin results in activation of both these endogenous genes but not of the myf5 gene (Braun *et al.* 1989b; Thayer *et al.* 1989).

The experimental strategy used to isolate MyoD1 was based on the assumption that one master regulatory determination locus is responsible for the myogenic conversion of 10T1/2 fibroblasts. (Davis *et al.* 1987). Now that four related sequences have been characterized with similar activities, the question arises as to whether they are involved in different key steps of myogenesis. Analysis of their expression in cell culture systems has shown that MyoD1 and myf5 are present in dividing myoblasts, whereas myogenin accumulates as muscle cells differentiate (Wright *et al.* 1989; Montarras *et al.* 1989; Braun *et al.* 1989a).

In this paper, we investigate the expression of these factors during myogenesis *in vivo*, in order to examine their potential roles in myogenic determination and differentiation. In birds and mammals, all skeletal muscles of the body are derived from the somites (Chevallier *et al.* 1977; Christ *et al.* 1977). In the mouse embryo, somites form from about 8 days *p.c.* in an anteroposterior gradient by segmentation of the paraxial mesoderm on each side of the neural tube and then mature along the same axis (Rugh, 1990). The early somite consists of a ball of epithelial-like cells surrounding a coelomic cavity (Ede and El-Gadi, 1986). The dorsal part is referred to as the dermomyotome, which will form the dermatome and the myotome when the somite matures and subdivides. The ventral part of the somite will form the sclerotome. The myotome that matures into the first skeletal muscle mass in the embryo is believed to form as a result of cells migrating from the dorsal region of the dermomyotome adjacent to the neural tube to lie in a ventral position under the dermatome (Kaehn *et al.* 1988; Ede and El-Gadi, 1986). Limb muscles are thought to originate from cells that migrate out from the ventrolateral edge of the dermomyotome before myotome formation takes place (Christ *et al.* 1986).

We had previously shown that myogenin and MyoD1 are both expressed in myotomes and in limb buds in the mouse embryo, but that the onset of their expression differs (Sassoon *et al.* 1989). Myogenin transcripts accumulate in the myotomes of somites from 8.5 days *p.c.*, whereas MyoD1 transcripts are not detectable in the myotomes before 10.5 days *p.c.* The expression of myogenin coincides with that of cardiac actin, one of the first muscle structural gene transcripts to be detected (Sassoon *et al.* 1988; Lyons *et al.* 1990). In the developing limb buds, myogenin and MyoD1 are present together from 11.5 days. Subsequently they continue to be co-expressed in skeletal muscles of the

developing mouse. Recently, qmf1 transcripts have been shown to accumulate in myotomes of quail somites. Onset of qmf1 expression coincides with that of muscle troponin C, but the distribution of transcripts is different and leads the authors to propose that qmf-1 is expressed in the dermomyotome (Charles de la Brousse and Emerson, 1990). *Xenopus* MyoD expression has been detected before somite formation, in early mesoderm where it precedes cardiac actin expression by a few hours (Hopwood *et al.* 1989; Harvey, 1990; Scales *et al.* 1990).

Here we describe the expression of the murine homologue of myf5 during mouse embryogenesis. The spatial and temporal distribution of *myf-5* transcripts has been analysed by *in situ* hybridization in parallel with myogenin, and compared with results obtained for MyoD1 and for certain muscle structural genes. We show that *myf-5* transcripts are detectable in the first somites from 8 days *p.c.* prior to myogenin or any other muscle marker sequence examined. *myf-5* is expressed in the dermomyotome of somites before formation of the myotome. Transcripts are present in cells distributed over the dermomyotome, but are especially concentrated in the dorsal region adjacent to the neural tube. *myf-5* is also an early marker of pre-muscle cells that have migrated from somites to the visceral arches and limb buds. From 11.5 days *p.c.*, *myf-5* expression decreases rapidly throughout the embryo, in contrast to myogenin and MyoD1, which continue to be expressed at a high level in embryonic and foetal skeletal muscles. The early expression of *myf-5* is discussed in the context of its possible role in myogenic lineage determination.

## Materials and methods

### Preparation of tissue sections for hybridization

C3H and BALB/c mice were used in this study, and embryos dated according to Rugh (1990) with post coital (*p.c.*) day 0.5 counted as the morning when the vaginal plug was detected. In order to determine the state of development of the embryos, somites were often counted before embedding. Sections were prepared as described in Sassoon *et al.* (1988). Briefly, embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline, dehydrated and infiltrated with paraffin. The paraffin blocks were cut to give parasagittal, frontal or transverse sections of the embryo of 5–7 microns. Our standard procedure for comparing sections was as follows. Two immediately adjacent sections are hybridized per slide and the next slide in the series contains the two following adjacent sections (serial sections). When the section in the series does not immediately follow, it is referred to as 'parallel'. 1–3 serial sections were mounted on subbed slides (Gall and Pardue, 1971), deparaffinized in xylene and rehydrated. They were then treated with proteinase K, post-fixed, treated with triethanolamine/acetic anhydride, washed and dehydrated as already described (Sassoon *et al.* 1988).

### Hybridization and washing procedures

The hybridization and post-hybridization procedures are based on Wilkinson *et al.* (1987). Briefly sections were hybridized overnight at 52°C in hybridization solution containing 50% deionized formamide, 0.3 M NaCl, 20 mM Tris-HCl pH 7.4, 5 mM EDTA, 10 mM NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 10%

dextran sulphate,  $1 \times$  Denhardt's,  $50 \mu\text{g ml}^{-1}$  total yeast RNA, 10 mM DTT, and  $50\text{--}75,000 \text{ cts min}^{-1} \mu\text{l}^{-1}$  of  $^{35}\text{S}$ -labelled riboprobe. Sections were washed at  $65^\circ\text{C}$  in 50% formamide,  $2 \times$  SSC, 10 mM DTT and rinsed in phosphate-buffered saline before treatment with  $20 \mu\text{g ml}^{-1}$  RNAase A at  $37^\circ\text{C}$  for 30 min. They were then washed in  $2 \times$  SSC and  $0.1 \times$  SSC at  $37^\circ\text{C}$  for 15 min, dehydrated and dipped in undiluted Kodak NTB-2 nuclear track emulsion. After exposure for 7 days in light-tight boxes with desiccant at  $4^\circ\text{C}$ , slides were developed in Kodak D-19, and analyzed using light- and dark-field optics on a Zeiss Axiophot microscope.

### Probes

The MyoD1 probe used was that described in Sassoon *et al.* (1989) and corresponds to a fragment of the 3' untranslated sequence of the mRNA from nucleotides 751 to 1785 cloned into Henikoff-modified Bluescribe.

The myogenin probe used was also described in Sassoon *et al.* (1989) and corresponds to a 3' untranslated fragment of the mRNA from nucleotides 791 to 1486. A shorter myogenin probe corresponding to the 5' end of the mRNA (nucleotides 176–264) was also prepared in order to have a more exact comparison with the short *myf-5* probe. Similar results to those shown in this paper with the 3' probe were obtained with the 5' region. In both cases, the sequences are myogenin specific.

The *myf-5* probes used were derived from the 5' end of the mouse *myf-5* gene (Bober *et al.* unpublished data). This was isolated by screening a mouse lambda genomic library with a human *myf5* cDNA. The cap site and exonic/intronic structure of the 5' end of the mouse gene were established by sequencing and comparison with the human *myf5* sequence (Braun *et al.* 1989a). Two fragments were subcloned into Bluescribe from the 5' end of the gene sequence: first, a *BalI*–*ApaI* fragment of 311 nucleotides from nucleotides 15 to 326 relative to the putative transcriptional start site of the mouse gene; 5' CCAACAGGCATCTGTCCTTGTTAATTACAGAGAGACAGTCCCAAACCTCCGGGAGCTCCGCC-TGGATTTGCTGGCCTGCAGCAGCCAGGGACTCGG-TGTCTCCCTCTCTGCTGAATCCAGGTATCCCACCT-GCTTCTCTGAAGGATGGACATGACGGACGGCTGC-CAGTTCTCCCCTTCTGAGTACTTCTATGAAGGCTCC-TGTATCCCCTCACCAGAGGATGAGTTTGGGGACCA-GTTTGAGCCAAGAGTAGCAGCCTTCGGAGCACAC-AAAGCTGAGCTGCAGGGCTCAGACGATGAGGAG-CACGTGCG 3' secondly, a shorter probe the *SalI*–*PstI* fragment of 103 nucleotides located within exon I, extending from nucleotides 94 to 197 of the mouse gene, 5'-AC-TTCTATGAAGGCTCCTGTATCCCCTCACCAGAGGA-TGAGTTTGGGGACCAGTTTGAGCCAAGAGTAGCA-GCCTTCGGAGCACACAAAGCTGAGCTGCAG-3'.

The specificity of these two probes was verified by sequence comparison. No significant homologies were seen with the other myogenic factors. On northern blots, these probes detect distinct *myf-5* mRNA, only in skeletal muscle (E. Bober, unpublished observations).

In initial experiments, the shorter *myf-5* probe was used. A similarly short myogenin probe was generated in order to have probes of comparable size. In most of the experiments reported here (see Figure legends), the longer *myf-5* (300) probe was used. Only results with the longer myogenin probe are shown.

### Results

For this study, sections of mouse embryos at different

stages were examined by *in situ* hybridization using a  $^{35}\text{S}$ -labelled antisense riboprobe corresponding to a 5' region of the mouse *myf-5* coding sequence. This hybridization probe is specific for *myf-5* and does not cross-react with other myogenic factor messenger RNAs (see Methods). In all experiments, serial sections to those used for *myf-5* were hybridized with the myogenin probe. In some cases MyoD1 or cardiac actin probes were also used.

#### (1) *myf-5* expression is localized in somites and derivatives

Maximal accumulation of *myf-5* transcripts is visible in embryos between 10.5 and 11 days of development. Fig. 1 shows serial transverse sections of somites from the hindlimb region of an 11 day *p.c.* embryo. Hybridization with the *myf-5* probe (Fig. 1A and C) gives a strong signal in somites comparable to that obtained on a serial section hybridized with the myogenin probe (Fig. 1B and D). At this stage, the somite is already mature and both transcripts are concentrated in the myotome, which is the first skeletal

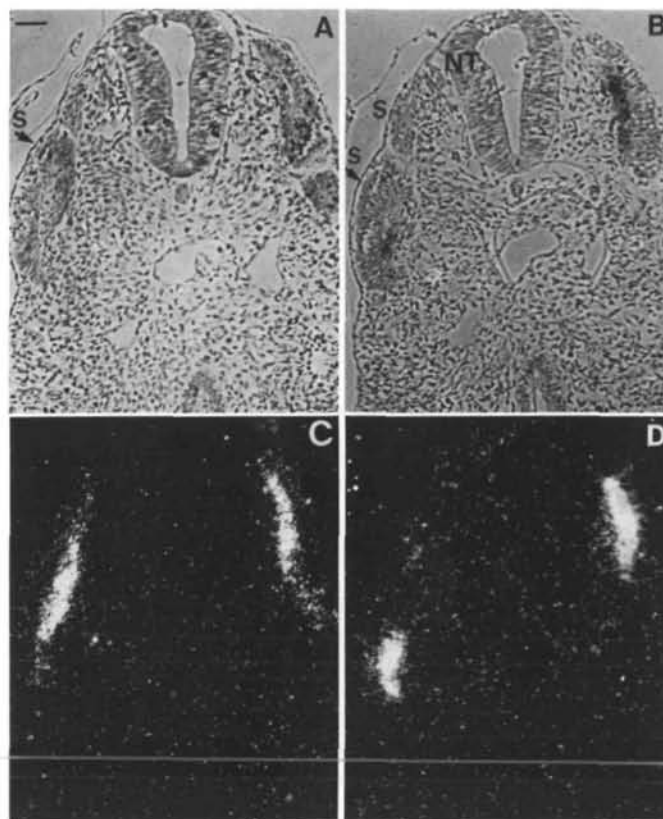
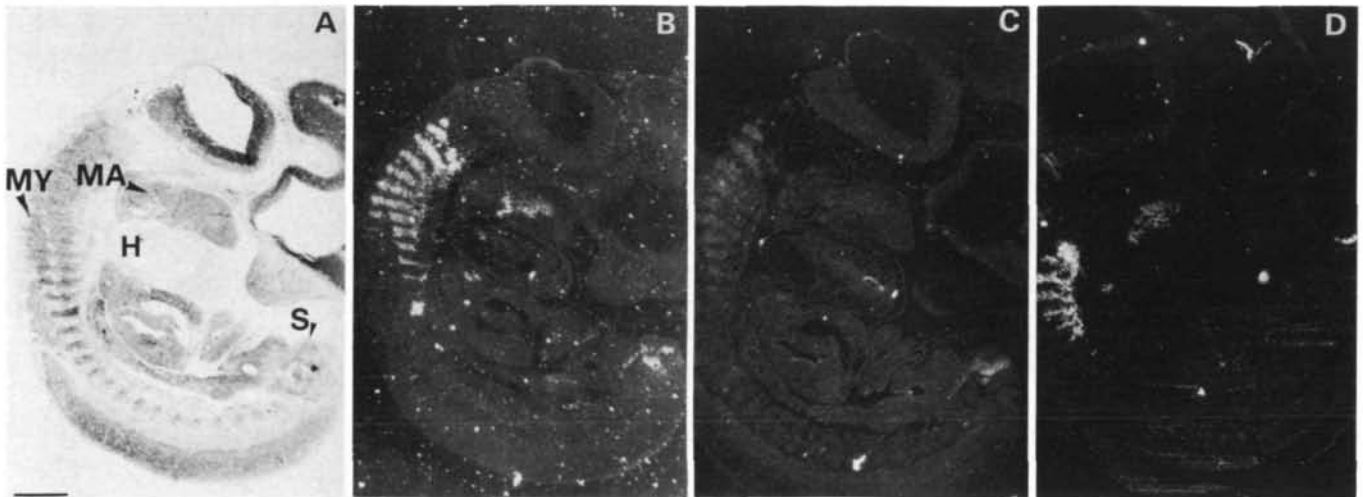


Fig. 1. Serial transverse sections of an 11 day *p.c.* (40–44 somites) embryo, showing somites at the level of the hindlimbs. (A) Phase-contrast micrograph of a section hybridized with the *myf-5* (300) probe. (B) Photomicrograph of a serial section to that shown in A, hybridized with the myogenin probe. (C) Dark-field micrograph of A, showing *myf-5* hybridization. (D) Dark-field micrograph of B, showing myogenin hybridization. NT, neural tube; S, somite. Scale bar: 50 microns for A and B.



**Fig. 2.** Parasagittal sections of an 11.5 day *p.c.* mouse embryo. (A) Phase-contrast micrograph of a section; (B) dark-field micrograph of A hybridized with the MyoD1 probe; (C) dark-field micrograph of a parallel section hybridized with the *myf-5* probe (100 nucleotides); (D) dark-field micrograph of a parallel section hybridized with the myogenin probe. Three serial sections were present on each slide; successive slides (B–D) were hybridized with each probe. H, heart; M.A., mandibular arch; MY, myotomes; S, caudal somite. Scale bar=500 microns.

muscle to form. However, we observe frequently in transverse sections of somites at this stage that the *myf-5* signal appears to extend further dorsally and ventrally than myogenin. A similar difference in spatial distribution is seen with transcripts of other muscle genes and can be correlated with differences in the time of onset of their expression (Lyons *et al.* 1990).

Fig. 2 shows parallel parasagittal sections of an 11.5 day *p.c.* embryo hybridized with probes for MyoD1 (Fig. 2B), *myf-5* (Fig. 2C) and myogenin (Fig. 2D). Myotomal muscle masses (MY) have already formed in the rostral region while somites are still present in the caudal region (S). One such somite is seen in Fig. 2A in a section that is not quite transverse due to the curvature of the tail in the plane of section. *myf-5* transcripts have accumulated in the myotomes and caudal somites. Transcripts of myogenin and MyoD1 are also present as already demonstrated by Sassoon *et al.* (1989). A signal is visible in the mandibular arch, where muscle cells that have migrated from the somites will contribute to the formation of the musculature of the tongue and jaw. As in the case of myogenin and MyoD1, there is no expression of *myf-5* in the heart (H) at this or any other stage examined.

The *myf-5* signal already differs quantitatively at 11.5 days *p.c.* from that of the two other myogenic sequences. In the mandible, *myf-5* transcripts are barely detectable and the hybridization in myotomes is weaker than that of MyoD1 or myogenin. In contrast to what is observed for myogenin or MyoD1, the signal in the caudal somite with the *myf-5* probe is stronger than the signal seen in myotomes. Thus, at this stage, *myf-5* expression is lower in mature myotomes and is higher in more recently formed somites. Despite these quantitative differences, all three myogenic sequences have a similar spatial distribution in the embryo.

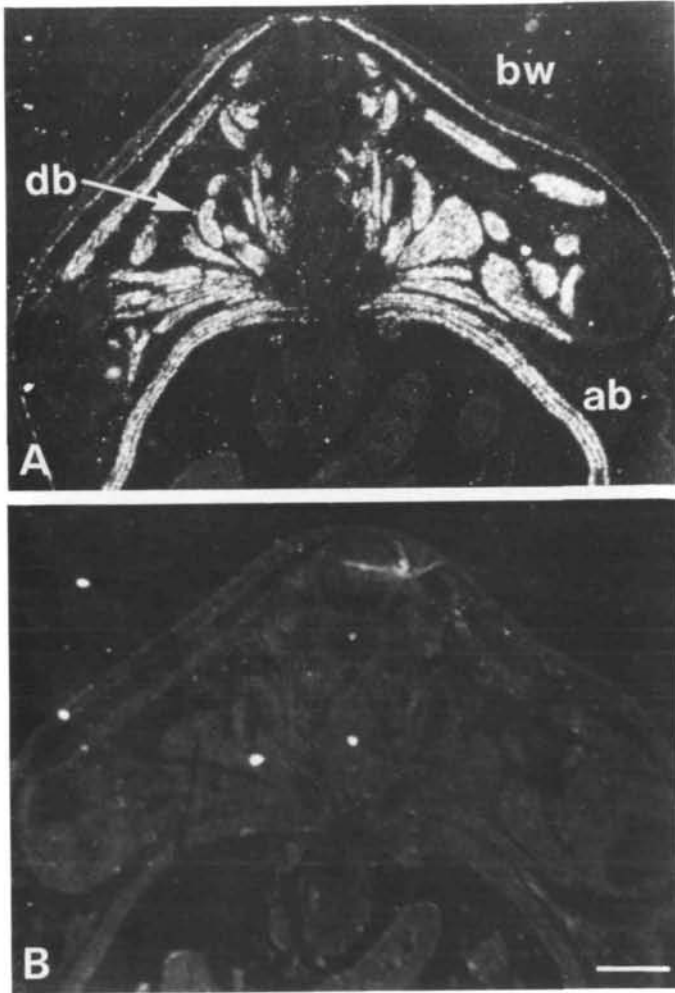
### (2) *The limited time course of myf-5 expression*

In contrast to myogenin and MyoD1 whose transcripts are clearly detectable until birth, *myf-5* expression is limited to embryonic development. By 11.5 days *p.c.*, *myf-5* transcripts accumulate to a lesser extent in the anterior part of the embryo where myotomes have matured (Fig. 2). Already at 12.5 days, the *myf-5* signal is very faint and is virtually undetectable at 14 days (data not shown). Fig. 3 shows a transverse section through the abdomen of a 16 day *p.c.* fetus. Myogenin transcripts (Fig. 3A) are abundant in skeletal muscles (abdominal, body wall and deep back muscles), but no *myf-5* transcripts are detectable (Fig. 3B). Later stages of development *in utero* and at two weeks after birth have been examined and are also negative (results not shown).

### (3) *Early expression of myf-5 in the dermomyotome*

*myf-5* expression, as detected by *in situ* hybridization, is confined to the earliest stages of myogenesis in the mouse embryo. As soon as the first somites form, a signal is distinguishable over them with the *myf-5* probe. A representative example of this is shown in Fig. 4A in a transverse section of an 8 day *p.c.* embryo in which the neural tube is visible, flanked by two somites: At this stage (4 somites, Fig. 4A) the somites still have a ball shape, with cells of an epithelial-like morphology surrounding a central coelemic cavity. No subdivision into dermatome, myotome and sclerotome is yet visible. With the *myf-5* probe, a signal is visible in the somites (Fig. 4B), primarily in the more dorsal part adjacent to the neural tube (Fig. 4C,D). The myogenin probe on a serial section (Fig. 4E) gives uniformly distributed background hybridization. As described





**Fig. 3.** Transverse sections through the abdominal region of a 16 day *p.c.* mouse. (A) Dark-field micrograph of a section hybridized with the myogenin probe. (B) Dark-field micrograph of a serial section hybridized to the *myf-5* (300) probe. ab, abdominal muscle; bw, body wall muscle; db, deep back muscles. Scale bar=500 microns.

previously, myogenin first accumulates in the myotomes of somites at 8.5 days (Sassoon *et al.* 1989), when transcripts of the  $\alpha$ -cardiac muscle isoform of actin are also detected (Sassoon *et al.* 1988). In parallel sections of the embryo shown in Fig. 4, cardiac actin transcripts had accumulated in the embryonic heart but not yet in the somites.

Fig. 5 shows transverse sections in the caudal part of a 9.5 day *p.c.* (21–29 somites) embryo. In this region, the somites are still immature, with no clear myotome compartment yet formed. *myf-5* transcripts are already abundant in the dermomyotome, mainly in the dorsal region adjacent to the neural tube (Fig. 5B). In a parallel section myogenin transcripts are not yet detectable (Fig. 5D). However, in a parasagittal section of a littermate embryo, myogenin and cardiac actin transcripts are present in the myotomes of the first rostral somites although again not in more caudal

somites, whereas *myf-5* transcripts are clearly present both in rostral and in caudal somites (data not shown).

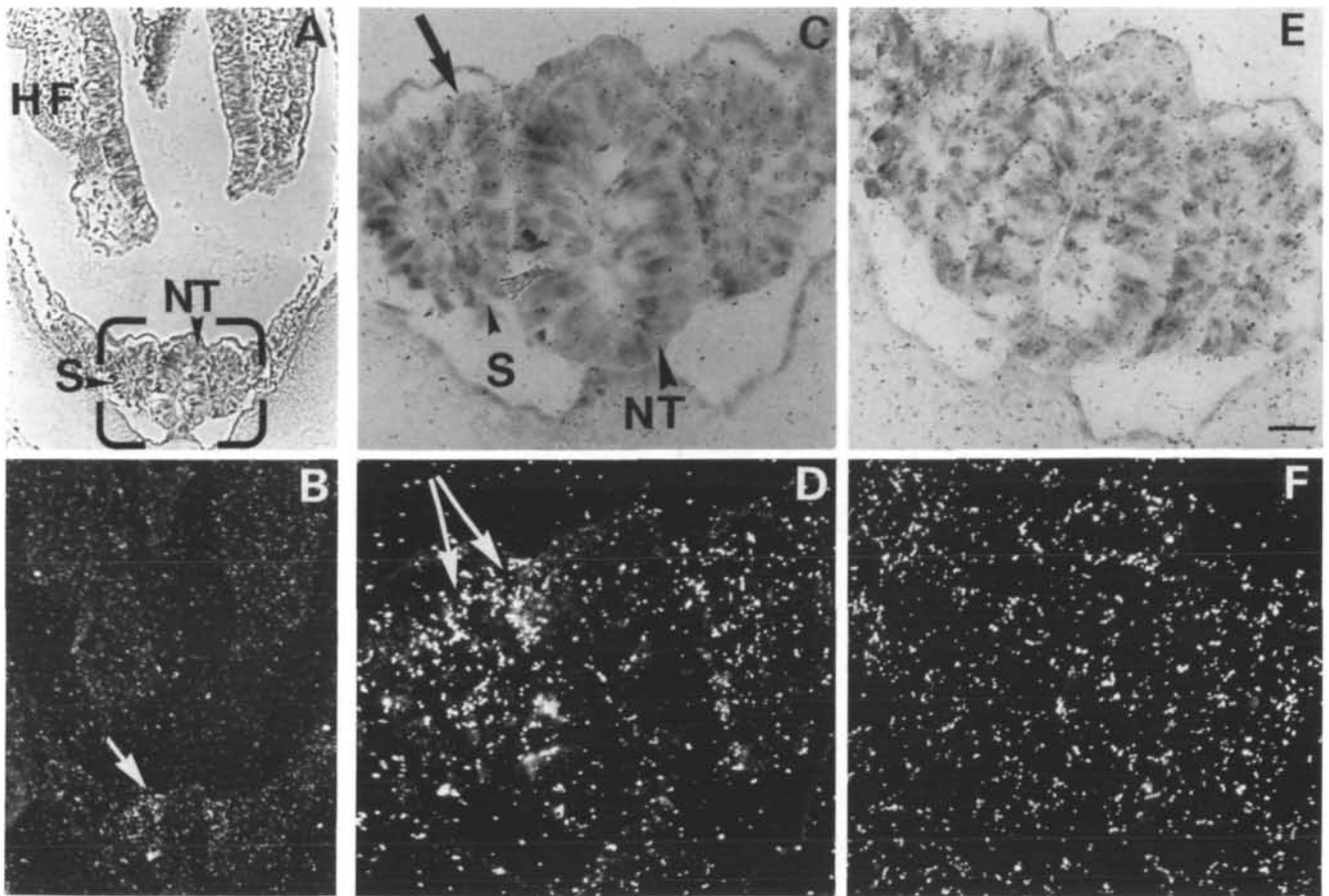
#### (4) *myf-5* expression follows a rostrocaudal gradient

As we have demonstrated, *myf-5* expression precedes that of myogenin not only as the first somites form in the embryo, but also at later stages, following the anterior posterior gradient of somite maturation. This is illustrated in Fig. 6, which shows serial transverse sections of a 10.5 day *p.c.* embryo hybridized alternately with *myf-5* (A, C, D) and myogenin (B). Due to the curvature of the embryo, the same section goes through more rostral somites, where myotome has already formed, and through immature caudal somites. The rostral somites show a strong signal when hybridized with either *myf-5* or myogenin. As noted previously (Fig. 1), the *myf-5* signal is rather more extensive. Signal in the caudal somites differs according to the probe used: a signal is distinguishable with *myf-5* (Fig. 6A,D) while no signal above background is visible with myogenin (Fig. 6B). Again, this shows that *myf-5* transcripts are present in the dermomyotome of the immature caudal somites, before formation of the myotome and expression of myogenin. The accumulation of *myf-5* transcripts, therefore, shows a rostrocaudal gradient in the embryo. A similar gradient is seen as *myf-5* expression declines and caudal somites retain higher level expression (Fig. 2).

#### (5) *myf-5* expression is also detected before myogenin in the visceral arches and limb buds

Fig. 7 shows transverse sections of a 9.25 day *p.c.* (18 somites) embryo, cut at the level of the visceral arches. Serial sections have been hybridized with *myf-5* (A–D) and myogenin (E,F). *myf-5* transcripts have already accumulated in the hyoid arch at this stage, whereas only background levels of hybridization are seen with myogenin. At a later stage (30–34 somites, 10 days *p.c.*), *myf-5* transcripts also precede those of myogenin in the mandibular arch (results not shown). At 11.5 days of development, both myogenin and MyoD1 transcripts are present in the mandible, whereas *myf-5* expression is declining (Fig. 2).

*myf-5* expression also has an early onset in the limb buds. Fig. 8 shows a section through a hindlimb bud of an 11 day *p.c.* embryo. *myf-5* transcripts have already accumulated in the proximal/dorsal region of the limb bud shown here (Fig. 8D,E) whereas at 10.5 days (Fig. 6A,D) they were not yet detectable. In the phase-contrast photograph shown in Fig. 8A, hybridization of myogenin transcripts can be detected in the myotomes, but there is no hybridization above background in the hindlimb bud at this stage (Fig. 8B,C). As demonstrated previously, MyoD1 and myogenin transcripts are accumulated at high levels at 11.5 days (Sassoon *et al.* 1989) while the *myf-5* signal rapidly declines. At 11 days, myogenin as well as *myf-5* is detectable in the forelimb bud which develops more rapidly following the anteroposterior gradient of somite formation. *myf-5* transcripts are detectable in the forelimb bud from 10.5 days (data not shown).



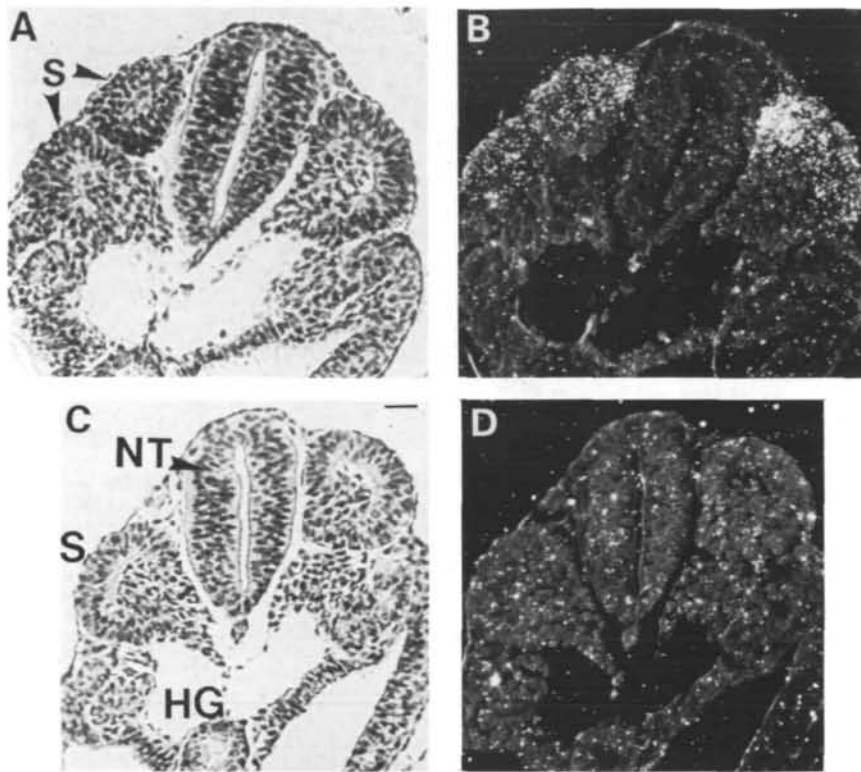
**Fig. 4.** Sections of an embryo (4 somites) at about 8 days *p.c.* A Photomicrograph of the embryo traversing the head fold and neural tube hybridized with the *myf-5* (300) probe; (B) Dark-field micrograph of A. (C) Enlargement of the boxed area shown in A, containing the neural tube flanked by two somites. The arrow points to the dorsal medial lip of one of the somites, also indicated in B. (D) Dark-field micrograph of C, showing the signal obtained with the *myf-5* probe in the upper part of the somite (arrows); (E) Adjacent serial section to that shown in A, where the photomicrograph shows an enlargement of the same region as C hybridized with the myogenin probe. (F) Dark-field micrograph of E. HF, head fold, NT, neural tube, S, somites. Scale bar=20 microns.

## Discussion

The experiments reported here define the spatial and temporal pattern of expression of *myf-5*, during mouse embryogenesis. The results are compared with those already obtained for myogenin and MyoD1 (Sassoon *et al.* 1989). The underlying questions are whether these related sequences play different roles during myogenesis, and whether any of them are associated *in vivo* with muscle cell determination rather than differentiation.

*myf-5* expression, like that of MyoD1 and myogenin, is restricted to developing skeletal muscle. However, in contrast to the two other myogenic regulatory sequences, *myf-5* transcripts are detectable in the dermomyotome of somites, prior to the formation of the myotome and prior to the expression of any other muscle gene examined. As shown in Table 1, which summarizes our observations, *myf-5* transcripts accumulate in the first somites before the appearance of myogenin and well before those of MyoD1. It is difficult

to define the lower limit of resolution of the *in situ* hybridization technique. However, experiments (in collaboration with D. Montarras and C. Pinset, manuscript in preparation) using the polymerase chain reaction (PCR) which is a highly sensitive method for detecting mRNA transcripts (Montarras *et al.* 1989) confirm the results on the early onset of *myf-5* expression prior to that of myogenin and MyoD1 shown in Table 1. *myf-5* is the only signal detected at 8 days in whole embryos. The level of *myf-5* transcripts begins to decrease from 11.5 days onwards, which is the time at which MyoD1 has reached its maximum level of expression. The onset of MyoD1 expression at 10.5 days correlates with the timing of an effect of the neural tube on somite maturation (Vivarelli and Cossu, 1986; see Sassoon *et al.* 1989) which also just precedes the down regulation of the *myf-5* gene. *myf-5* is barely detectable by *in situ* hybridization at 14.5 days *p.c.* and no longer detectable at 16 days *p.c.* in the bulk of the embryo, while myogenin and MyoD1 continue to be expressed at a high level until birth. This decrease



**Fig. 5.** Transverse sections from the caudal region of a 9.5 day *p.c.* (21–24 somites according to Rugh, 1990) embryo. (A) Phase-contrast photomicrograph showing the neural tube and flanking somites, hybridized with the *myf-5* (300) probe. (B) Dark-field micrograph of A. (C) Phase-contrast micrograph of a parallel section to A, hybridized with the myogenin probe. (D) Dark-field micrograph of C. Two sections were hybridized per slide and gave identical results. The section shown in C is two serial sections away from that shown in A. NT, neural tube; S, somite; HG, hind gut. Scale bar=50 microns.

follows the same anteroposterior sequence, according to the gradient of somite formation, as does the onset of *myf-5* expression.

The early expression of *myf-5* is seen not only in the somites and myotomes, it is also the first myogenic regulatory sequence to be detected in limb buds (Fig. 8) and in the visceral arches (Fig. 7) where myogenic cells are found that have migrated from the somites (see Milaire, 1976). Here too, its expression is transitory: in the limb buds, for example, *myf-5* transcripts are only accumulated at detectable levels from 10.5–11 days and subsequently decrease rapidly. Myogenin and MyoD1 begin to be expressed together slightly later (from 11.5 days) (Sassoon *et al.* 1989) but remain major transcripts in the muscle masses of the limbs, jaw and tongue throughout prenatal development. Expression of the three factors is also observed in the extraocular muscles, again with *myf-5* transcripts detectable first (data not shown). This observation is of particular interest since facial skeletal muscles may be partially derived from the prechordal plate mesoderm as well as from the most rostral somites (Wachtler and Jacob,

1986, for review), suggesting that *myf-5* is expressed in both types of myogenic precursor.

The distinct temporal patterns of expression of *myf-5*, myogenin and MyoD1 during myogenesis *in vivo* raise a number of questions in relation to their potential roles as transcriptional factors. First, the autoactivation phenomenon between this family of genes demonstrated in cell culture systems (Braun *et al.* 1989b) does not seem to operate in the *in vivo* situation. *myf-5* and myogenin are expressed at high levels for two days before the onset of MyoD1 expression, and *myf-5* is subsequently down regulated while MyoD1 and myogenin expression is maintained. Second, there is the question of the activation of expression of muscle structural genes. Transcripts of the muscle structural genes first appear in myotomes asynchronously over a period of several days (Lyons *et al.* 1990) and one can conclude that activation of muscle structural genes at any given time is taking place with a subset of myogenic regulatory factors. Initially *myf-5* is expressed in the absence of muscle gene transcription. If the *myf-5* protein is also present, either a second myogenic factor

**Table 1.** Transcript accumulation of the myogenic regulatory sequences during the formation of myotomes and body musculature in the mouse

	Days post coitum									
	8	8.5	9.25	9.5	10.5	11.5	12.5	14.5	16	17.5
Number of somites	1–7	8–12	18–25	21–29	35–39	45	50	64		
<i>myf-5</i>	+	+	++	++	++	++	+	+/-	-	-
myogenin	-	++	+++	+++	+++	+++	+++	+++	+++	+++
MyoD1	-	-	-	-	++	+++	+++	+++	+++	+++
Number of embryos examined	3	2	2	3	3	2	2	1	1	2



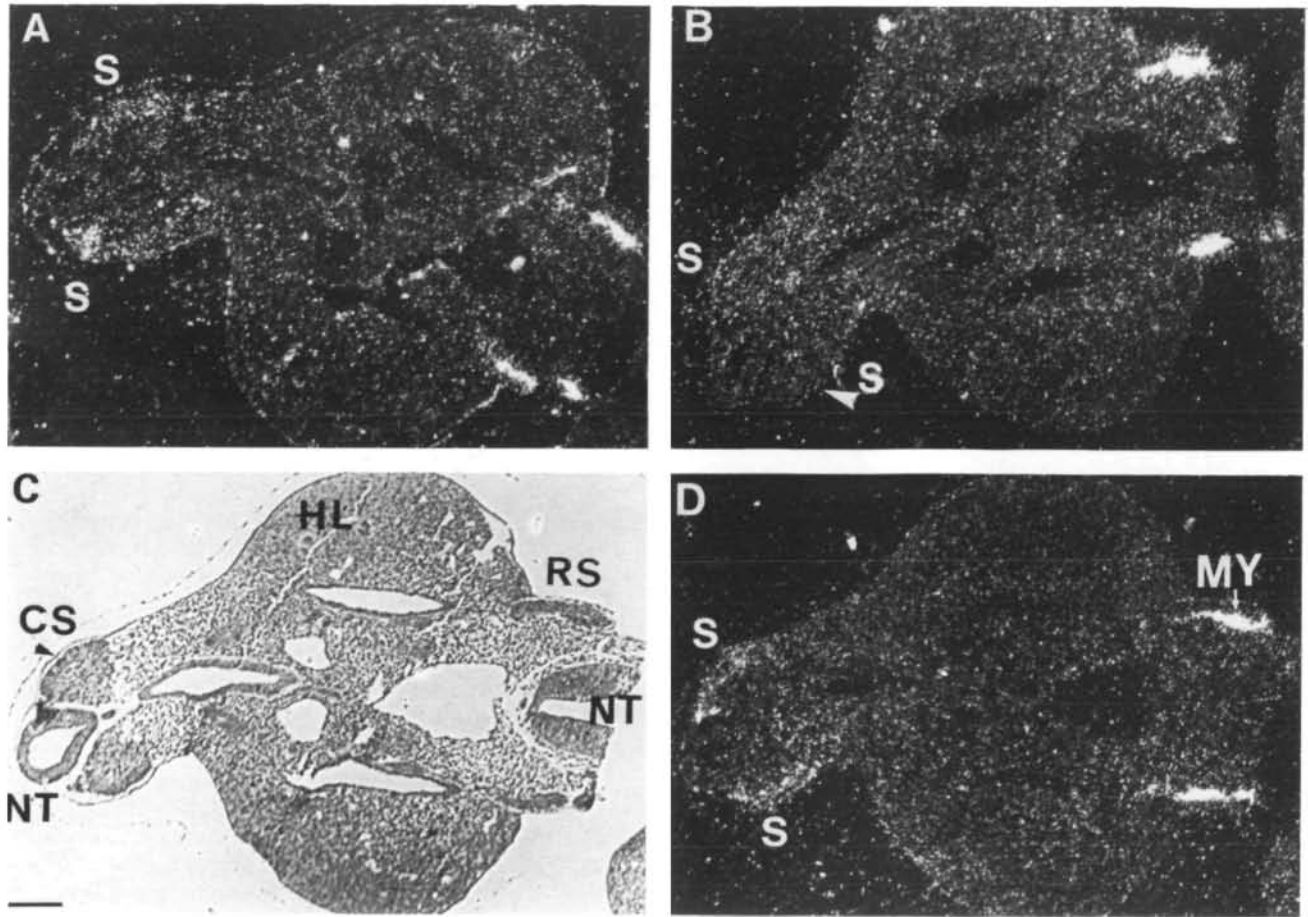


Fig. 6. Serial transverse sections of a 10.5 day *p.c.* embryo hybridized alternatively with *myf-5* (300) (A,D) or myogenin B; C is the phase contrast micrograph of the dark-field photograph shown in D. RS, rostral somites; CS, caudal somites; NT, neural tube; S, somite; MY, myotome; HL, hindlimb bud. Scale bar=100 microns.

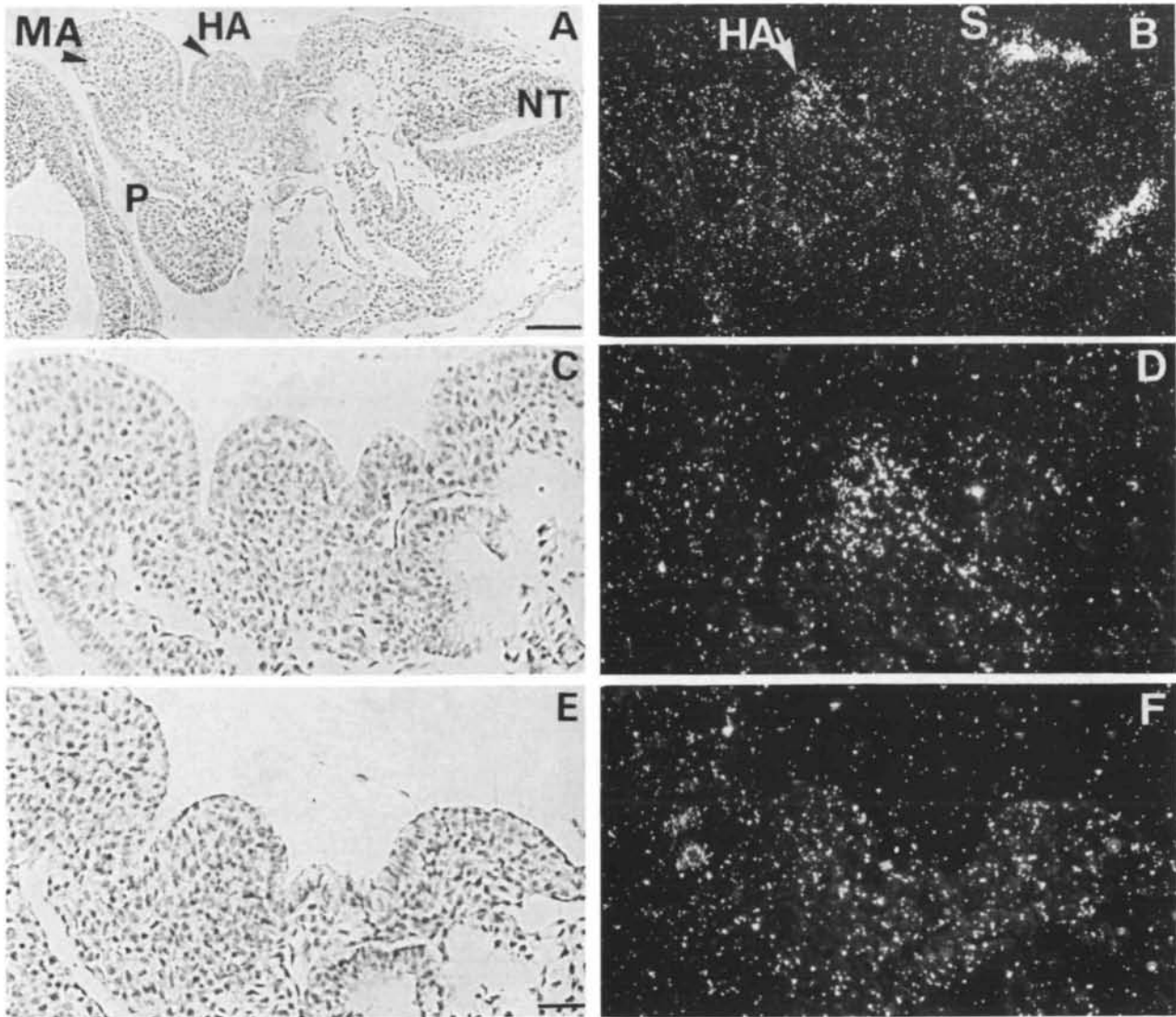
such as myogenin is required, or the levels of a negative regulator such as Id (Benezra *et al.* 1990) are sufficiently high to prevent *myf-5* binding to DNA. *myf-5*, like myogenin and MyoD1, is not detectable in the heart, although many of the muscle structural genes expressed during skeletal muscle development are also expressed in cardiac muscle (Minty *et al.* 1982; Lyons *et al.* 1990). Presumably different regulatory sequences interacting with different regulatory factors are involved (Mar *et al.* 1988).

The early appearance of *myf-5* transcripts in the mouse embryo suggests that it may be involved in muscle cell determination, prior to the activation of myogenic differentiation *in vivo*. This view is supported by the initial localization of *myf-5* expression. By *in situ* hybridization, we do not detect any *myf-5* transcripts prior to segmentation of somites from the paraxial mesoderm. However, as soon as somites form, initially as a ball of epithelial-like cells, *myf-5* transcripts are detectable. As the somites begin to mature, the dermomyotome in the dorsal part can be distinguished from the ventral sclerotome. Intense labelling with *myf-5* is seen at this stage over cells in the dorsomedial lip region of the dermomyotome adjacent to the neural tube. Based on embryological observations, it is

thought that cells from here will migrate under the dermomyotome to form the myotome (see Kaehn *et al.* 1988). The fact that a myogenic regulatory gene is expressed in the cells of the dorsomedial lip and later in the myotome is in keeping with this proposal. *myf-5* is thus a marker of dermomyotomal cells that will form the myotome. Cells migrate out from the dermomyotome, particularly from the ventral lateral edge (Christ *et al.* 1977; Milaire, 1976; Ede and El-Gadi, 1986) prior to the formation of myotome to found other skeletal muscle masses of the body and limbs. Transcripts of *myf-5* are not confined to the dorsomedial lip but are also present in some cells throughout the dermomyotome, which may correspond to such migratory muscle precursor cells. *myf-5*-positive cells are not detectable in extrasomitic locations initially, although they accumulate in the early arches and limb buds. Our observations suggest that myogenic precursor cells can be distinguished from other cells in the limb bud at 10.5–11 days of development by their expression of *myf-5*.

Recently, the expression of an avian myogenic sequence, *qmfl*, related to both MyoD1 and *myf-5* has been described in the myotomes of quail embryos (Charles de la Brousse and Emerson, 1990). Its pattern





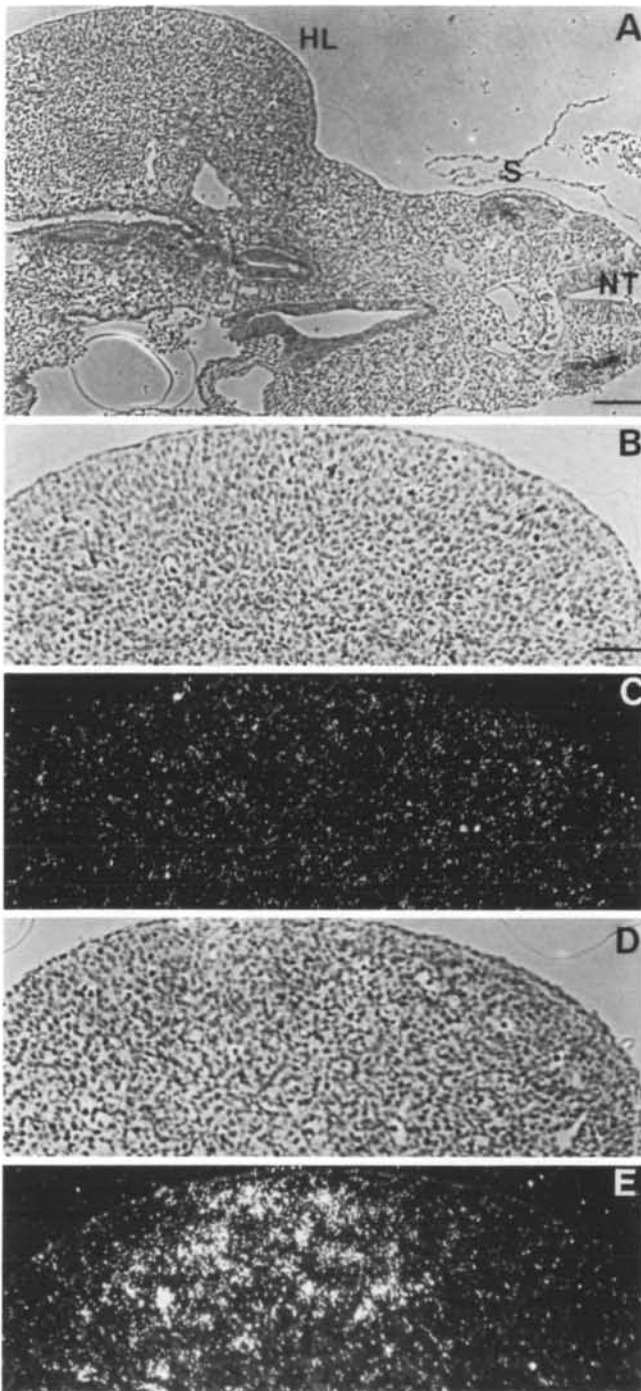
**Fig. 7.** Transverse sections of a 9.25 day *p.c.* (18 somites) embryo in the region of the pharynx showing the visceral arches. (A) Phase-contrast micrograph of a section hybridized with the *myf-5* (300 probe); (B) Dark-field micrograph of A, showing *myf-5* accumulation in the hyoid arch (arrow); (C) Enlargement of the visceral arches shown in A; (D) Dark-field micrograph of C; (E) Phase-contrast micrograph of a serial section hybridized with the myogenin probe; (F) Dark-field micrograph of E; MA, mandibular arch; HA, hyoid arch; P, pharynx; NT, neural tube; S, somites. Scale bar=50 microns.

of expression appears to differ from that of MyoD1 or *myf-5* in the mouse. *Qmf1* transcripts accumulate in the dorsomedial lip of the myotome, but have not been described prior to myotome formation and are absent from less mature caudal somites. Furthermore, the authors do not observe *qmf1* expression before that of the muscle structural gene troponin T. The earliest marker of myogenesis described to date is the sequence XMyoD which in *Xenopus* is expressed at gastrulation shortly after mesodermal induction. It is expressed subsequently in somites. In *Xenopus*, cardiac actin transcripts also first accumulate before somites form, and are detectable a few hours after XMyoD (see Hopwood *et al.* 1989; Harvey, 1990; Scales *et al.* 1990). It is difficult to make a direct comparison between this

situation and that in higher vertebrates where these events take place more slowly.

Results obtained with muscle cells in culture are in keeping with a potential role of *myf-5* at an early stage in the myogenic programme. Most cultured muscle cells are derived from late foetal or adult muscle and generally have a low level of *myf-5* expression (Braun *et al.* 1989*a,b*). However, *myf-5* transcripts are present in the inducible C2 cell line, prior to induction of these cells for terminal differentiation, at a stage when both MyoD1 and myogenin are absent (Montarras *et al.* 1989; C. Pinset and D. Montarras, personal communication). MyoD1 and particularly myogenin seem to be more closely associated with muscle cell differentiation.

In conclusion, during embryogenesis in the mouse,



**Fig. 8.** Serial sections of the hindlimb region of an 11 day *p.c.* embryo. (A) Phase-contrast micrograph of a section hybridized with myogenin; (B) Enlargement of A showing a portion of the hindlimb bud; (C) Dark-field micrograph of B; (D) Enlargement of a similar region of the hindlimb bud to that shown in B from a phase contrast micrograph of a serial section to that in A hybridized with the *myf-5* (300) probe. (E) Dark-field micrograph of D. HL, hindlimb; NT, neural tube; S, somite. Scale bar=50 microns.

the expression of *myf-5* is consistent with a role during myogenic determination, and certainly strongly suggests that it is an early determination marker,

specifying, in the dermomyotome, cells committed to follow the skeletal muscle cell differentiation pathway.

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