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Xenopus Myf-5 marks early muscle cells and can activate muscle genes ectopically in early embryos

N. D. HOPWOOD, A. PLUCK and J. B. GURDON

CRC Molecular Embryology Group, Department of Zoology, Downing Street, Cambridge, CB2 3EJ, UK

Summary

We have cloned a *Xenopus* cDNA that encodes a homologue of the human myogenic factor, Myf-5. *Xenopus* Myf-5 (XMyf5) transcripts first accumulate in the prospective somite region of early gastrulae. The pattern of XMyf5 expression is similar to that of the *Xenopus* MyoD (XMyoD) gene, except that XMyf5 transcripts are largely restricted to posterior somitic mesoderm even before any somites have formed. Transient ectopic expression of XMyf5 activates cardiac actin and XMyoD genes in animal cap cells, but does not cause full myogenesis, even in combination with XMyoD. These results suggest that XMyf5 acts together with XMyoD as one of the set of genes regulating the earliest events of myogenesis, additional factors being required for complete muscle differentiation.

Key words: helix-loop-helix proteins, mesoderm, muscle, myogenesis, *Xenopus*.

Introduction

The discovery of MyoD, a mouse cDNA that can convert cultured fibroblasts into myoblasts (Davis *et al.* 1987), has been followed by the isolation of three more mammalian myogenic factors related to MyoD: myogenin (Wright *et al.* 1989; Edmonson and Olson, 1989), Myf-5 (Braun *et al.* 1989*b*) and MRF4/Myf-6/herculin (Rhodes and Konieczny, 1989; Braun *et al.* 1990; Miner and Wold, 1990). They are all members of the helix-loop-helix family of DNA-binding proteins (Murre *et al.* 1989*a*) and can bind to some of the sequences required for transcription from muscle-specific promoters (Lassar *et al.* 1989; Braun *et al.* 1990; Brennan and Olson, 1990; Piette *et al.* 1990). Much has been learned about muscle development and the role of the MyoD family of myogenic factors from analysis of the differentiation of determined myoblasts in culture. However, it is important to find out to what extent conclusions drawn from work on tissue culture models of myogenesis also apply to the normal development of muscle in embryos.

The African clawed frog, *Xenopus laevis*, provides large, accessible embryos and much is already known about their development of mesoderm in response to an early cellular interaction (reviews by Smith, 1989; Hopwood, 1990). A *Xenopus* homologue of MyoD (XMyoD) is expressed as an early consequence of mesoderm induction in the myotomes, from which the axial musculature develops (Hopwood *et al.* 1989*a*; Harvey, 1990). XMyoD RNA accumulates above a low maternal level about two hours before muscle-specific actin transcripts first appear (Hopwood *et al.* 1989*a*; Harvey, 1990; Scales *et al.* 1990) and so is expressed at the right time and in the right place to be involved in the initiation of normal myogenesis. This is not so in mammals, and may not be in birds: in mouse embryos, myogenin and cardiac actin transcripts were detected at 8½ days post coitum, but MyoD transcripts did not appear until two days later (Sassoon *et al.* 1989); transcripts encoding a quail relative of MyoD, qmf1, first appear at about the same time as cardiac actin RNA (de la Brousse and Emerson, 1990). MRF4 appears to be expressed even later in mammalian development than MyoD (Rhodes and Konieczny, 1989). The pattern of embryonic expression of the Myf-5 gene has not been described.

MyoD can activate muscle genes in various types of cultured cells, although in many experiments the frequency of activation has been low, and some cells were entirely refractory (Davis *et al.* 1987; Weintraub *et al.* 1989; Schäfer *et al.* 1990). The activity of XMyoD in normal embryonic cells was tested by expressing it ectopically in early embryos *via* microinjection of synthetic XMyoD mRNA. This caused ectopic activation of the cardiac actin gene in animal cap cells (Hopwood and Gurdon, 1990), which normally form ectodermal structures. XMyoD is therefore sufficient, together with cellular components already present in animal caps, to activate a muscle gene in embryonic cells of a non-muscle lineage. However, these XMyoD-injected animal caps, which expressed the cardiac actin gene at a similar level to myotomal cells of early neurulae, did not subsequently differentiate any muscle (Hopwood and Gurdon, 1990). They also failed to express the 12/101 antigen, a muscle marker detectable

from post-neurula stages onwards (Kintner and Brockes, 1984). This suggested that XMyoD alone was insufficient to cause full myogenesis, but might normally co-operate with other factors to do so. Possible co-operators are the other known members of the MyoD family, such as Myf-5.

We have cloned a *Xenopus* homologue of the human myogenic factor, Myf-5 (Braun *et al.* 1989*b*), and used the clone to analyze its expression and activity in early embryos. The specific expression of XMyf5 in the somites closely parallels that of XMyoD, but XMyf5 RNA is largely restricted to posterior somitic mesoderm even before any somites have formed. We have expressed XMyf5 ectopically by microinjecting synthetic mRNA into early embryos, and find that XMyf5, like XMyoD, can activate muscle genes in animal cap cells. However, even co-injection of XMyf5 and XMyoD did not cause full myogenesis.

Materials and methods

Characterization of a XMyf5 cDNA

A st17 cDNA library (Kintner and Melton, 1987) was screened with a random primed probe (Feinberg and Vogelstein, 1983) as described by Hopwood *et al.* (1989*a*). The XMyf5-2 cDNA was sequenced using the dideoxy chain termination method (Sanger *et al.* 1977; Biggin *et al.* 1983) on M13 templates (Messing, 1983). Templates for sequencing the 3′ *Eco*RI fragment (nt132–1134) were generated using the method of Henikoff (1984). The 5′ *Eco*RI fragment was sequenced separately, and the *Eco*RI junction sequenced as part of the 5′ *Sac*I fragment of a clone of the whole XMyf5-2 cDNA made by partial *Eco*RI digestion of the λgt10 clone. The cDNA sequence was compiled using the DB programmes (Staden, 1982). Each base was sequenced at least twice on each strand.

Analysis of RNA

Northern blotting was carried out as described (Hopwood *et al.* 1989*a*), all blots being washed to 0.2×SSPE, 0.1% SDS at 65°C. A single-stranded DNA probe for XMyf5 transcripts was produced from the 3′ *Rsa*I–*Eco*RI fragment (nt887–1134) of XMyf5-2 cloned between the *Sma*I and *Eco*RI sites of M13mp19. A similar 3′ untranslated region (UTR) probe was used for XMyoD (Hopwood and Gurdon, 1990); cardiac actin RNA was detected as described (Hopwood and Gurdon, 1990) using the pSP21S RNA probe of T. J. Mohun; and EF-1α probes were made by random priming (Feinberg and Vogelstein, 1983) of a cDNA (Krieg *et al.* 1989). A single-stranded DNA probe for injected XMyoD and XMyf5 RNA was made from the *Bgl*II–*Eco*RI fragment of pSP64T containing the β-globin 3′ UTR (Krieg and Melton, 1984) cloned between *Bam*HI and *Eco*RI sites of M13mp19. The number of XMyf5 transcripts in 10 μg gastrula RNA was determined by the same method used previously for XMyoD (Hopwood *et al.* 1989*a*), using synthetic XMyf5 RNA transcribed with T7 RNA polymerase from the XMyf5-2 cDNA cloned in pSP73 (Krieg and Melton, 1987) after linearization with *Bgl*II.

In situ *hybridization*

This was done as described by Kintner and Melton (1987) and Hopwood *et al.* (1989*a*). In order to orient them more easily, wild-type rather than albino gastrulae were used. The pigment grains in sections from wild-type embryos were bleached by treatment for 16 h in 2% (v/v) hydrogen peroxide in 2×SSPE prior to hybridization. XMyf5 transcripts were detected using a probe made from the pSP73–XMyf5-2 clone after linearization with *Sac*II (includes 3′ UTR and last 45 nt of coding region). The XMyoD probe was that used previously (Hopwood *et al.* 1989*a*).

Transcription clones and RNA synthesis

The clones pSP64T–XMyoD and –XMyoD114P have been described (Hopwood and Gurdon, 1990). The plasmid pSP64T–XMyf5 was constructed by first using site-directed mutagenesis (Amersham kit, Version 2) to create a *Nco*I site around the XMyf5-2 initiation codon. The *Nco*I–*Rsa*I fragment containing the coding sequence and 28 bp of 3′ UTR was then transferred to pSP64–Xβm (Krieg and Melton, 1984), from which the β-globin coding sequence had been removed by digestion with *Nco*I and *Bst*EII (filled in). The point mutant derivative pSP64T–XMyf5–102P was produced by site-directed mutagenesis of the smaller *Sac*I–*Sac*I fragment of pSP64T–XMyf5 in M13mp19 using the oligonucleotide 5′GTGAACCAGCCTTTTGAAACGC3′. Sequencing of the mutant confirmed that no other bases had been changed. Capped, trace-labelled synthetic RNA was produced from these clones after *Xba*I digestion, using SP6 RNA polymerase (Krieg and Melton, 1987). Transcripts were capped by including m^7GpppG (New England Biolabs) in the reactions, and unincorporated nucleotides were removed by three rounds of ethanol precipitation.

In vitro *translation*

Synthetic RNAs (20 μg ml^{-1}) were translated *in vitro* in rabbit reticulocyte lysate (purchased from R. T. Hunt, Dept of Biochemistry, University of Cambridge) as described by Jackson and Hunt (1983). Translation products were resolved by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970).

Embryos and RNA injection

Xenopus laevis embryos were cultured as described (Gurdon, 1977). The neurula dissections were performed in MBS containing 3 mg ml^{-1} collagenase (Sigma C-2139). RNA at 0.5 mg ml^{-1} in distilled water was injected into the animal half of both cells of two-cell embryos (2–3 ng per cell) in 1×MBS, 2% Ficoll 400. After a couple of hours, embryos were transferred to 0.1×MBS for further culture.

Results

Cloning and sequence of a new Xenopus *myogenic factor*

A probe made from the whole rat myogenin cDNA (Wright *et al.* 1989) was used to screen a *Xenopus laevis* neurula cDNA library (Kintner and Melton, 1987) at low stringency. Forty hybridizing clones were plaque purified, of which 22 cross-hybridized at high stringency to the *Xenopus* MyoD cDNA described previously (XMyoD2–24; Hopwood *et al.* 1989*a*). All of these clones also cross-hybridized at high stringency to a probe containing only the 3′ part of the XMyoD2–24 3′ untranslated region (UTR), and gave rise to restriction fragments diagnostic of XMyoD cDNAs (data not shown). Of the remaining clones, ten cross-hybridized to each other at high stringency, two had very small or no inserts, and the hybridizing regions of the other six

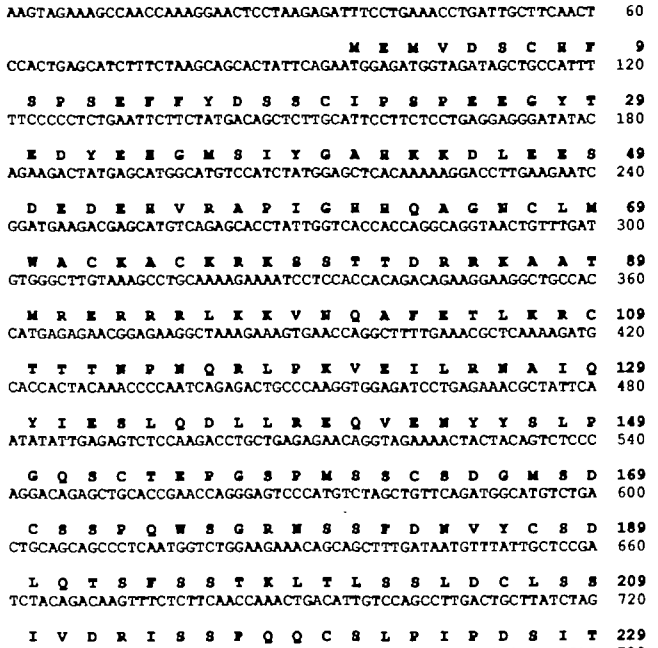

Fig. 1. Sequence of the XMyf5–2 cDNA. The asterisk indicates the translation stop, and the poly(A)-addition signal is underlined.

Fig. 2. Comparison of the predicted XMyf5 protein sequence to those of representatives of the four mammalian myogenic factors, human Myf-5 (Braun *et al.* 1989*b*), mouse MyoD (Davis *et al.* 1987), rat MRF4 (Rhodes and Konieczny, 1989) and rat myogenin (Wright *et al.* 1989). The box surrounds the basic-helix-loop-helix domain; amino acid residues identical to those of XMyf5 are shown in bold. XMyf5 is more closely related to Myf-5 than to the other factors, showing extensive similarity outside the basic-helix-loop-helix region.

were not significantly homologous at the level of predicted protein to the myogenin probe (data not shown). We have further analysed the class of ten clones, which apparently all represent transcripts from the same gene, choosing one of the longest representatives (XMyf5–2) for sequencing.

The cDNA sequence of the clone XMyf5–2 contains a single long open reading frame, preceded upstream by in-frame stop codons (Fig. 1). The cDNA is, at 1134 bp, a similar size to the transcripts it detects on northern blots (see below), and ends in a poly(A) tail. The predicted protein is much more similar to the human myogenic factor, Myf-5 (Braun *et al.* 1989*b*), and to its bovine homologue (Clark *et al.* 1990), than to any of the other known mammalian members of this class, including myogenin, which was used as the probe (Fig. 2). All of the known myogenic factors are very similar in the helix-loop-helix and DNA-binding domains, but they show only limited similarity to each other outside these regions. However, the predicted protein sequence of the new *Xenopus* factor is very like that of Myf-5 in many regions where there is no relationship between Myf-5 and any of the other factors. Seventy per cent of amino acid residues are identical between the frog and human proteins, and a further 10% represent conservative substitutions. The *Xenopus* factor, whilst it may not have identical properties to the human protein, is therefore probably a homologue of Myf-5, and so we designate it XMyf5.

Accumulation of XMyf5 transcripts during development

We have analyzed the accumulation of XMyf5 transcripts in early *Xenopus* development, comparing the pattern to that of transcripts from the XMyoD gene(s). In order to compare accurately the times of onset of accumulation of XMyoD and XMyf5 transcripts, RNA was extracted from blastula and gastrula embryos at timed intervals following fertilization. Specific probes made from the 3′ untranslated regions of the XMyf5–2 and XMyoD2–24 cDNAs (see Materials and methods) were then hybridized successively to the RNA on a northern blot. The XMyf5 probe detected two sizes of transcripts of about 1.2 and 1.3 kb (not resolved in the autoradiographs presented), which were first clearly detectable in late (st9½) blastulae, and then accumulated rapidly during gastrulation (Fig. 3A). We do not know the origin of the two XMyf5 (and XMyoD) transcript size classes, but one possible explanation is that the transcripts from the (non-allelic) duplicated genes in the tetraploid genome of *Xenopus laevis* (Kobel and DuPasquier, 1986) are slightly different sizes.

Determination of the absolute number of XMyf5 transcripts in gastrula (st11) RNA (see Materials and methods) allowed values obtained from densitometry of

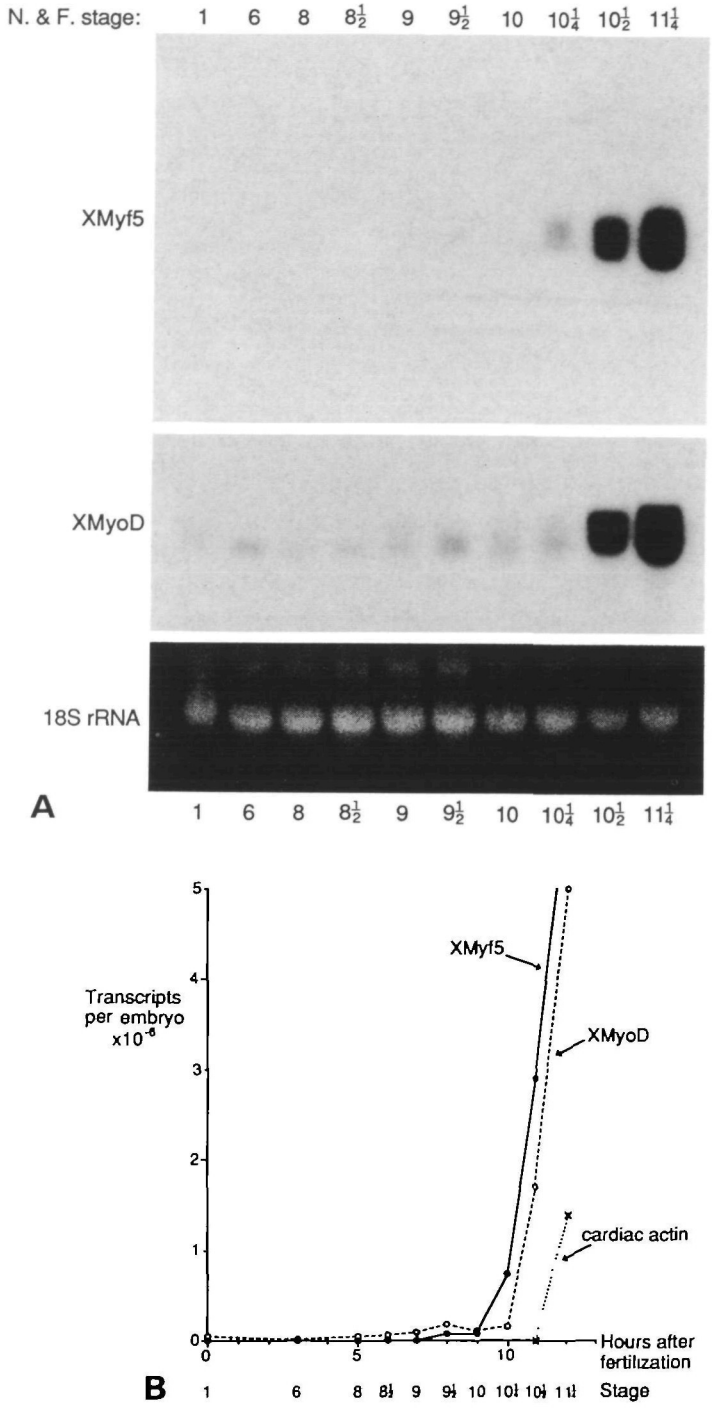

Fig. 3. XMyf5 expression in early development.
(A) Northern blot of RNA from early embryos (two per lane) at timed stages after fertilization, probed successively for XMyf5, XMyoD and cardiac actin (not shown) transcripts (see Materials and methods). (B) Graph plotted from densitometry of appropriate autoradiographic exposures of the blot shown in A, using transcript number measurements for XMyf5 (see Materials and methods) and XMyoD (Hopwood *et al.* 1989*a*). The content of XMyf5 and XMyoD transcripts increases before transcripts of the cardiac actin gene first appear. Stages are those of Nieuwkoop and Faber (1967). Embryos were cultured at 23 °C.

the autoradiographs to be compared to those for XMyoD, and a graph to be plotted (Fig. 3B). It shows that the first substantial increase in the number of transcripts occurs at about the same time for both XMyoD and XMyf5 in early gastrulae. This precedes by about two hours the first accumulation of transcripts from the cardiac actin gene (Mohun *et al.* 1984; Cascio and Gurdon, 1986) and represents the earliest known cell type-specific activity of embryonic muscle cells. Unlike for XMyoD (Hopwood *et al.* 1989*a*; Harvey, 1990), we did not detect any maternal XMyf5 transcripts in this or other similar experiments. Using a more sensitive RNAase protection assay we could still see none, and estimate that there are fewer than 5×10^4 transcripts per egg that fully protect a XMyf5–2 probe (data not shown). XMyf5 transcripts accumulate to a peak in neurulae, and then their concentration declines in later development slightly earlier than the similar decline in the content of XMyoD transcripts (data not shown). We conclude that the timing of accumulation of XMyf5 RNA in whole embryos is similar to that for XMyoD.

XMyf5 transcripts are somite-specific

We have used northern blots of dissected embryonic tissues and *in situ* hybridization to determine the location of XMyf5 transcripts in early embryos. *In situ* hybridization to sections of gastrulae and neurulae showed that XMyf5 transcripts are present only in the somites, from which the axial musculature develops. Transverse sections of late gastrulae and late neurulae were hybridized with either XMyf5 or XMyoD probes: they both labelled the same region of the embryos that contains cardiac actin transcripts (Hopwood *et al.* 1989*a*), namely the somites (Fig. 4A–D). *In situ* hybridization to tadpole (st33–34) heart sections showed no labelling above background with a XMyf5 probe; the somites in tail sections from the same embryos on the same slides were labelled (data not shown).

The somite-specificity of XMyf5 transcripts was also shown by dissecting late neurulae into component parts and analyzing RNA from the different tissues by northern blotting. XMyf5 transcripts were detectable only in the somites and in the 'tail', the posterior part of the embryo that is difficult to separate into different germ-layer components (Fig. 5). The blot was reprobed for cardiac actin transcripts, which are known to be somite-specific (Mohun *et al.* 1984), and for EF-1α transcripts, which encode a translation factor found in all cells and so show the relative amounts of total RNA in each sample (Krieg *et al.* 1989). Cardiac actin transcripts were, as expected, also present only in somite and tail RNA but were differently distributed between the two fractions from XMyf5 transcripts: most cardiac actin RNA is in the somites in the trunk of the embryo, whereas most XMyf5 RNA is in the posterior 'tail' region (see below). We conclude that XMyf5, like XMyoD (Hopwood *et al.* 1989*a*; Harvey, 1990), is expressed only in the somites of early embryos.

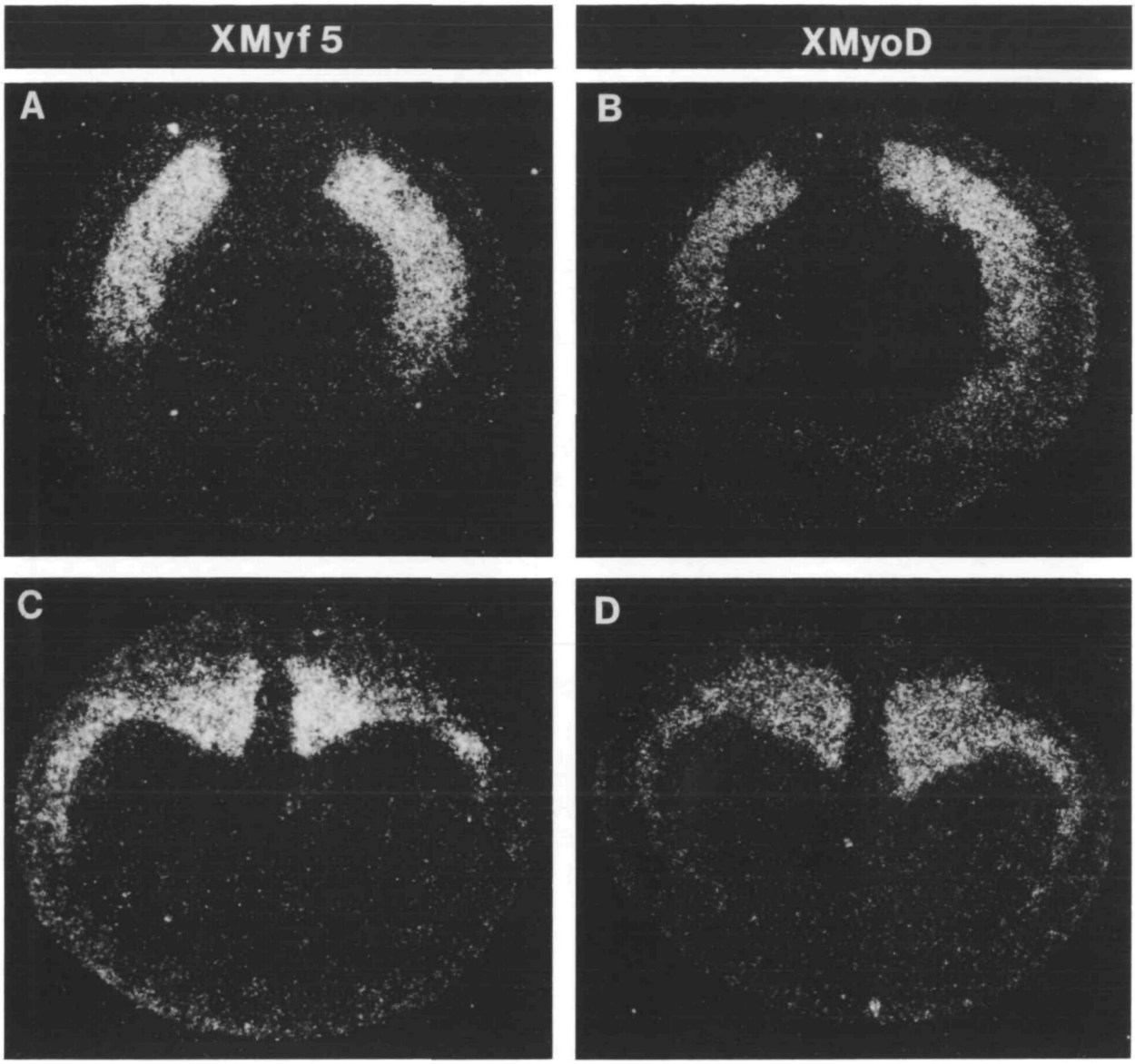

Fig. 4. XMyf5 transcripts are somite-specific. *In situ* hybridizations to transverse sections through the posterior parts of late (st11½) gastrulae (A,B) and late (st18) neurulae (C,D), probed for XMyf5 (A,C) or XMyoD (B,D). Note that the neurula sections are from the 'tail' region used for the northern analysis in Fig. 5 below, and show somite-specific expression in this part of the embryo which was not dissected into its component tissues. The gastrula sections, from wild-type embryos, were bleached with hydrogen peroxide (see Materials and methods); the neurula sections were from albino embryos. Probe concentrations (and exposure times) were: (A,B) 400 cts $min^{-1}\mu l^{-1}$ (one month); (C) 750 cts $min^{-1}\mu l^{-1}$ (one week); (D) 600 cts $min^{-1}\mu l^{-1}$ (two weeks).

XMyf5 RNA is restricted to posterior somitic mesoderm before XMyoD RNA

The concentration of XMyoD RNA in the somites falls after they have formed (Hopwood *et al.* 1989*a*). Since they differentiate from anterior to posterior, XMyoD RNA becomes restricted to progressively more posterior somites, being in tadpoles (st33–34) confined largely to the still developing tail muscle. Analysis of RNA from dissected neurulae (st18; Fig. 5) showed that XMyf5 transcripts are found even at this stage mainly in posterior tissue ('tail'), when a significant amount of XMyoD RNA is still present in the somites of the trunk (Hopwood *et al.* 1989*a*). We have therefore investigated the anterior–posterior location of XMyf5 RNA at earlier stages, comparing it to both XMyoD and cardiac actin transcripts.

Late gastrulae (st12½), and mid- (st15) and late (st18) neurulae were divided into three pieces along the anterior–posterior axis, and RNA from these parts analyzed by northern blotting successively for transcripts from XMyf5, XMyoD, cardiac actin and EF-1α genes (Fig. 6). At st12½ the anterior sample contained very little of any of the muscle-specific RNAs. They were divided between the middle and posterior

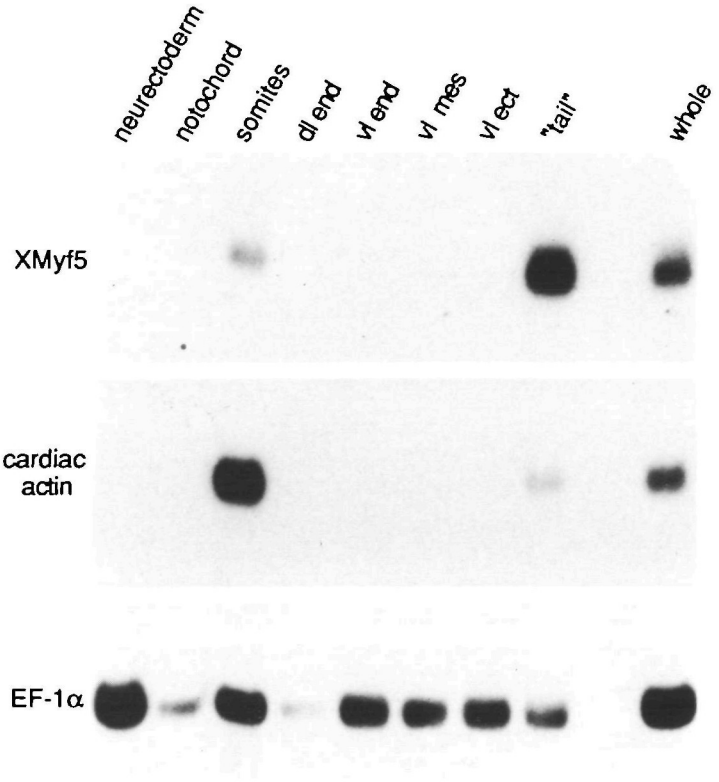

Fig. 5. Northern blot of RNA from dissected parts of late (st18) neurulae, probed successively for transcripts from the XMyf5, cardiac actin (Mohun *et al.* 1984), and EF-1α (Krieg *et al.* 1989) genes. Cardiac actin is a striated muscle marker, and EF-1α shows the relative amounts of total RNA in each lane. Parts were pooled from five dissected embryos; RNA from one whole embryo was analysed for comparison. The dorsal endoderm and notochord lanes were relatively underloaded in this experiment. Two other experiments, in which relatively more endodermal RNA was used, showed that the endoderm does not contain a significant concentration of XMyf5 RNA at st13 or at st18 (data not shown). That the notochord does not express XMyf5 was shown by *in situ* hybridization (Fig. 4A,C). Dl, dorsal; vl, ventral; end, endoderm; mes, mesoderm; ect, ectoderm.

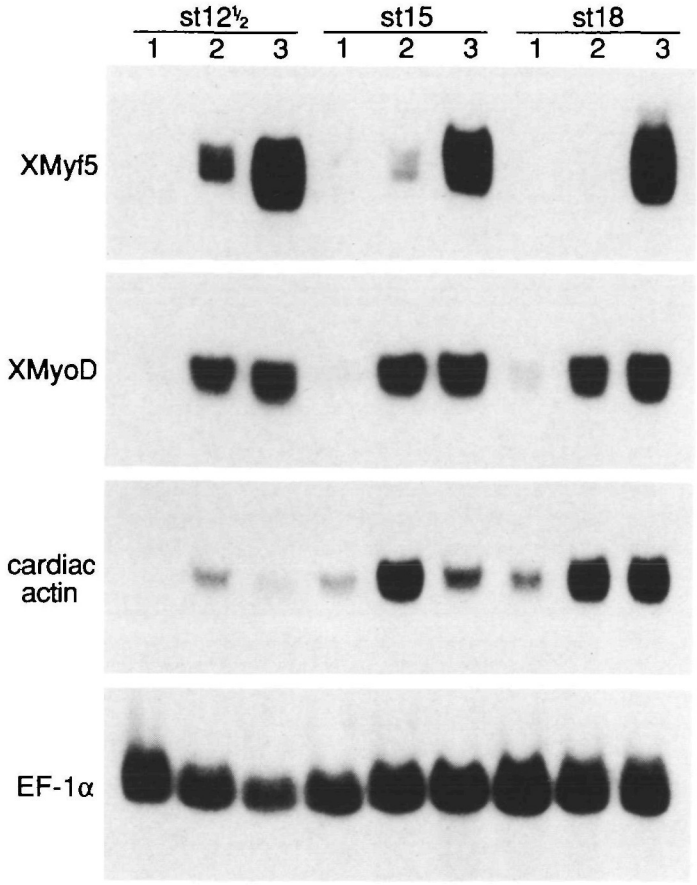

Fig. 6. XMyf5 transcripts become restricted to posterior somites before those from the XMyoD gene. Northern blot of RNA from anterior (1), middle (2) and posterior (3) parts of late gastrulae (st12½), mid-neurulae (st15) and late neurulae (st18) probed successively for transcripts from XMyf5, XMyoD, cardiac actin and EF-1α genes. Parts from six embryos were loaded in each lane.

fractions as follows: over 90% of XMyf5 RNA was in the posterior fraction; XMyoD RNA was fairly evenly distributed, with slightly more in the posterior fraction; and most cardiac actin transcripts were in the middle fraction. In the neurulae, XMyf5 transcripts became even more strikingly restricted to posterior tissue, as the amount in the middle pieces declined to a barely detectable level. The distribution of XMyoD RNA changed little: at st18 there were still roughly equal amounts of XMyoD RNA in middle and posterior fractions. It becomes restricted to the forming tail only in later embryos (Hopwood *et al.* 1989*a*). Cardiac actin RNA accumulated first in the middle pieces (st15) and then also in the posterior pieces (st18).

There are two possible interpretations of these results: either XMyf5 is always restricted to posterior somitic mesoderm, or it is expressed in anterior tissue prior to the late gastrula stage, but expression there declines before that of XMyoD. We have not been able to eliminate one of these possibilities by analysis of dissected parts of embryos, because XMyf5 transcripts are already restricted to posterior tissue at the earliest stage at which we can cleanly separate anterior and posterior mesoderm. However, for several reasons, we believe the latter to be more likely. *In situ* hybridizations to st11½ gastrulae show similar labelling by XMyoD and XMyf5 probes, XMyoD transcripts being detectable only a few sections more anterior than XMyf5 RNA (Fig. 4A,B, and data not shown). Furthermore, in whole embryos the content of XMyf5 transcripts declines before that of XMyoD (data not shown), and the content of XMyf5 RNA in the trunk declines progressively from st12½ to st18 (Fig. 6). We therefore believe that XMyoD and XMyf5 are activated at about the same time (Fig. 3), probably in the whole somitic mesoderm, but that XMyf5 transcripts disappear sooner than those of XMyoD from the anterior tissue that differentiates first.

Ectopic expression of XMyf5

Myf-5 function has to date been tested only in C3H10T½ fibroblasts and their derivatives, which it can convert to myoblasts (Braun *et al.* 1989*b*). It is, however, important to know what the myogenic factors can do in normally developing embryonic cells at the time when muscle differentiation is first being established. We have previously expressed XMyoD ectopically in early embryos by microinjection of synthetic mRNA, and found that it can activate some muscle genes, but not cause complete muscle differentiation (Hopwood and Gurdon, 1990). We wished to find out to what extent the activity of XMyf5 is similar to that of XMyoD, and whether co-injection of the two factors, which are normally co-expressed in very early muscle cells, would cause more complete muscle differentiation.

Preliminary experiments showed that capped RNA produced from the XMyf5–2 cDNA cloned in pSP73 (Krieg and Melton, 1987) did not activate the cardiac actin gene in embryos, although the RNA was stable enough that a high concentration remained at the time of normal activation of the gene (data not shown). RNA transcribed from an equivalent XMyoD clone had also been ineffective (N.D.H. and J.B.G., unpublished data), but a template in which the XMyoD coding region (from which all of the 5′ UTR and most of the 3′ UTR had been removed) was inserted between the 5′ and 3′ UTRs of a *Xenopus* β-globin gene (Krieg and Melton, 1984) allowed production of active mRNA (Hopwood and Gurdon, 1990). We therefore constructed a similar template for XMyf5 (Fig. 7A). (We note that RNAs transcribed from templates in which the β-globin UTRs flanked XMyoD or XMyf5 sequences, but in which fusion to the β-globin 5′ UTR was made within the 5′ UTR of the myogenic factor cDNAs, and the 3′ UTR was not removed, caused no detectable activation of the cardiac actin gene.)

A control for the non-specific effects of RNA injection was made by mutating a single G residue to a C, thus changing an alanine codon to one for a proline in the predicted first helix of the helix-loop-helix domain (Fig. 7A). This minimal change in the physical structure of the RNA would be expected to inactivate the encoded XMyf5 protein by preventing oligomerization, which is required for DNA binding. Equivalent mutations in mouse and frog MyoD proteins prevent biological activity (Davis *et al.* 1990; Hopwood and Gurdon, 1990). XMyf5 and the XMyf5–102P mutant RNAs are translated *in vitro* with similar efficiency, and with similar efficiency to XMyoD and XMyoD114P mutant RNAs, producing proteins of apparent M_r about 39000 compared to the predicted M_r of 28000 (Fig. 7B). Similarly aberrant migration in SDS gels has been observed for mouse, chicken and frog MyoD proteins (Tapscott *et al.* 1988; Lin *et al.* 1989; Scales *et al.* 1990; Hopwood and Gurdon, 1990) and for myogenin (Brennan and Olson, 1990).

We first tested the effect of XMyf5 RNA injection in animal cap cells of blastulae, which, although they can be induced experimentally to form muscle (Sudarwati and Nieuwkoop, 1971), normally develop only into

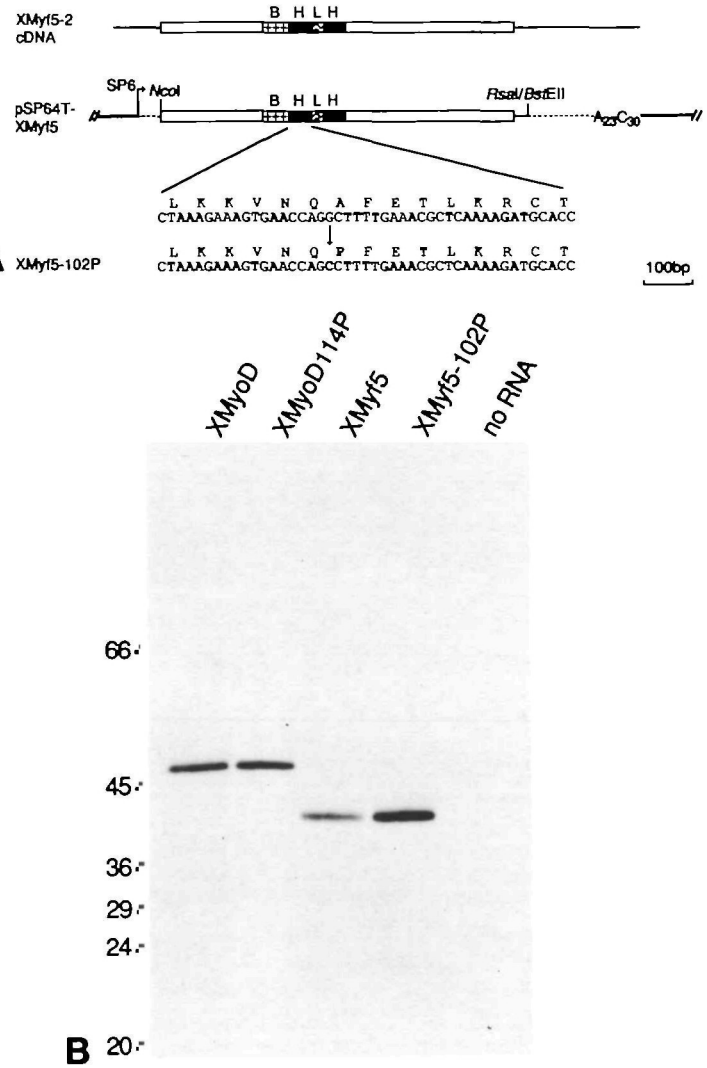

Fig. 7. (A) Clones for expression of XMyf5 in embryos, and point mutant control. Their construction is described in the Materials and methods. BHLH, basic-helix-loop-helix region; $A_{23}C_{30}$, poly(dA)–poly(dC) tracts; dotted line *Xenopus* β-globin UTRs. (B) Translation in a rabbit reticulocyte lysate of XMyoD, XMyoD114P, XMyf5 and XMyf5–102P synthetic mRNAs. Autoradiograph of ^{35}S-labelled protein. Numbers are $M_r \times 10^{-3}$ of marker proteins. The mutant controls are translated as efficiently as the non-mutated mRNAs.

ectodermal structures. Synthetic mRNAs were injected into both cells of two-cell embryos, from which the animal caps were removed at the late blastula stage (st9). They were cultured until sibling embryos passed the stage at which muscle actin genes are normally activated, and then analyzed by northern blotting for transcripts from the cardiac actin gene (Fig. 8). Injection of XMyf5 RNA activated this normally muscle-specific gene as effectively as injection of an equivalent amount of XMyoD RNA, that is to the same level as in the myotomes of uninjected early neurulae (Hopwood and Gurdon, 1990). The mutant XMyf5–102P RNA did not, thus confirming the validity

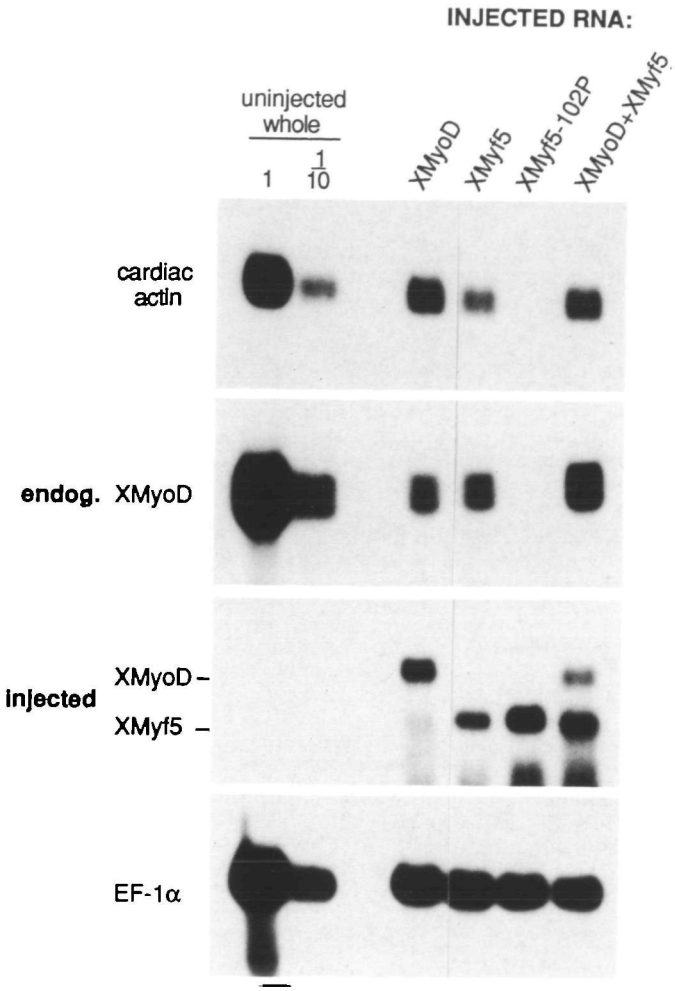

Fig. 8. XMyf5 can activate cardiac actin and XMyoD genes in animal cap cells. Animal caps were dissected from RNA-injected embryos and cultured until sibling embryos became late neurulae (st18), when they were frozen for analysis by northern blotting. RNA from 12 animal caps was pooled and RNA from two animal cap equivalents probed successively for cardiac actin transcripts, injected RNA, and EF-1α transcripts. The injected RNA was detected using a probe that recognizes the β-globin 3′ UTR that is part of the injected transcripts, but is not present in uninjected embryos at the stage of analysis; injected XMyf5 transcripts are distinguishable from injected XMyoD RNA, because they are smaller. The remaining 10 animal cap equivalents were probed for *trans*-activated transcripts from the endogenous (endog.) XMyoD gene(s). Note, therefore, that for this panel, five times more total RNA was used in the animal cap samples relative to the whole embryo standards than is indicated by the EF-1α analysis shown. The cardiac actin and XMyoD genes were activated in XMyoD- and XMyf5-injected animal caps, but not in animal caps injected with the point mutant XMyf5–102P RNA. Co-injection of XMyoD and XMyf5 RNAs did not show any synergy in activating the XMyoD and cardiac actin genes.

of the result. XMyf5 also activated the endogenous XMyoD gene(s) to the same extent as XMyoD did (to about one-fortieth of the transcript level per myotomal cell; Hopwood and Gurdon, 1990) (Fig. 8). In contrast, we have detected no activation by either XMyoD or XMyf5 of the endogenous XMyf5 gene(s). Using a RNAase protection assay, we estimate that XMyf5 gene activation by either XMyoD or XMyf5, if it occurs, is at least fivefold less effective than activation of the XMyoD gene (data not shown). When injected together, XMyoD and XMyf5 RNA acted additively rather than synergistically in activating both cardiac actin and XMyoD genes (Fig. 8).

XMyf5-injected embryos developed into fairly normal tadpoles (data not shown). XMyf5-injected animal caps, like those injected with XMyoD, did not differentiate any histologically recognizable muscle, and failed to stain with the 12/101 anti-muscle antibody (Kintner and Brockes, 1984) (data not shown). Co-injection of XMyoD and XMyf5 RNAs also failed to produce differentiated muscle in animal caps. It is possible that muscle did not form because we could not maintain a high enough concentration of XMyf5 protein as the injected RNA was progressively degraded, but we believe this to be unlikely for the following reasons. The concentration of XMyf5 RNA remaining at st18 was several-fold greater than that normally found in the somites, and we note that the concentration of XMyf5 RNA *normally* falls later in embryogenesis. More importantly, XMyf5 RNA injection was, by comparison to our previous estimate for the efficiency of XMyoD activation, able to activate the cardiac actin gene in animal cap cells to about its normal level in the myotomes of early neurulae.

We conclude from these experiments that, although unable to cause complete and stable myogenesis, XMyf5 is sufficient, together with components present in animal cap cells, to activate transcription of the cardiac actin gene. Taken together with the pattern of expression of XMyf5 that we have described, this result argues that XMyf5 is likely to be involved in the normal embryonic activation of muscle-specific gene expression.

Discussion

We have shown that XMyf5, like XMyoD (Hopwood *et al.* 1989*a*; Harvey, 1990; Scales *et al.* 1990), is expressed in embryonic cells that will form skeletal muscle prior to the accumulation of cardiac actin mRNA, itself a very early marker of myogenesis (Mohun *et al.* 1984; Cascio and Gurdon, 1986). These two factors are expressed, as shown by *in situ* hybridization, uniformly in the same region of the embryo and therefore probably in the same cells. The main clear difference between the patterns of transcript accumulation from the two genes is that XMyf5 transcripts are restricted to the posterior part of the somitic mesoderm many hours before XMyoD transcripts become similarly restricted, indeed before any somites have formed. This suggests that XMyf5 might act largely during the very earliest stages of myogenesis.

Microinjection of synthetic XMyf5 and XMyoD

mRNA into early embryos activates cardiac actin and XMyoD genes in animal cap cells. This muscle gene activation is likely to depend on the specific properties of these proteins, rather than being a relatively non-specific effect of high levels of any helix-loop-helix protein, because ectopic expression of the Xtwi protein (Hopwood *et al.* 1989*b*) did not activate the cardiac actin gene (N.D.H., unpublished results). In addition to the results described above, XMyoD and XMyf5 activate with similar efficiency the synthesis of the muscle-specific intermediate filament protein, desmin, in animal cap cells (unpublished results). The activity of XMyf5 in this assay is thus, with respect to the properties we have tested, indistinguishable from that of XMyoD (Hopwood and Gurdon, 1990).

We have not detected activation by either XMyoD or XMyf5 of the endogenous XMyf5 gene(s), although both proteins can *trans*-activate XMyoD, albeit weakly. This means that XMyf5 could be acting, at least in part, through XMyoD, or indeed through other factors. However, the action of XMyf5 could be direct, since XMyf5 protein synthesized *in vitro* can, like XMyoD, participate in the formation of a specific complex with a region of the cardiac actin promoter that is required for transcription (M. V. Taylor, T. J. Mohun, N.D.H. and J.B.G., unpublished results). That XMyf5 can activate the XMyoD gene suggests that XMyf5 might normally play a part in the transcriptional activation of this gene. Our results on auto- and cross-activation of XMyoD and XMyf5 genes in animal cap cells are qualitatively comparable to the results of transfection experiments in 10T½ fibroblasts and their methylcholanthrene-transformed derivatives (Thayer *et al.* 1989; Braun *et al.* 1989*a*). These showed that both MyoD and Myf-5 can activate the MyoD gene, but that in the clones analyzed MyoD did not activate the Myf-5 gene, and in most cases Myf-5 did not autoactivate. If the XMyf5 gene is as refractory to *trans*-activation in normal somitic cells as it is in animal caps, then this could conceivably account for the relative transience of XMyf5 expression compared to that of XMyoD.

Our results suggest that XMyoD and XMyf5 are insufficient, even in combination, to cause complete and stable myogenesis in animal cap cells. What else is required for full myogenic conversion of these cells, which can respond to mesoderm inducers by forming muscle? Perhaps another *Xenopus* member of the MyoD family can synergise with XMyoD and/or XMyf5. Two other, unrelated factors can also convert cultured fibroblasts into myoblasts, but it is not known if they are involved in normal myogenesis: the uncharacterized genomic locus, *myd* (Pinney *et al.* 1988), and the oncogene, v-*ski* (Colmenares and Stavnezer, 1989). A possible role for another cell type-specific factor has been suggested by the recent discovery of Id, a helix-loop-helix protein which lacks the basic region that is believed to mediate DNA binding in other members of the family (Benezra *et al.* 1990). A protein such as Id could sequester in non-functional complexes either members of the MyoD family, or their necessary non-muscle-specific partners (*e.g.* the human immunoglobulin enhancer-binding protein, E12; Murre *et al.* 1989*a*,*b*), and so be a buffer against the effects of XMyoD or XMyf5 in animal cap cells. Myogenesis might require the inactivation of negative regulators like Id.

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References

Benezra, R., Davis, R. L., Lockshon, D., Turner, D. L. and Weintraub, H. (1990). The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. *Cell* **61**, 49–59.

Biggin, M. D., Gibson, T. J. and Hong, G. F. (1983). Buffer gradient gels and ^{35}S label as an aid to rapid sequence determination. *Proc. natn. Acad. Sci. U.S.A.* **80**, 3963–3965.

Braun, T., Bober, E., Buschhausen-Denker, G., Kotz, S., Grzeschik, K.-H. and Arnold, H. H. (1989*a*). Differential expression of myogenic determination genes in muscle cells: possible autoactivation by the *Myf* gene products. *EMBO J.* **8**, 3617–3625.

Braun, T., Bober, E., Winter, B., Rosenthal, N. and Arnold, H. H. (1990). *Myf-6*, a new member of the human gene family of myogenic determination factors: evidence for a gene cluster on chromosome 12. *EMBO J.* **9**, 821–831.

Braun, T., Buschhausen-Denker, G., Bober, E., Tannich, E. and Arnold, H. H. (1989*b*). A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T½ fibroblasts. *EMBO J.* **8**, 701–709.

Brennan, T. J. and Olson, E. N. (1990). Myogenin resides in the nucleus and acquires high affinity for a conserved enhancer element on heterodimerization. *Genes and Dev.* **4**, 582–595.

Cascio, S. and Gurdon, J. B. (1986). The timing and specificity of actin gene activation in early *Xenopus* development. *UCLA Symp. Mol. Cell Biol.* **51** (ed. Firtel, R. A. and Davidson, E. H.), pp. 195–204. New York: Alan R. Liss Inc.

Clark, T. G., Morris, J., Akamatsu, M., McGraw, R. A. and Ivarie, R. (1990). A bovine homolog to the human myogenic determination factor *myf-5*: sequence conservation and 3′ processing of transcripts. *Nucleic Acids Res.* **18**, 3147–3153.

Colmenares, C. and Stavnezer, E. (1989). The *ski* oncogene induces muscle differentiation in quail embryo cells. *Cell* **59**, 293–303.

Davis, R. L., Cheng, P.-F., Lassar, A. B. and Weintraub, H. (1990). The MyoD DNA binding domain contains a recognition code for muscle-specific gene activation. *Cell* **60**, 733–746.

Davis, R. L., Weintraub, H. and Lassar, A. B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* **51**, 987–1000.

de la Brousse, F. C. and Emerson, C. P. (1990). Localized expression of a myogenic regulatory gene, *qmf1*, in the somite dermatome of avian embryos. *Genes and Dev.* **4**, 567–581.

Edmonson, D. G. and Olson, E. N. (1989). A gene with homology to the *myc* similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. *Genes and Dev.* **3**, 628–640.

Feinberg, A. P. and Vogelstein, B. (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Analyt. Biochem.* **132**, 6–13.

Gurdon, J. B. (1977). Methods for nuclear transplantation in amphibians. *Meth. Cell Biol.* **16**, 125–138.

Harvey, R. P. (1990). The *Xenopus MyoD* gene: an unlocalized maternal mRNA predates lineage-restricted expression in the early embryo. *Development* **108**, 669–680.

Henikoff, S. (1984). Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene* **28**, 351–359.

Hopwood, N. D. (1990). Cellular and genetic responses to mesoderm induction in *Xenopus*. *BioEssays* **12**, 465–471.

HOPWOOD, N. D. AND GURDON, J. B. (1990). Activation of muscle genes without myogenesis by ectopic expression of MyoD in frog embryo cells. *Nature* **347**, 197–200.

HOPWOOD, N. D., PLUCK, A. AND GURDON, J. B. (1989*a*). MyoD expression in the forming somites is an early response to mesoderm induction in *Xenopus* embryos. *EMBO J.* **8**, 3409–3417.

HOPWOOD, N. D., PLUCK, A. AND GURDON, J. B. (1989*b*). A *Xenopus* mRNA related to *Drosophila twist* is expressed in response to induction in the mesoderm and the neural crest. *Cell* **59**, 893–903.

JACKSON, R. J. AND HUNT, T. (1983). Preparation and use of nuclease-treated rabbit reticulocyte lysates for the translation of eukaryotic messenger RNA. *Meth. Enzymol* **96**, 50–74.

KINTNER, C. R. AND BROCKES, J. P. (1984). Monoclonal antibodies identify blastemal cells derived from dedifferentiating muscle in newt regeneration. *Nature* **308**, 67–69.

KINTNER, C. R. AND MELTON, D. A. (1987). Expression of *Xenopus* N-CAM RNA in ectoderm is an early response to neural induction. *Development* **99**, 311–325.

KOBEL, H. R. AND DUPASQUIER, L. (1986). Genetics of polyploid *Xenopus*. *TIG* **2**, 310–315.

KRIEG, P. A. AND MELTON, D. A. (1984). Functional messenger RNAs are produced by SP6 *in vitro* transcription of cloned cDNAs. *Nucleic Acids Res.* **12**, 7057–7071.

KRIEG, P. A. AND MELTON, D. A. (1987). *In vitro* RNA synthesis with SP6 RNA polymerase. *Meth. Enzymol.* **155**, 397–415.

KRIEG, P. A., VARNUM, S. M., WORMINGTON, W. M. AND MELTON, D. A. (1989). The mRNA encoding elongation factor 1-α (EF-1α) is a major transcript at the midblastula transition in *Xenopus*. *Devl Biol.* **133**, 93–100.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

LASSAR, A. B., BUSKIN, J. N., LOCKSHON, D., DAVIS, R. L., APONE, S., HAUSCHKA, S. D. AND WEINTRAUB, H. (1989). MyoD is a sequence-specific DNA binding protein requiring a region of *myc* homology to bind to the muscle creatine kinase enhancer. *Cell* **58**, 823–831.

LIN, Z., DECHESNE, C. A., ELDRIDGE, J. AND PATERSON, B. M. (1989). An avian muscle factor related to MyoD1 activates muscle-specific promoters in nonmuscle cells of different germ-layer origin and in BrdU-treated myoblasts. *Genes and Dev.* **3**, 986–996.

MESSING, J. (1983). New M13 vectors for cloning. *Meth. Enzymol.* **101**, 20–79.

MINER, J. H. AND WOLD, B. (1990). Herculin, a fourth member of the MyoD family of myogenic regulatory genes. *Proc. natn. Acad. Sci. U.S.A.* **87**, 1089–1093.

MOHUN, T. J., BRENNAN, S., DATHAN, N., FAIRMAN, S. AND GURDON, J. B. (1984). Cell type-specific activation of actin genes in the early amphibian embryo. *Nature* **311**, 716–721.

MURRE, C., MCCAW, P. S. AND BALTIMORE, D. (1989*a*). A new DNA binding and dimerization motif in immunoglobulin enhancer binding, *daughterless*, MyoD and *myc* proteins. *Cell* **56**, 777–783.

MURRE, C., MCCAW, P. S., VAESSIN, H., CAUDY, M., JAN, L. Y., JAN, Y. N., CABRERA, C. V., BUSKIN, J. N., HAUSCHKA, S. D., LASSAR, A. B., WEINTRAUB, H. AND BALTIMORE, D. (1989*b*). Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common sequence. *Cell* **58**, 537–544.

NIEUWKOOP, P. D. AND FABER, J. (1967). *Normal Table of* Xenopus laevis *(Daudin)*, 2nd ed. Amsterdam: North-Holland.

PIETTE, J., BESSEREAU, J.-L., HUCHET, M. AND CHANGEUX, J.-P. (1990). Two adjacent MyoD1-binding sites regulate expression of the acetylcholine receptor α-subunit gene. *Nature* **345**, 353–355.

PINNEY, D. F., PEARSON-WHITE, S. H., KONIECZNY, S. F., LATHAM, K. E. AND EMERSON, C. P. (1988). Myogenic lineage determination and differentiation: evidence for a regulatory gene pathway. *Cell* **53**, 781–793.

RHODES, S. J. AND KONIECZNY, S. F. (1989). Identification of MRF4: a new member of the muscle regulatory gene family. *Genes and Dev.* **3**, 2050–2061.

SANGER, F., NICKLEN, S. AND COULSON, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463–5467.

SASSOON, D., LYONS, G., WRIGHT, W. E., LIN, V., LASSAR, A., WEINTRAUB, H. AND BUCKINGHAM, M. (1989). Expression of two myogenic regulatory factors myogenin and MyoD1 during mouse embryogenesis. *Nature* **341**, 303–307.

SCALES, J. B., OLSON, E. N. AND PERRY, M. (1990). Two distinct *Xenopus* genes with homology to MyoD1 are expressed before somite formation in early embryogenesis. *Molec cell. Biol.* **10**, 1516–1524.

SCHÄFER, B. W., BLAKELY, B. T., DARLINGTON, G. J. AND BLAU, H. M. (1990). Effect of cell history on response to helix-loop-helix family of myogenic regulators. *Nature* **344**, 454–458.

SMITH, J. C. (1989). Mesoderm induction and mesoderm-inducing factors in early amphibian development. *Development* **105**, 665–677.

STADEN, R. (1982). Automation of the computer handling of gel reading data produced by the shotgun method of DNA sequencing. *Nucleic Acids Res.* **10**, 431–451.

SUDARWATI, S. AND NIEUWKOOP, P. D. (1971). Mesoderm formation in the anuran *Xenopus laevis* (Daudin). *Wilhelm Roux' Arch. EntwMech. Org.* **166**, 189–204.

TAPSCOTT, S. J., DAVIS, R. L., THAYER, M. J., CHENG, P.-F., WEINTRAUB, H. AND LASSAR, A. B. (1988). MyoD1: a nuclear phosphoprotein requiring a Myc homology region to convert fibroblasts to myoblasts. *Science* **242**, 405–411.

THAYER, M. J., TAPSCOTT, S. J., DAVIS, R. L., WRIGHT, W. E., LASSAR, A. B. AND WEINTRAUB, H. (1989). Positive autoregulation of the myogenic determination gene MyoD1. *Cell* **58**, 241–248.

WEINTRAUB, H., TAPSCOTT, S. J., DAVIS, R. L., THAYER, M. J., ADAM, M. A., LASSAR, A. B. AND MILLER, A. D. (1989). Activation of muscle-specific genes in pigment, nerve, fat, liver and fibroblast cell lines by forced expression of MyoD. *Proc. natn. Acad. Sci. U.S.A.* **86**, 5434–5438.

WRIGHT, W. E., SASSOON, D. A. AND LIN, V. K. (1989). Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. *Cell* **56**, 607–617.

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Note added in proof:

The accession number in the EMBL database of the sequence shown in Fig. 1 is X56738.