Transient expression of XMyoD in non-somitic mesoderm of Xenopus gastrulae

DALE FRANK and RICHARD M. HARLAND*
Department of Molecular and Cellular Biology, Division of Biochemistry and Molecular Biology, 401 Barker Hall, University of California, Berkeley, CA 94720, USA

*Corresponding author

Summary
XMyoD is the earliest marker of muscle development in Xenopus embryos and is expressed in presumptive somites in the late gastrula. In the early gastrula, in situ hybridization showed XMyoD transcripts in precursors of both muscle and non-muscle mesoderm. Embryos ventralized by UV irradiation made no muscle, but expressed XMyoD transiently. Embryo explants that differentiated ventral mesoderm also expressed XMyoD transiently. These results show that the initiation of XMyoD expression is not sufficient to convert cells to muscle and suggest that XMyoD is expressed in response to a general mesodermalizing signal; expression is stabilized and enhanced only in muscle precursors that have received a dorsaling signal.

Key words: MyoD, Xenopus, mesoderm induction.

Introduction
In amphibian embryos, mesoderm is induced in the equatorial region (marginal zone) of the embryo (reviewed by Gurdon, 1987; Smith, 1989). Fate maps of the 32-cell embryo and gastrula show that the most dorsal marginal zone makes notochord and the first muscle to develop derives from the lateral marginal zone (Keller, 1976; Dale and Slack, 1987a; Moody, 1987). The ventral mesoderm gives rise predominantly to lateral plate mesoderm, mesenchyme and blood, though a small amount is recruited at later stages to form tail muscle (Keller, 1976; Keller, personal commun.). Although it is possible that there are numerous inductive signals that induce different tissues, much of the experimental evidence is consistent with a simple three-signal model (Dale and Slack, 1987b). In this model, early vegetal cells exist in two states, dorsal or ventral. These induce either the most dorsal mesoderm (Spemann organizer), or ventral mesoderm in the marginal zone of the blastula (Sudarwarti and Nieuwkoop, 1971; Slack and Forman, 1980; Gimlich and Gerhart, 1984). The further differentiation of the lateral marginal zone is due to a dorsaling signal emanating from the organizer, and acting predominantly in the late blastula and gastrula (Dale and Slack, 1987b; Stewart and Gerhart, 1990).

In this paper, we address how early inductions activate the expression of muscle-specific gene regulators, and the consequences of this activation for differentiation of cells into muscle. We have assayed the spatial distribution of transcripts of the XMyoD gene, and asked whether activation of the endogenous XMyoD genes is sufficient to cause determination of cells as muscle.

The MyoD gene product is a member of a family of basic helix-loop-helix (bHLH) DNA-binding proteins which can commit cells to the myogenic lineage (reviewed by Weintraub et al. 1991). MyoD belongs to an extended family of muscle-specific bHLH proteins; all of these proteins have a striking structural similarity in their bHLH DNA-binding domains, and they all convert non-myoogenic cell lines to muscle when expressed by transfection (Weintraub et al. 1989; Wright et al. 1989; Braun et al. 1989a; Rhodes and Koniezny, 1989; Miner and Wold, 1990). These mammalian proteins are restricted to muscle cell lineages during normal embryonic development (Sasoon et al. 1989; Ott et al. 1991), suggesting an active role for these genes in controlling muscle cell differentiation in vivo.

In the amphibian Xenopus laevis, the XMyoD gene is the earliest identified marker for mesoderm induction. The gene is activated when transcription initiates at the mid blastula transition (Hopwood et al. 1989; Harvey, 1990; Scales et al. 1990). Initial zygotic transcription levels are low, with levels rising sharply from gastrula to neurula stages. XMyoD is expressed in mesodermal cells during gastrula stages, and by late gastrula the transcripts are localized to the presumptive somites (Hopwood et al. 1989, 1991). The localized expression of XMyoD in somite precursor cells precedes the
appearance of mRNAs such as cardiac actin by about 2h. The temporal and regional patterns of MyoD expression suggest that it may establish muscle cell identity in developing somites in a trans-dominant manner as in the mammalian cell culture systems.

While XMyoD may indeed orchestrate myogenesis in vivo, not all experimental evidence confirms this premise. Ectopic expression of microinjected XMyoD mRNA in animal cap cells (presumptive ectoderm) did not convert them into muscle, though these cells did transiently express cardiac actin genes (Hopwood and Gurdon, 1990). Thus, a transient burst of XMyoD during early development may not suffice to convert presumptive ectodermal cells into muscle. In addition, low level maternal transcripts found ubiquitously throughout the pre-gastrula embryo do not interfere with normal development (Harvey, 1990; Scales et al. 1990). It has also been shown that the initial phase of new expression, when XMyoD genes turn on at a low level at the mid-blastula stage, is not restricted to marginal zone, but will occur in isolated animal and vegetal cells (Rupp and Weintraub, 1991). This early phase of expression is not stable, the transcripts disappear from animal cap cells at gastrulation, and the activation of the genes at this low level is not sufficient to cause muscle formation (Rupp and Weintraub, 1991). If XMyoD regulates myogenesis in a trans-dominant manner, regulatory mechanisms must exist to prevent both the maternal and early zygotic transcripts from converting non-mesodermal cell types into muscle.

In this paper, we re-examine the dramatic increase in XMyoD transcripts that occurs at the onset of gastrulation. Two issues are addressed. First, is expression of the XMyoD gene induced in cells that will not make muscle in the embryo? Although XMyoD transcripts are present in earlier embryos, the induction of expression during gastrulation might be the important step in stabilizing the muscle phenotype. Second, how does this phase of expression relate to the inductive signals that subdivide the embryo, and in what way does it relate to the three-signal model? If XMyoD was only expressed in lateral marginal zone, we would suggest that the gene was turned on by dorsalizing signals emanating from the organizer; on the other hand, if the gene were turned on throughout the lateral and ventral marginal zones, then its activation would be more consistent with induction by the ventral-vegetal signal.

We have used three approaches to address these questions. In order to localize endogenous transcripts, we have improved upon previous methods of in situ hybridization to whole amphibian embryos (Hemmati-Brivanlou et al. 1990), so that we can detect XMyoD transcripts in the early gastrula. To examine the time course of XMyoD mRNA expression in non-muscle mesoderm, we have used ventralized embryos; these embryos are generated by UV irradiation of the egg (Scharf and Gerhart, 1983; reviewed by Gerhart et al. 1989), and extreme forms make no muscle. Finally, we have examined XMyoD expression in regions of marginal zone explanted at the onset of gastrulation; while dorsal and lateral marginal zones are already specified to make muscle tissue at this stage, ventral marginal zone is not specified to differentiate muscle tissues or dorsal cell behaviour (Dale and Slack, 1987a,b; Keller and Danilchik, 1988). Our results show that XMyoD is turned on in both lateral and ventral marginal zones at the onset of gastrulation and induction of XMyoD expression is not sufficient to cause muscle differentiation. Already, three phases of XMyoD expression have been defined; maternal expression (Harvey, 1990; Scales et al. 1990), ubiquitous expression at the mid-blastula transition (Rupp and Weintraub, 1991), and induction-dependent expression in the presumptive somites of the gastrula (Hopwood et al. 1989; Harvey, 1990). Here, we show that the phase of expression that starts at gastrulation should be further divided into general expression throughout the ventral and lateral marginal zone, followed by expression restricted to presumptive muscle cells. These results are consistent with the view that the ventral vegetal signal activates XMyoD expression at the onset of gastrulation, the expression is then stabilized and increased further by dorsalizing signals from the organizer. As a result of the combination of inductions, muscle tissue is formed.

Materials and methods

Embryos

Xenopus laevis were obtained from the Berkeley colony maintained by the laboratory of J. C. Gerhart. For in situ hybridization, albino embryos were used. Ovulation of females, in vitro fertilization, UV irradiation and embryo culture were carried out as described previously (Condie and Harland, 1987). Embryos were staged according to Nieuwkoop and Faber (1967). Embryos for in situ hybridization were pretreated for 5–10 min with 5 μg ml⁻¹ proteinase K to facilitate removal of the vitelline membrane during gastrula and neurula stages. Often these embryos are slow to close the blastopore so embryos were staged relative to untreated controls. The dorsal-anterior index of UV irradiated embryos was determined at stage 41 according to Kao and Elison (1988). Gastrulating embryos were dissected at stage 10°. All dissections were carried out in 1/3× Modified Ringers (1/3×MR pH6.9; Condie and Harland, 1987). Vitelline membranes were manually removed with watchmakers forceps, and embryos were dissected with eyebrow knives.

Whole-mount in situ hybridization

The whole-mount in situ hybridization protocol detailed in Hemmati-Brivanlou et al. (1990) was modified as follows (a detailed protocol is available from R.M.H. on request; Harland, 1991). After proteinase K treatment, (which increases the hybridization signal but is not essential for detection of most transcripts) embryos were rinsed twice in 0.1 M triethanolamine pH7–8. Acetic anhydride (12.5 μl/5 ml triethanolamine buffer) was added and after 5 min was added again (Lynn et al. 1983). The hybridization buffer was modified by the addition of 1×Denhardt’s buffer, 0.1 % CHAPS and Torula RNA to 1 mg ml⁻¹. Prehybridization was a minimum of 6h. The washes that followed hybridization contained 0.3 % CHAPS. Prior to protein blocking and antibody incubation CHAPS was removed by rinsing in PBT.
Fig. 1. Albino embryos were examined by whole-mount hybridization using digoxigenin-labeled XMyoD antisense and sense probes as described in the methods. (A) Hybridization to a family of developing *Xenopus* embryos. From top to bottom: stage 13, stage 24, stage 32, stage 40, and stage 40 (sense control). (B) Negative control hybridization to a stage 11 gastrula with a sense probe. (C) View from the vegetal pole of a stage 10.25 gastrula. Arrows point to the limit of the dorsal lip. Note the grainy signal above background levels throughout the lateral and ventral marginal zones. (D–F) Stage 11 gastrula. (D) Dorsal view, (E) Lateral view, (F) Ventral view. Figures D to I are oriented with the blastopore at the bottom of the picture. The blastopore lip is arrowed in D–I. (G,H) *In situ* hybridization to stage 13 neurulae. (G) Dorsal view, (H) Ventral view, (I) Stage 15 neurula.
The chromogenic reaction is stopped by replacing the solution with MEMFA (Hemmati-Brivanlou et al. 1990) or Bouins fixative. MEMFA stabilizes the stain and embryos can be stored in it. We have not carried out a systematic determination of which changes are important; however, it is clear that the changes not only decrease the background, but also substantially boost the signal obtained in a short chromogenic reaction. The combination of increased signal with long chromogenic incubations (the animals in Fig. 1 were incubated overnight at 18°) substantially increases the sensitivity of the method.

Sense and antisense RNA probes containing digoxigenin were synthesized with T3 and T7 polymerase from a full-length XMyoD cDNA (p3) linearized with either XbaI or HindIII (kindly provided by R. Rupp).

Northern blot analysis

Total RNA was isolated as described previously (Condie and Harland, 1987). Unless otherwise stated, one embryo equivalent of RNA was fractionated on a formaldehyde gel and blotted onto a nylon membrane. Filters were hybridized sequentially with the complementary DNA probes of XMyoD (0.55 kb BamH I–EcoR I, from pSP73-M24/3, Hopwood et al. 1989), EF1α (0.4 kb Pst–SsrI, Krieg et al. 1989), or the muscle-specific cardiac actin (m-act in figures; 0.5 kb PvuH–EcoK1, from pAC100, Dworkin-Rastl et al. 1986). Although EF1α mRNA increases in abundance during development, it is used as a positive standard for comparing levels of RNA loaded per well at any given stage. The XMyoD probe detects transcripts from both XMyoD genes (Harvey, 1990). DNA probes were made by random primer extension with specific activities of an early gastrula (Fig. 1C) where the region above the blastopore (arrowed) has no staining. The ventral expression is more striking in the mid-gastrula (stage 11); Fig. 1D, E and F shows dorsal, lateral and ventral views of the same animal. A belt of XMyoD-positive cells extends around the lateral and ventral marginal zone, just above the blastopore lip (arrowed). A sense probe control of stage 11 (Fig. 1B) shows no similar staining. Although the band of XMyoD staining is fairly uniform throughout the lateral and ventral marginal zones, fate mapping of the early gastrula shows that the most ventral regions give rise mostly to non-somatic mesodermal tissue (Keller, 1975, 1976). In hybridizations to sectioned embryos, XMyoD mRNA was not detected in most dorsal or ventral marginal zones at stage 11.5 (Hopwood et al. 1991); this variation may be due to slight differences in stage, or the difficulty of obtaining a section through all the expressing tissues. One advantage of whole-mount staining is that the picture of expression is not dictated by the plane of section.

As gastrulation proceeds, staining becomes more intense in the presumptive somites. Fig. 1G and H show dorsal and ventral views of a stage 13 embryo, and Fig. 1J shows a dorsal view of a midneurula. Thus the early expression of XMyoD in the ventral marginal zone is not increased to the same extent as in the lateral marginal zone. In early and mid gastrulae, the presence of XMyoD transcripts in the most ventral marginal zone strongly suggests that the XMyoD gene is transiently transcribed in mesodermal lineages fated for non-somatic tissues such as lateral plate mesoderm and blood.

Expression of XMyoD in UV-irradiated ventralized embryos

If XMyoD is transcribed in non-muscle tissues, then embryos that do not make muscle should express the XMyoD gene transiently. To test this, we used ventralized embryos; UV irradiation early in the first cell cycle disrupts a cortical rotation and leads to loss of dorsal development (reviewed by Gerhart et al. 1989). These embryos have significantly reduced levels of somitic mesoderm and extreme ventralized embryos develop no axial structures and no detectable muscle (Scharf and Gerhart, 1983; Condie and Harland, 1987; Kao and Elinson, 1988). Pools of ventralized embryos at different stages were analyzed for XMyoD expression by RNA blot hybridization. Our methods do not detect the small amount of maternal XMyoD transcript (Harvey, 1990; Scales et al. 1990) so that the results reflect the new transcription of the XMyoD gene. As a control the filters were rehybridized with the ubiquitously expressed EF1α RNA (Fig. 2). Siblings were allowed to develop to tadpole stages to assess the efficiency of the UV treatment by morphological criteria (Scharf and Gerhart, 1983; Kao and Elinson, 1988); 70% of these embryos developed no axial structures (see Table 1). The early phase of XMyoD expression (at stages 10.75 to 11.5) is unaffected by the
Fig. 2. XMyoD mRNA is expressed in ventralized gastrulae. Total RNA from two pools of five control and UV ventralized embryos were isolated at stages 9 (lanes 1, 2), 10+ (lanes 3, 4), 10.75 (lanes 5, 6), 11.5 (lanes 7, 8), 12.5 (lanes 9, 10) and stage 22 (lanes 11, 12). One embryo equivalent of RNA was electrophoresed on a formaldehyde gel and blotted onto a nylon membrane. The filter was hybridized sequentially with complementary DNA probes of XMyoD and EF1c as described in the methods. 

irradiation, but the later phase of expression (at stage 22) is reduced to less than 15% of the control in such pools (Fig. 2). The residual XMyoD RNA in the later pools could be due to some incompletely ventralized embryos, so in parallel, individual embryos were assayed for XMyoD expression (Fig. 3). Even though it is unreliable to assess the degree of ventralization before the late neurula stage, the individuals should include embryos fated to make no muscle. Embryos were selected at random and will represent different degrees of ventralization. However, 70% of the embryos would give no axial structure if allowed to develop further (Table 1). 

Fig. 3A shows that at early stages (11 and 12) all embryos express XMyoD whether UV treated or not. However, by stage 14 (early to mid neurula) the UV-treated embryos fall into two classes; those that express both XMyoD and muscle-specific actin, and those that express neither (Fig. 3A). The stage 14 individuals that fail to express XMyoD (6/9) are present at a similar frequency to the extreme ventralized embryos scored at the tadpole stage (70% see Table 1); thus, at this developmental stage, the lack of XMyoD expression may be a direct measure of ventralization.

To correlate expression of XMyoD further with morphological criteria, individual late neurula embryos were scored for degree of dorsoanterior development (Kao and Elinson, 1988) and were analyzed for mRNA. Fig. 3B shows that the expression of both XMyoD and muscle-specific actin closely follows the morphological prediction, with the extreme ventralized embryos (DAI 0) expressing neither XMyoD nor muscle-specific actin.

---

**Table 1. The dorsal anterior deficiency index (DAI) of members of the batch of irradiated embryos used in figures 2 and 3 was determined at stage 41 according to Kao and Elinson (1988), with grade 0 being the most axial deficient and grade 5 being normal.**

<table>
<thead>
<tr>
<th>DAI</th>
<th>Dorsal Anterior Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>63 16 8 2 0 1</td>
</tr>
</tbody>
</table>

In these experiments the DAI population average was less than 0.5. UV-irradiated embryos for northern analysis were randomly picked to ensure a representative sampling of the population.
We also analyzed the embryos for expression of the XMyf-5 gene, another member of the myogenic helix-loop-helix family. Rehybridizing the filters in Fig. 3A with an XMyf-5 probe (Hopwood et al. 1991) shows that XMyf-5 expression correlates with that of XMyoD in ventralized embryos (data not shown). By in situ hybridization, we have also observed radially symmetric expression of XMyoD throughout the marginal zone of UV ventralized early gastrulae (data not shown).

The results presented in Fig. 3 suggest that the large increase in XMyoD expression that occurs during gastrulation is not strictly correlated with the formation of muscle tissue until after stage 12; and much of the RNA in the early gastrula is expressed in non-muscle precursors. These results demonstrate that XMyoD, XMyf-5, and perhaps other bHLH myogenic transcription factors are transiently expressed in ventral mesodermal tissues during gastrulation.

**Transient expression of XMyoD in the ventral marginal zone of normal embryos**

A strong prediction from these findings is that isolated ventral marginal zones from normal embryos should express XMyoD transiently, even though they do not differentiate into muscle. Although part of the ventral marginal zone will generate some muscle in normal embryos, the tissue is not specified as muscle at stage 10 and in isolation will develop into ventral structures (Dale and Slack, 1987b). In contrast, the lateral marginal zone, which makes most of the muscle, is more strongly specified to make muscle at this stage. Normal gastrulating embryos were dissected to determine whether XMyoD is transiently expressed in the ventral marginal zone (VMZ) as well as the dorsal and lateral marginal zone (DMZ and LMZ). Embryos were dissected at the 10+ stage when a pigment line is formed at the dorsal lip by involuting bottle cells, thus enabling a clear identification of the presumptive dorsal and ventral sides. A 45°–60° region of VMZ and DMZ was isolated with the remainder taken as LMZ (Fig. 4A).

Dissected tissues from each region were incubated until controls reached stages 11 or 20 and then assayed for XMyoD expression. As can be seen in Fig. 4B, XMyoD is expressed in all three regions at stage 11. The relative levels of zygotic XMyoD transcripts seen at stage 11 are remarkably similar in the VMZ and LMZ explants (determined by densitometry with reference to EF1α to control for RNA loading). At stage 20, both XMyoD expression and muscle-specific actin RNA expression are observed in the LMZ and DMZ explants, but are almost absent in the VMZ explant. These results confirm that the stage 10+ VMZ is not yet specified to form muscle, but in striking similarity to the UV ventralized embryos, XMyoD is transiently expressed in this non-muscle tissue.

**Discussion**

Although the MyoD gene is a strong activator of muscle differentiation, we have shown here that the XMyoD gene is transcriptionally active in gastrula tissues that do not make muscle. The methods included in situ hybridization to normal embryos, and RNA analysis of both ventralized embryos and ventral marginal zones, neither of which make muscle. Transient expression of XMyoD is found in ventral tissue of the gastrula; stable expression is associated with somitic mesoderm by the early neurula stage.

Previously, it had been shown that there are three phases of XMyoD expression; our results show that there are at least four. The first phase is maternal (Harvey, 1990; Scales et al. 1990) and the second is a low ubiquitous expression at the mid-blastula transition (Rupp and Weintraub, 1991). We have shown that activation of XMyoD at the gastrula stage consists of a third phase of expression throughout the ventral and lateral marginal zones and a fourth, where expression is concentrated in future muscle cells. Ectopic expression of MyoD has been shown to convert many non-muscle mammalian cell lines into...
myoblasts (Weintraub et al. 1989). Recent studies have also shown that ectopic MyoD expression is not always sufficient to convert cells to muscle lineages (Weintraub et al. 1989; Schafer et al. 1990; Hopwood and Gurdon, 1990). The HepG2 hepatocyte cell line cannot respond to MyoD’s myogenic effects even when the transcript is translated (Schafer et al. 1990). In other studies, a myogenically deficient myoblast cell line appears to contain a trans-dominant repressor capable of inhibiting the biological activity of MyoD and other bHLH proteins (Peterson et al. 1990). In this study, we have not examined the translation of the XMyoD protein product and it is possible that non-somatic mesoderm can repress the myogenic effect of XMyoD by preventing its translation. Even if XMyoD was translated in non-somatic mesoderm, other mechanisms could be present to prevent its biological activity, such as a lack of the proper combinatorial amounts of other bHLH proteins (Murre et al. 1989), or trans-dominant repression by factors such as an Id protein (Benezra et al. 1990) and cellular oncogenes (Miner and Wold, 1991). It may be necessary for MyoD to activate a cascade of other myogenic proteins in order to fix the myogenic state. The various muscle bHLH proteins demonstrate variability in their potentials for autoactivation and transactivation of the other myogenic bHLH proteins in mammalian tissue culture systems (Thayer et al. 1989; Braun et al. 1989b; Miner and Wold, 1990). In animal caps ectopically expressing injected XMyoD mRNA, endogenous XMyf-5 is not activated, while ectopic XMyf-5 expression activates endogenous XMyoD but not endogenous XMyf-5 (Hopwood et al. 1991). Perhaps non-somatic ventral mesoderm is not fixed into the myogenic fate because of some limitation in the autoactivation cascade of these muscle bHLH proteins. Some transcription factors are inhibited by phosphorylation (Cherry et al. 1989; Luscher et al. 1990; Boyle et al. 1991). MyoD is a known phosphoprotein (Tapscott et al. 1989) and perhaps phosphorylation restricts its biological activity in a regionalized manner. Interestingly, in two invertebrate species, Caenorhabditis elegans and Drosophila melanogaster, the gene is activated in some cell lineages whose progeny may not develop as muscle (Krause et al. 1990; Michelson et al. 1990).

In Xenopus, animal caps expressing XMyoD ectopically from injected mRNA do express cardiac actin but do not acquire muscle morphology (Hopwood and Gurdon, 1990). XMyoD mRNA is present at low levels in maternal RNA (Harvey, 1990; Scales et al. 1990) and it has recently been shown that a low level of transcription of the XMyoD gene is activated in animal and vegetal pole cells at the mid-blastula stage (Rupp and Weintraub, 1991). These previous studies examined transcripts that are expressed ubiquitously and do not require mesoderm induction. In contrast, we have found that transcripts expressed at the onset of gastrulation require cell contact. The XMyoD gene was not expressed detectably at stage 11 in dissociated embryos, whereas two control genes which are newly expressed in the late blastula were expressed at normal levels (Frank and Harland, in preparation). The studies reported here show that the large initial increase in XMyoD mRNA during gastrulation (Hopwood et al. 1989; Harvey, 1990; Scales et al. 1990) is not confined to presumptive muscle mesoderm tissue. In addition we have shown that the failure of XMyoD gene expression to cause muscle development is a property of normal embryonic cells, as well as tissue culture models. Therefore, the induction of muscle development must involve events other than activation of XMyoD gene transcription.

XMyoD is thought to be one of the earliest markers for somitic induction during early amphibian development. The data presented here suggest that XMyoD can be turned on by general mesoderm-inducing signals. Stable and high level expression is only achieved in future muscle cells, which suggests that the gene responds to further inductive signals. Although other explanations are possible, activation of XMyoD expression is consistent with the ‘three-signal’ model of mesoderm induction (Dale and Slack, 1987b) where XMyoD is transiently activated throughout the mesoderm by a ventral type of induction from vegetal cells. Dorsal vegetal cells induce an ‘organizer’ (Gerhart et al. 1989), which would then ‘dorsalize’ the mesoderm to enhance and stabilize XMyoD expression in the lateral marginal zone. Thus, formation of pattern in the mesoderm is not a simple, one step event, but a series of processes that result in correct tissue-specific gene expression and axial patterning in the embryo.

We are grateful to Nick Hopwood and members of the Weintraub group for plasmids. We thank Ralph Rupp and Hal Weintraub for communicating results prior to publication. D.F. was supported by Rothschild Foundation and University of California Cancer Research Coordinating Committee Fellowships. This work was supported by NIH grant GM 42341 to R.M.H.

References


