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MyoD expression in the forming somites is an early response to mesoderm induction in *Xenopus* embryos

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We describe the cloning, cDNA sequence and embryonic expression of a *Xenopus* homologue of MyoD, a mouse gene encoding a DNA-binding protein that can activate muscle gene expression in cultured cells. The predicted *Xenopus* MyoD protein sequence is remarkably similar to mouse MyoD. Zygotic expression of MyoD begins in early gastrulae, but there is a low level of unlocalized maternal message. Northern blot analysis of dissected embryos and *in situ* hybridization show that MyoD RNA is restricted to the gastrula mesoderm and to the somites of neurulae and tailbud embryos. The time and place of MyoD expression are consistent with a role for MyoD in the activation of other muscle genes in the somites of the frog embryo. However, MyoD is skeletal muscle-specific and is not expressed even in the early embryonic heart, which co-expresses cardiac and skeletal actin isoforms. Striated muscle genes can therefore be activated in some embryonic tissues in the absence of MyoD. The concentration of MyoD in the somites falls once they have formed, suggesting that MyoD may act there transiently to establish muscle gene expression. MyoD transcription is activated following mesoderm induction, and is the earliest muscle-specific response to mesoderm-inducing factors so far described.

Key words: mesoderm induction/muscle/MyoD/myogenesis/*Xenopus*

Introduction

Recent work on tissue culture models of myogenesis has identified genes that may regulate muscle development (Davis *et al.*, 1987; Pinney *et al.*, 1988; Braun *et al.*, 1989; Wright *et al.*, 1989). C3H10T½ mouse fibroblasts can be converted by 5-azacytidine treatment to give rise to clones of myoblasts, chondroblasts and adipoblasts (Taylor and Jones, 1979), presumably by changing patterns of DNA methylation. Davis, Weintraub and Lassar (1987) used differential cDNA library screening, enhanced by subtractive hybridization, to isolate clones corresponding to muscle-specific transcripts expressed in 10T½ aza-myoblasts but not in proliferating 10T½ cells. Expression of one cDNA, MyoD, transfected into 10T½ fibroblasts could convert them into stable myoblasts. MyoD is related to the proto-oncogene c-*myc* and is believed to encode a sequence- specific DNA-binding protein (Davis *et al.*, 1987; Murre *et al.*, 1989). These results make it likely that MyoD is a regulator of muscle development, but its specific role is currently unknown.

The mesoderm in the frog *Xenopus* is formed as a result of vegetal cells of the blastula inducing overlying animal hemisphere cells (reviewed by Gurdon, 1987; Smith, 1989). The mesoderm of the early gastrula is a ring of deep cells in the equatorial region of the embryo. During gastrulation the dorsal mesoderm, which includes the cells which will form the somites, undergoes extensive movements to bring the somites to lie on either side of the dorsal midline. The earliest known molecular response to induction is the accumulation of cardiac and skeletal muscle actin transcripts (Mohun *et al.*, 1984; Gurdon *et al.*, 1985), which are first detectable in mid-gastrulae (st11; Cascio and Gurdon, 1986). One way to find out more about mesoderm development is to identify gene products expressed in the mesoderm at earlier stages in its differentiation. These molecules, apart from possibly being involved in activating the expression of genes like the α-actins, would also provide markers for mesodermal cells at early stages in the process of commitment.

The work described here builds a bridge between the mouse cell culture systems which have been used so effectively to identify molecules likely to be involved in the determination of muscle, and the *Xenopus* embryo, in which the earliest stages of mesoderm (including muscle) formation are particularly accessible to analysis. We describe the cloning, cDNA sequence and embryonic expression of a *Xenopus* homologue of MyoD. We find that MyoD is expressed as an early response to mesoderm induction, and its transcripts appear in future muscle cells well before those of muscle actins.

Results

Cloning and sequence of a Xenopus MyoD cDNA

We screened a *Xenopus laevis* gastrula cDNA library (Krieg and Melton, 1985) at low stringency with a probe made from the whole mouse MyoD cDNA (Davis *et al.*, 1987). Purified positive cDNAs all hybridized to each other at high stringency and were arranged by limited DNA sequencing into an overlapping series. No single cDNA spanned the length of the transcripts estimated from Northern blots (see below), and so two overlapping cDNAs extending furthest at the 5′ and 3′ ends, respectively (XmyoD2 and XmyoD24) were chosen for sequencing (see Materials and methods). There are no differences in the region of overlap (nucleotides 283–573 of the composite sequence shown in Figure 1), but we cannot exclude the possibility that these two cDNAs originated from transcripts of different structure. They were joined at the *Sph*I site (position 383 in Figure 1) to make a composite cDNA (XmyoD2–24), which was used to prepare probes for the expression studies described below. The composite cDNA is 1.45 kb, similar to the sizes of the transcripts it detects on Northern blots (see below) and so must be nearly full-length.

Figure 1 shows the composite cDNA and deduced protein

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gaattccgGTGAGAGCTGCCAAGAGTCCAGATTTCCTACAACGGGGCAAGGTGACTGTGCGTGGGCATTCGGACTGGACCCCAGAGCTGT
        10        20        30        40        50        60        70        80        90

                                                  M  E  L  L  P  P  P  L  R  D  M  E  V  T  E
GGATCTTTTAAACCTTCGCTGGGAGTAGTGACTTTGCGCTGTTTCTATGGAGCTGTTGCCCCCACCACTGCGGGACATGGAAGTCACTGA
       100       110       120       130       140       150       160       170       180

  G  S  L  C  A  F  P  T  P  D  D  F  Y  D  D  P  C  F  N  T  S  D  M  S  F  F  E  D  L  D
GGGATCTCTCTGCGCCTTCCCGACCCCCGACGACTTCTACGACGACCCCTGTTTCAATACCTCAGACATGAGTTTCTTTGAAGACCTGGA
       190       200       210       220       230       240       250       260       270

  P  R  L  V  H  V  T  L  L  K  P  E  E  P  H  H  N  E  D  E  H  V  R  A  P  S  G  H  H  Q
CCCCAGGCTGGTGCACGTGACTCTTCTGAAACCAGAGGAACCCCACCATAACGAGGATGAGCACGTGAGGGCCCCCAGTGGGCACCATCA
       280       290       300       310       320       330       340       350       360

  A  G  R  C  L  L  W  A  C  K  A  C  K  R  K  T  T  N  A  D  R  R  K  A  A  T  M  R  E  R
GGCTGGCAGGTGCTTGTTGTGGGCATGCAAAGCCTGTAAGAGAAAGACAACCAATGCCGACAGGAGGAAGGCCGCCACTATGAGGGAGAG
       370       380       390       400       410       420       430       440       450

  R  R  L  S  K  V  N  E  A  F  E  T  L  K  R  Y  T  S  T  N  P  N  Q  R  L  P  K  V  E  I
AAGGAGACTCAGCAAGGTCAATGAAGCGTTTGAGACCCTGAAGCGATACACCTCAACTAACCCCAACCAAAGGCTCCCCAAAGTGGAGAT
       460       470       480       490       500       510       520       530       540

  L  R  N  A  I  R  Y  I  E  S  L  Q  A  L  L  H  D  Q  D  E  A  F  Y  P  V  L  E  H  Y  S
CCTGCGCAACGCGATTCGCTACATAGAGAGCCTCCAGGCTCTGCTCCACGACCAGGATGAGGCTTTCTACCCGGTTCTGGAACATTACAG
       550       560       570       580       590       600       610       620       630

  G  D  S  D  A  S  S  P  R  S  N  C  S  D  G  M  M  D  Y  N  S  P  P  C  G  S  R  R  R  N
TGGGGACTCAGATGCCTCAAGCCCCAGGTCCAACTGCTCCGATGGCATGATGGATTATAACAGCCCCCCCTGCGGCTCCAGGAGAAGGAA
       640       650       660       670       680       690       700       710       720

  S  Y  D  S  S  F  Y  S  D  S  P  N  D  S  R  L  G  K  S  S  V  I  S  S  L  D  C  L  S  S
CAGCTACGACAGCAGCTTCTACAGTGACAGCCCAAATGACTCGAGACTTGGGAAAAGTTCAGTGATCTCCAGCCTTGACTGCCTCTCCAG
       730       740       750       760       770       780       790       800       810

  I  V  E  R  I  S  T  Q  S  P  S  C  P  V  P  T  A  V  D  S  G  S  E  G  S  P  C  S  P  L
CATCGTAGAGCGGATCTCCACCCAAAGCCCCAGCTGCCCCGTCCCCACAGCTGTGGATAGTGGATCCGAGGGCAGTCCCTGTTCTCCCCT
       820       830       840       850       860       870       880       890       900

  Q  G  E  T  L  S  E  R  V  I  T  I  P  S  P  S  N  T  C  T  Q  L  S  Q  D  P  S  S  T  I
GCAGGGGGAGACATTGAGCGAGAGAGTAATCACCATCCCTTCTCCCAGCAATACCTGCACTCAACTGTCCCAGGACCCCAGCAGCACCAT
       910       920       930       940       950       960       970       980       990

  Y  H  V  L  *
CTATCACGTCTTATAGGCTTCAGCCTGCCTCCTGCTGGTTGCTGAATTTCCATTAACAGGATTCTCTCCTAATTCCTTCCCAATCCATGA
      1000      1010      1020      1030      1040      1050      1060      1070      1080

ACTTCCCCTTTATTTATTGGTTTGTCCTGGCCAAAGGATTCTGCCATATTTCCAATGTAAATAACCAAGCCCCTCCCAATATCCAATCAG
      1090      1100      1110      1120      1130      1140      1150      1160      1170

ATTGCAGCTGGTGTTGAAGGACAGACCACTCTGGAACCTTAGGGGTTACATGACCTGCCAATGTTGTGTTGAGCACGGACAATGGGGCAA
      1180      1190      1200      1210      1220      1230      1240      1250      1260

TTCCCCCCTGAGGCCAAAGGAAACTTGCGGACCACTTTTTGTAAGATTTTTTTATAGATTTGTAAATAAGAGGTGATTGTGCCTTATTTA
      1270      1280      1290      1300      1310      1320      1330      1340      1350

TGTGCTTGGTGTGTTGTGCCAGATGCTCCTTTATATATTTATACTGTGTGAATGCTCCGGATGCTTCCAGAATATTCTAAATAAAGTACC
      1360      1370      1380      1390      1400      1410      1420      1430      1440

TTATTTATAAAAAAAAAAAAAcggaattc
      1450      1460
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Fig. 1. cDNA sequence of *Xenopus* MyoD. The sequence shown is a composite of two cDNAs (XmyoD2 and 24) which overlap between nucleotides 283 and 573. The poly(A) addition signal is underlined, and the *Eco*RI linkers are in lower case letters. *, translation stop.

sequence of the *Xenopus* XmyoD2−24 clones. There is only one long open reading frame, preceded upstream by in-frame stop codons, and this encodes a protein so similar to mouse MyoD that we consider it to be the *Xenopus* homologue. The 3′ end of this sequence is judged to represent the 3′ end of the transcript because of the presence of a poly(A) addition signal preceding a poly(A) tract. We have found no sequence similarity between mouse and frog clones in non-coding regions, with the exception of the 12 nucleotide motif TGTAAATAAGAG in the 3′ untranslated regions (nucleotides 1321−1332 in Figure 1; nucleotides 1657−1668 of Davis *et al.*, 1987). We have no clue to the significance, if any, of this short sequence.

There is extensive similarity between the *Xenopus* and mouse MyoD proteins (Figure 2). Seventy-three percent of the amino acid sequence in the frog protein is identical in the mouse one. Frog MyoD contains 289 amino acid residues compared to the 318 of mouse MyoD, a difference accounted for by several short insertions in the mouse protein relative to the frog one. There are only two changes, one of them conservative, in the basic and Myc-like regions (Davis *et al.*, 1987), which are sufficient for myogenic conversion of 10T½ cells and are thought to mediate DNA-binding and dimerization (Tapscott *et al.*, 1988; Murre *et al.*, 1989). However, there are long runs of identities outside these regions, suggesting that the functions of the MyoD protein may be more diverse and subtle than revealed by the transfection assay. There are many shared amino acids throughout the acidic amino-terminal region, but in the carboxy-terminal half of the proteins the identities are

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M.m. MELLSPPLRDIDLTGPDGSLCSFETADDFYDDPCFDSPDLRFFEDLDPRLVHVGALLKPE  60
     :::: ::::: ..:   :::: : : ::::::::: . :  ::::::::::::  :::::
X.l. MELLPPPLRDMEVTE--GSLCAFPTPDDFYDDPCFNTSDMSFFEDLDPRLVHVT-LLKPE  57

M.m. EHAHFSTAVHPGPGARED EHVRAPSGHHQAGRCLLWACKACKRKTTNADRRKAATMRERR 120
     :  :     :      :::::::::::::::::::::::::::::::::::::::::::
X.l. EP-H-----H-----NEDEHVRAPSGHHQAGRCLLWACKACKRKTTNADRRKAATMRERR 106

M.m. RLSKVNEAFETLKRCTSSNPNQRLPKVEILRNAIRYIEGLQALLRDQDAAPPGAAAFYAP 180
     :::::::::::::: ::.:::::::::::::::::::::::::::.::: :      :: :
X.l. RLSKVNEAFETLKRYTSTNPNQRLPKVEILRNAIRYIESLQALLHDQDEA------FY-P 159

M.m. GPLPPGRGSEHYSGDSDASSPRSNCSDGMMDYSGPPSGPRRQNGYDTAYYSEAVRESRPG 240
       :      ::::::::::::::::::::::::  :: : :: : ::. .::.   .:: :
X.l. V-L------EHYSGDSDASSPRSNCSDGMMDYNSPPCGSRRRNSYDSSFYSDSPNDSRLG 212

M.m. KSAAVSSLDCLSSIVERISTDSPAAPALLLADAPPE-SPPGPPEGASLSDTEQGTQTPSP 299
     :: ..:::::::::::::::: ::  :.  ..:   : ::  :  : .::  :     :::
X.l. KSSVISSLDCLSSIVERISTQSPSCPVPTAVDSGSEGSPCSPLQGETLS--ERVITIPSP 270

M.m. DAAPQCPAGSNPNAIYQVL 318
                    :: ::
X.l. SNTCTQLSQDPSSTIYHVL 289
```

Fig. 2. Comparison of the predicted protein sequences of the *Xenopus* and mouse (Davis *et al.*, 1987) MyoD cDNAs. The box surrounds the basic and Myc-like regions, which are sufficient for myogenic conversion of 10T½ cells (Tapscott *et al.*, 1988). Note that there is extensive similarity even outside these regions.

clustered in two regions, mouse amino acids 190–212 and 246–260. Two other cDNAs, myogenin (Wright *et al.*, 1989; Edmonson and Olson, 1989) and Myf-5 (Braun *et al.*, 1989), have been discovered recently that can convert 10T½ fibroblasts to myoblasts, and encode proteins containing basic and Myc-like domains similar to those of MyoD (see Discussion). The two blocks of conserved sequence in the carboxy-terminal halves of mouse and frog MyoD proteins cover two blocks of identities between MyoD and Myf-5. These two regions do not appear to be conserved in the rat and mouse myogenin cDNAs.

MyoD RNA accumulates in early gastrulae from a low maternal level

We have examined the accumulation of MyoD RNA during early *Xenopus* development using the XmyoD2–24 cDNA to make probes for Northern blots. Southern blots indicate that the XmyoD2–24 probe hybridizes under the high stringency conditions used for Northern blots and *in situ* hybridizations to non-repeated sequences in the tetraploid *X.laevis* genome (Kobel and Du Pasquier, 1986). We detected only two fragment sizes in *Bam*HI (2.8 and 2.3 kb) and *Eco*RI (10 and 8.4 kb) digests, and a single size (20 kb) in a *Hin*dIII digest (data not shown). We therefore believe that our Northern blots and *in situ* hybridizations are specific for MyoD. Figure 3A shows a blot which has been probed successively with MyoD and a cardiac actin gene-specific sequence (Mohun *et al.*, 1984). There are two sizes of MyoD transcripts around 1.5 kb. Only the smaller one is present in unfertilized eggs, cleavage stages, and blastulae (faint bands in Figure 3A). The larger one first appears in early (st10) gastrulae, from which time the abundance of the two classes of transcript increases rapidly, presumably as a result of transcriptional activation. It peaks in late neurulae and then declines in later embryos. Cardiac actin transcripts are first detectable in mid-gastrulae (st11) and then accumulate quickly through the whole period of early development (Mohun *et al.*, 1984; Cascio and Gurdon, 1986).

We wished to establish whether or not MyoD transcripts were present sufficiently early that their protein product could activate the cardiac actin gene, transcription of which is the earliest known muscle-specific response to mesoderm induction. In order to make our analysis independent of probe sensitivities, absolute amounts of MyoD RNA were measured by comparing hybridization of a MyoD probe to embryo RNA and a dilution series of *in vitro* synthesized XmyoD2–24 RNA (data not shown; see Materials and methods). Values of 1×10^5 transcripts per unfertilized egg and 4×10^6 of each transcript class per gastrula were obtained. A graph was then plotted of numbers of MyoD transcripts through early development, based on densitometry of autoradiographs of the Northern blot (Figure 3B). This was compared with a graph of numbers of cardiac actin transcripts obtained in the same way, assuming that a tailbud embryo contains 2.5×10^8 transcripts (T.J.Mohun, personal communication). We conclude that new MyoD transcripts appear ~2 h before cardiac actin RNA begins to accumulate. This would be enough time for MyoD protein to be made and for it to activate transcription from the cardiac actin gene.

MyoD RNA is restricted to the developing somites

We have used Northern blot analysis of RNA from dissected tissues and *in situ* hybridization to find out in which early embryonic structures MyoD is expressed. These techniques are complementary: Northern blots are very sensitive and easy to quantify and *in situ* hybridization gives greater spatial resolution. We dissected late neurulae (st20) and analysed the parts for MyoD RNA by Northern blotting (Figure 4).

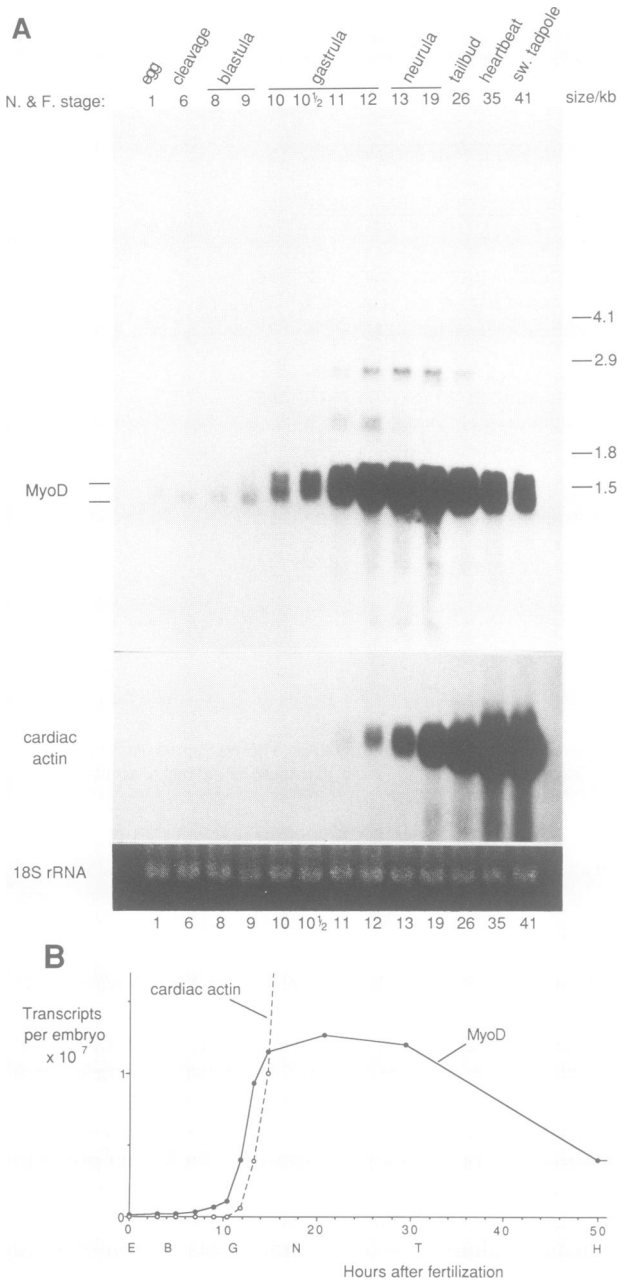


Fig. 3. The accumulation of MyoD RNA during early *Xenopus* development. (**A**) Northern blot containing 10 μg of total staged (Nieuwkoop and Faber, 1967) embryo RNA per lane probed with random-primed fragments made from the XmyoD2−24 cDNA. The blot was then stripped and re-probed with a cardiac actin gene-specific probe (Mohun *et al.*, 1984) for comparison. Ethidium bromide staining of the 18S rRNA shows roughly equal loading. (**B**) Graph of numbers of MyoD and cardiac actin transcripts per embryo through development based on densitometry of appropriately exposed autoradiographs of the blot shown in (**A**). Absolute numbers of MyoD transcripts in eggs and st11 embryos were measured as described in the Materials and methods. Values for MyoD refer to the smaller transcript class only; in embryos older than early gastrulae the two sizes of transcript are present in roughly equal numbers, and so the total content of MyoD mRNA is double the number plotted. E, egg; B, blastula; G, gastrula; N, neurula; T, tailbud; and H, heartbeat.

The blot was reprobed with a cardiac actin gene-specific sequence (Mohun *et al.*, 1984) for comparison, and with EF-1α (Krieg *et al.*, 1989), a translation factor found in all cells, to show the relative amounts of RNA loaded. MyoD transcripts are present only in the somites. There is a trace

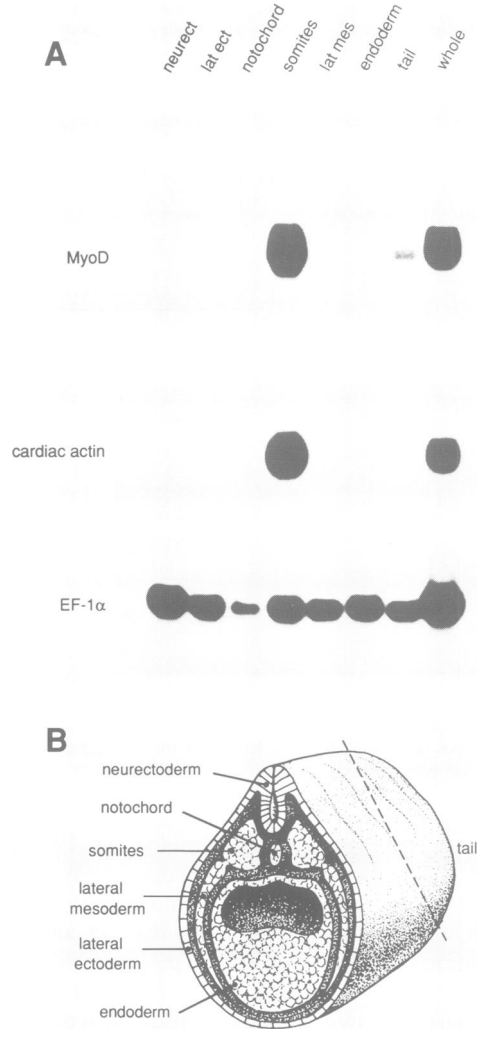
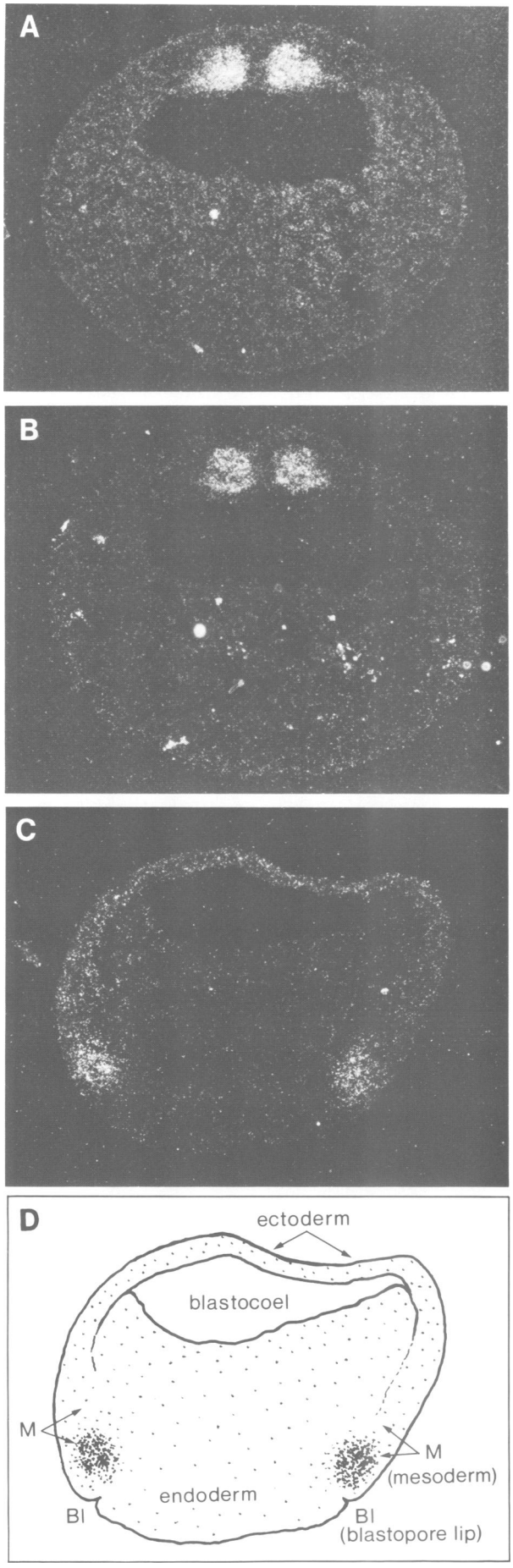


Fig. 4. MyoD is expressed specifically in the somites of neurula embryos. (**A**) Northern blot of RNA extracted from dissected tissues of st20 embryos probed for MyoD and cardiac actin as in Figure 3A, and for EF-1α (Krieg *et al.*, 1989) to show the relative amounts of total RNA in each lane. Neurect, neurectoderm; lat ect, lateral ectoderm, and lat mes, lateral mesoderm. (**B**) Diagram showing the location of the tissues dissected for analysis in (**A**).

of MyoD RNA in the lateral plate mesoderm, but a similar trace of cardiac actin RNA suggests that this is a result of slight contamination during dissection from the adjoining somites. We have also analysed mid-neurula (st15) and tailbud (st26) dissections with similar results (data not shown).

In situ hybridization of sections of embryos using ^{35}S-labelled RNA probes (Kintner and Melton, 1987; Krieg and Melton, 1987; and see Materials and methods) confirms the results from the analysis of dissections. We used a probe containing sequences from the entire XmyoD2−24 cDNA in order to maximize the hybridization signal. We washed at high stringency to reduce the possibility of cross-hybridization to MyoD-related RNAs; the Southern blot referred to above suggests that under these conditions we would detect only MyoD RNA. Figure 5A and B shows equivalent transverse sections from a st18 neurula probed with cardiac actin and MyoD, showing in each case intense labelling over the somites but none over other structures, including mesodermal derivatives such as notochord and



lateral plate mesoderm. We conclude that MyoD expression is restricted to the developing somites of early *Xenopus* embryos. In *Xenopus* the myotome occupies most of the somite, the dermatome and sclerotome components being very small and hard to distinguish in sections of early embryos. We therefore cannot exclude the possibility that MyoD is expressed in these minor components, which will form the connective tissue layer of the skin and skeleton respectively. However, myotome-specific expression seems likely, since MyoD is not known to activate any non-muscle genes.

MyoD expression is specific to the mesoderm of the gastrula

New MyoD transcripts appear in early gastrulae (Figure 3). We have used *in situ* hybridization to find out where these first new transcripts are. Figure 5C shows a section of a st11 mid-gastrula hybridized with a MyoD probe. Two patches of labelled deep cells are visible in an equatorial position above the margins of the yolk plug and these correspond to at least part of the prospective mesoderm (Figure 5D). The apparent slight labelling of the animal ectoderm in Figure 5C is non-specific, because we detect no MyoD transcripts in these cells on Northern blots (data not shown). We were not able to orient the albino embryos used for *in situ* hybridizations exactly with respect to the dorsal–ventral axis during embedding, so we cannot specify precisely the plane of section of the embryo shown. Judging from the symmetry of the section, it appears to be perpendicular to the mid-sagittal plane.

The high concentration of MyoD RNA in gastrulae (8×10^6 transcripts per embryo at st11) has enabled us to show mesoderm-specific gene expression *in situ* significantly earlier than has hitherto been possible. This is either because the few known mesodermal markers are activated only later in development, e.g. cardiac actin, or because they are expressed at levels so far undetectable by *in situ* hybridization, e.g. Xhox3 (Ruiz i Altaba and Melton, 1989) and Xhox-1A (Harvey and Melton, 1988). We believe it likely, in view of our results from later embryos, that only those cells which will subsequently form somites express MyoD at the gastrula stage, but confirmation of this point awaits a careful comparison of the expression of MyoD and other early mesodermal markers with the well established fate map for this stage (Keller, 1975, 1976).

The localization of new MyoD transcripts in the gastrula raises the question whether the maternal RNA is also localized. RNA was extracted from the animal, equatorial and vegetal pieces of st8½ blastulae and probed on Northern blots with MyoD and EF-1α. All parts contained similar concentrations of MyoD transcripts (data not shown). Animal caps and vegetal pieces, which will not form muscle, therefore contain MyoD transcripts at a comparable level to equatorial pieces, from which muscle will later develop. We conclude that there is no prelocalization of maternal MyoD transcripts to the presumptive muscle.

Fig. 5. Location of MyoD transcripts by *in situ* hybridization. **(A)** and **(B)** are equivalent transverse sections of st18 neurulae hybridized with cardiac actin and MyoD probes respectively. Both label only the somites. **(C)** is a gastrula (st11) section hybridized with MyoD, and **(D)** an explanatory diagram. Labelling is restricted to mesodermal cells, most likely only those which will form the somites. Exposure times were (A) 2 days, (B) 10 days and (C) 10 days.

MyoD is expressed in skeletal, but not in cardiac or smooth muscle

We have further investigated the specificity of MyoD expression using Northern blots of dissected adult frog tissues and *in situ* hybridization to later embryos. The dissections (Figure 6) show that MyoD transcripts are found only in leg (skeletal) muscle, not in tissues such as heart or stomach, which contain cardiac and smooth muscle respectively. This result is in agreement with that of Davis *et al.* (1987) for tissues from new-born and adult mice. They suggested that the MyoD RNA in adult mouse muscle could be in myogenic stem cells.

It is of particular interest to establish whether or not the early embryonic heart expresses MyoD, because it co-expresses cardiac and skeletal muscle proteins, which in adult frogs are absolutely restricted to heart and skeletal muscle respectively (Mohun *et al.*, 1984). Equivalent transverse sections of st33 embryos were hybridized with MyoD and cardiac actin probes (Figure 7). Whilst the actin probe labels both the heart and the anterior somites, the MyoD section shows no labelling above background. In the same embryo, MyoD was expressed in the posterior somites (see below). The lack of MyoD expression in the early heart has been confirmed by more sensitive Northern analysis of dissections (data not shown). The heart has just started to beat at st33-34 (Nieuwkoop and Faber, 1967) and so it could be argued, especially in view of our results on the transience of MyoD expression in the axial muscle (see below), that MyoD RNA could have been present earlier in heart development. Although we cannot exclude very brief expression in an early heart-forming region, we have seen no hybridization of MyoD probes to the heart anlage at any stage. The absence of MyoD in the developing heart argues that MyoD is not essential for the expression of striated muscle genes (see Discussion).

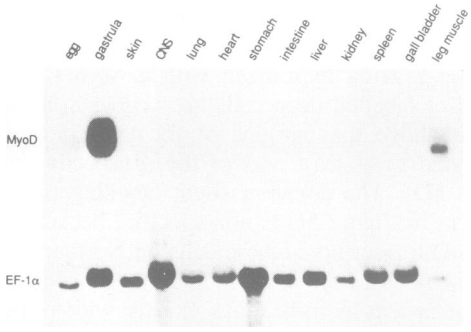


Fig. 6. MyoD is skeletal muscle-specific in adult frogs. Northern blot of RNA from a selection of tissues from a newly metamorphosed froglet probed with MyoD, and with EF-1α to show the relative amounts of total RNA in each lane. Unfertilized egg and st11 gastrula samples are included for comparison. Note that the leg muscle RNA, of which least was loaded, is the only adult sample to show a MyoD signal above background.

The concentration of MyoD RNA in the somites falls once they have formed

The abundance of MyoD transcripts in whole embryos declines after the late neurula stage (Figure 3). Northern blots of dissected st33 embryos showed a higher concentration of MyoD RNA in the posterior than in the middle or anterior parts (data not shown). *In situ* hybridization has extended these results. Longitudinal sections of st22 and st33 embryos were probed with MyoD (Figure 8). The labelling over the somites changes from being uniformly high at st22 to being undetectable except in the posterior region at st33. The

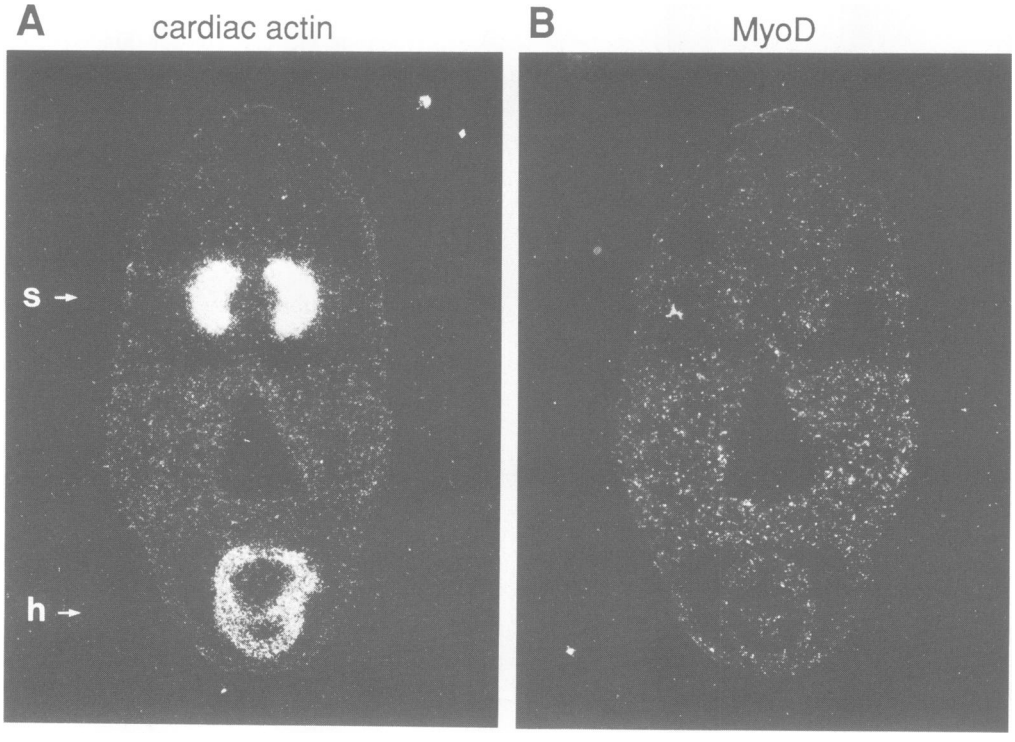


Fig. 7. MyoD is not expressed in embryonic heart. Equivalent transverse sections through the somites and heart of st33 embryos hybridized with (**A**) a cardiac actin gene-specific probe (Mohun *et al.*, 1984), and (**B**) MyoD. Note that MyoD is no longer expressed in the anterior somites at this stage; tail sections of the same embryo did show hybridization to the somites. s, somites; h, heart. Exposure times were (A) 2 days and (B) 10 days.

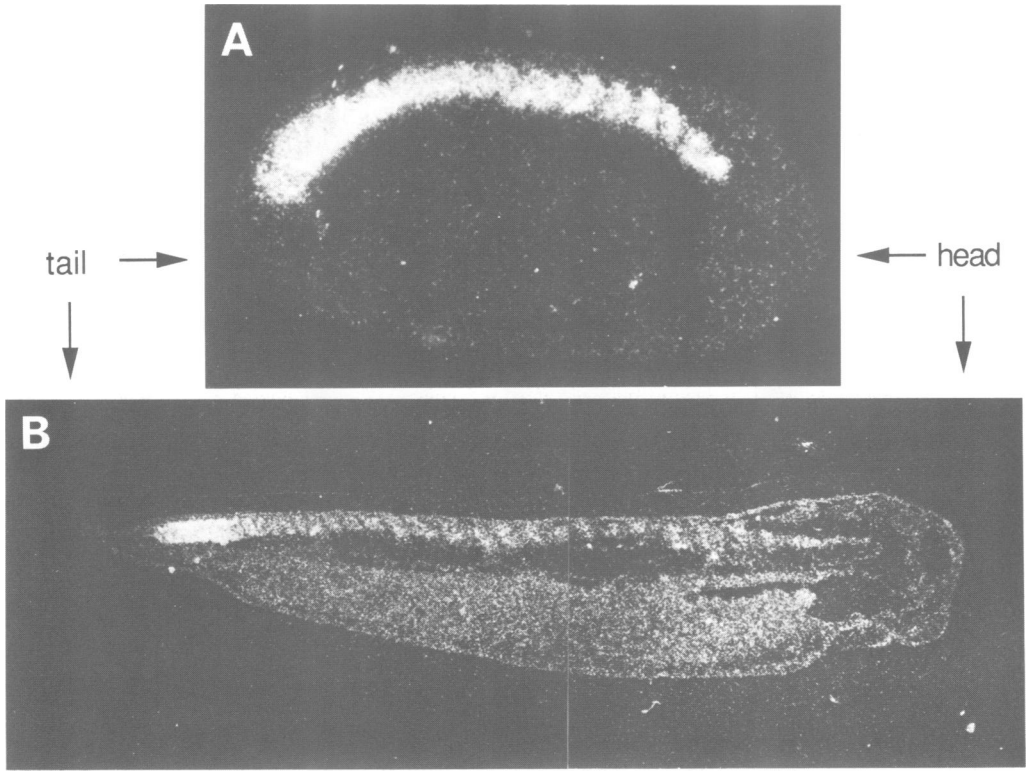


Fig. 8. The abundance of MyoD RNA in the somites falls once they have formed. Longitudinal sections of (**A**) a st22 and (**B**) a st33 embryo hybridized with MyoD. All of the somites are labelled at st22, but at st33 MyoD RNA is undetectable by *in situ* hybridization except in the posterior somites, which develop later than the anterior ones. Apparent hybridization to the anterior thorax of the st33 embryo is an artefact of uneven illumination during photomicroscopy.

MyoD RNA concentration has declined in the anterior somites, which differentiated first, whilst remaining high in the tail somites, where muscle is still forming at st33. By examining all serial sections of these embryos we have ensured that this result is not an artefact of the plane of section.

These *in situ* hybridization results suggest that a high concentration of MyoD is needed only for the initial activation of muscle gene expression. However, our analysis of dissections showed that anterior and middle parts of embryos still contain a small amount of MyoD RNA even at st33, and dissected adult leg muscle also contains MyoD transcripts (Figure 7). The persisting low concentration of MyoD RNA may be required for the maintenance of muscle gene expression, it may be restricted to myogenic stem cells (Davis *et al.*, 1987), or it may have no function.

MyoD is activated following mesoderm induction

We have begun the analysis of how MyoD expression is activated in early gastrulae by making tissue conjugates between parts of blastulae. Much previous work has shown that, when cultured alone, equatorial pieces form mesoderm, including muscle, but animal and vegetal parts do not. However, when combined in a conjugate, vegetal tissue induces the animal cap cells to form muscle and activates muscle-specific gene expression (e.g. Nieuwkoop, 1969; Gurdon *et al.*, 1985).

We divided st8½ blastulae into animal, equatorial and vegetal parts and cultured them in isolation until control whole embryos were late gastrulae (st12½), when they were frozen for analysis. We also made tissue conjugates of animal and vegetal pieces and cultured them in a similar way. RNA extracted from these explants was analysed for MyoD and

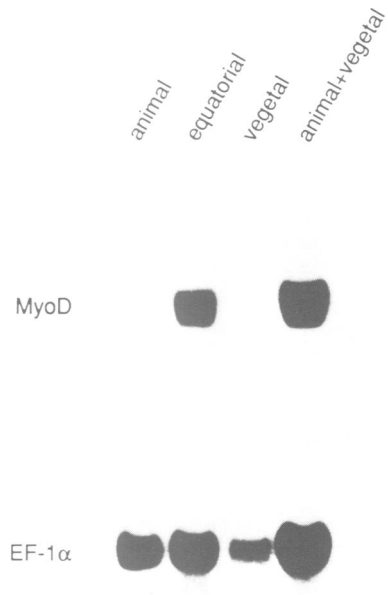


Fig. 9. MyoD RNA accumulates in response to mesoderm induction. St 8½ blastulae were dissected into animal, vegetal and equatorial pieces, which were cultured in isolation until control whole embryos became late (st12½) gastrulae. Animal and vegetal pieces were also cultured in combination. This Northern blot of RNA extracted from the explants was probed with XmyoD2−24 and then EF-1α. RNA from equal numbers of explants was loaded in each lane. Animal and vegetal pieces do not express MyoD when cultured alone, but do so when cultured in combination. The lack of even the low maternal content of MyoD RNA in animal and vegetal pieces dissected from blastulae and cultured to the late gastrula stage suggests that these transcripts have been degraded during the culture period.

EF-1α expression on a Northern blot; the result was that neither animal nor vegetal pieces cultured alone expressed MyoD, but equatorial pieces and animal:vegetal conjugates did so (Figure 9).

We conclude that MyoD expression is, like that of α-actins, activated by induction. Transcription of the MyoD gene in early gastrulae (Figure 3) is thus the earliest muscle-specific response to mesoderm induction yet described.

Discussion

What is the function of MyoD in muscle development? We have shown that the time and place of its expression in *Xenopus* embryos is consistent with a role in the activation of muscle genes in the somites. New MyoD transcripts appear in early gastrulae, ~2 h before cardiac actin RNA is first detectable. They are restricted to the mesoderm of mid-gastrulae, and to the somites of later embryos. However, MyoD is not expressed in the embryonic heart, in which both cardiac and skeletal actin genes are strongly transcribed (Mohun *et al.*, 1984). This means that although MyoD could turn on striated muscle genes in the somites, it cannot be essential for their activation in all embryonic tissues.

We have compared MyoD expression to that of the cardiac actin gene, because the latter is expressed relatively early in somite differentiation, and is the most thoroughly characterized muscle gene in *Xenopus*. Since MyoD is thought to be a DNA-binding protein (Murre *et al.*, 1989), it is reasonable to ask whether it could activate cardiac actin transcription. The *cis* sequences required for the embryonic activation of the *Xenopus* cardiac actin gene in the somites have largely been defined, and consist of a short region of promoter containing four repeated 'CArG' boxes (Minty and Kedes, 1986; Mohun *et al.*, 1986), the most proximal of which is essential for activation (Mohun *et al.*, 1989). A protein that binds to this sequence has been characterized and is indistiguishable from the serum-response factor of the c-*fos* gene which is present in both muscle and non-muscle cells (Taylor *et al.*, 1989). There is currently no evidence for a direct interaction of MyoD with this promoter. However, the timing of *Xenopus* MyoD transcription makes it a strong candidate to activate muscle genes either directly or indirectly.

Two other *myc*-related genes have been identified recently, both of which can convert 10T½ fibroblasts to myoblasts. Myogenin was found using subtractive hybridization to look for transcripts expressed during the differentiation of cultured rat myoblasts (Wright *et al.*, 1989), and the Myf-5 cDNA was isolated by screening a human fetal skeletal muscle library at low stringency using a MyoD probe (Braun *et al.*, 1989). The human genomic locus *myd*, which is different from MyoD and myogenin (Pinney *et al.*, 1988; Wright *et al.*, 1989) and possibly also Myf-5 (Braun *et al.*, 1989), also converts 10T½ fibroblasts to myoblasts. Particularly in view of the finding that MyoD is undetectable in some myogenic cell lines that express myogenin (Edmonson and Olson, 1989; Wright *et al.*, 1989), it is possible that the three related factors can function interchangeably in development. Alternatively, they might play equivalent roles in different developmental situations. Myogenin is expressed in the somites of mouse embryos at 8.5 days of gestation (Wright *et al.*, 1989), at about the same time as cardiac actin transcripts become detectable by *in situ* hybridization (Sassoon *et al.*, 1988), but it is not found in the embryonic mouse heart (Wright *et al.*, 1989). The relative roles of these different genes in somite development will be a subject for future work, but it is already clear that neither MyoD nor myogenin activates muscle genes in the heart; *myd*, Myf-5 or other myogenic genes yet to be discovered could have this function.

Xenopus embryos are a good system in which to study the function of MyoD in the context of the differentiation of the mesoderm as a whole. We have shown that transcription of the MyoD gene is a very early, muscle-specific response to mesoderm induction. It would be interesting now to find out how the processes initiated by mesoderm-inducing factors lead to the activation of genes such as MyoD in specific regions of the forming mesoderm.

Materials and methods

Library screening

A gastrula cDNA library in λgt10 (st11; Krieg and Melton, 1985) was probed at low stringency with random-primed fragments (Feinberg and Vogelstein, 1983) made from the mouse MyoD cDNA (Davis *et al.*, 1987). The screening followed Maniatis *et al.* (1982), except that hybridization was in a solution containing 50% formamide, 1 M NaCl, 1% SDS, 10% dextran sulphate and 100 μg/ml sheared, denatured salmon sperm DNA at 42°C for 16 h. Filters were washed in 2 × SSPE, 0.5% SDS at 45°C for 2 h.

Analysis of clones

Positively hybridizing clones were purified by standard methods. λDNA was analysed by Southern blotting and subcloned into plasmid and M13 vectors for restriction mapping, propagation and sequencing.

DNA sequencing used the dideoxy method (Sanger *et al.*, 1977; Biggin *et al.*, 1983) on templates prepared by limited exonuclease III digestion (Henikoff, 1984). Sequences were compiled using the DB programs of Staden (1982), each base being sequenced at least twice on each strand.

Embryos

Xenopus laevis embryos were dejellied in cysteine−HCl (pH 8.0) and cultured in modified Barth's solution (MBS) (Gurdon, 1977) at 23°C. Dissections were carried out in a 3 mg/ml solution of collagenase in MBS using sharpened watchmakers' forceps. Embryos and embryo fragments were frozen at −80°C before analysis.

Northern blots

RNA was extracted from 100 or more eggs or embryos by a LiCl−urea method (Auffray and Rougeon, 1980), and from dissected parts of embryos as described by Gurdon *et al.* (1985). Adult tissue RNA was extracted in the same way as from dissected embryonic tissues, but was further purified by precipitation from 2.5 M LiCl on ice. RNA was electrophoresed on 1.2% formaldehyde−agarose gels, transferred to GeneScreen (NEN) and baked for 1 h at 80°C. Blots were hybridized in GeneScreen buffer containing 330 mM sodium phosphate, pH 7.0, 5% SDS, 10 mM EDTA, 10% dextran sulphate and 250 μg/ml sheared, denatured salmon sperm DNA at 65°C. Probes were made by random priming of the XmyoD2−24 cDNA and of an EF-1α cDNA (Krieg *et al.*, 1989); cardiac actin RNA was detected using a single-stranded, gene-specific DNA probe (G2 of Mohun *et al.*, 1984). Washes were in 0.2 × SSPE, 0.1% SDS at 65°C for MyoD and EF-1α probes, and in 0.1 × SSPE, 0.1% SDS at 37°C for the short cardiac actin probe. Blots were stripped in 50% formamide, 0.1% SSPE at 70°C for 1 h before re-probing.

Measurement of the number of MyoD transcripts

Synthetic MyoD RNA was transcribed (Krieg and Melton, 1987) from a Bluescribe (Stratagene) template containing the XmyoD2−24 cDNA cloned into the *Eco*RI site and linearized with *Hin*dIII, using T7 RNA polymerase. The concentration of sense RNA produced was measured after DNase treatment and removal of unincorporated nucleotides. Dilutions of this sense RNA and 10 μg each of total unfertilized egg and st11 gastrula RNA were electrophoresed in a formaldehyde−agarose gel, transferred to GeneScreen and probed with XmyoD2−24 as described above. Densitometry of appropriately exposed autoradiographs of the blot allowed the mass and hence the number of transcripts of MyoD RNA in the embryo samples to be calculated.

In situ hybridization

In situ hybridization was by the method of Kintner and Melton (1987), with the following minor changes: 10 μm sections were dried on to slides treated with poly-L-lysine (Sigma) at 50 μg/ml in 10 mM Tris, pH 8.0; we used Ilford K5 emulsion; and the final wash after RNase digestion was in 2 × SSPE, 50% formamide at 50°C for 1 h.

RNA probes were made by *in vitro* transcription (Krieg and Melton, 1987) in the presence of [^{35}S]UTPαS at 400 Ci/mmol (Amersham). MyoD probes were a mixture of transcripts made from three subclones spanning the entire XmyoD2−24 cDNA. The SP73 (Krieg and Melton, 1987) templates contained the 5′ *Eco*RI−*Sph*I fragment (nucleotides 1-383), the internal *Sph*I−*Bam*HI fragment (nucleotides 384−872), and the 3′ *Bam*HI−*Eco*RI fragment (nucleotides 873−1469), and were linearized (and transcribed) using *Eco*RI (T7 RNA polymerase), *Hin*dIII (SP6) and *Bam*HI (SP6), respectively. The cardiac actin probe was made by transcription of the 3′ untranslated region of a cardiac actin gene (Mohun *et al.*, 1984) cloned as a blunt *Xmn*I−*Nco*I fragment (bases 6257−6472 of Mohun *et al.*, 1986) into the *Sma*I site of SP64 (Melton *et al.*, 1984). Probes were hydrolysed to an average of 150 nucleotides and used at 300−1000 c.p.m./μl hybridization solution.

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Note added in proof

The accession number in the EMBL database for the sequence shown in Figure 1 is *X*16106.