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Gene activation in the amphibian mesoderm

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Summary

Cell potency is progressively restricted in amphibian development by a series of cellular interactions called inductions. The mesoderm is believed to develop in response to the earliest known induction, in which vegetal cells of the blastula divert overlying animal hemisphere cells away from epidermal and towards mesodermal fates. We describe two early markers of mesodermal differentiation in *Xenopus laevis*, both mRNAs that encode DNA-binding proteins of the helix-loop-helix family. One is a frog homologue of MyoD, a gene that in transfection experiments can convert cultured fibroblasts into myoblasts. *Xenopus* MyoD (XMyoD) is expressed in the early myotomes, from which the axial musculature develops. The accumulation of XMyoD RNA precedes that of transcripts from the cardiac actin gene, until now the earliest known marker of the muscle lineage, this result indicating that XMyoD could play a role in initiating muscle differentiation in normal development. We show by microinjection of synthetic RNA that XMyoD can indeed activate muscle-specific gene expression in animal cap cells, which would normally form only ectoderm. However, the XMyoD-injected animal caps did not produce differentiated muscle, suggesting that additional specific factors are required for full myogenesis. The other mRNA is a relative of the *twist* gene of *Drosophila*, which is required for mesodermal differentiation in flies. This gene (Xtwi) is expressed widely in the early frog mesoderm, but not, however, in the myotomes, where XMyoD is expressed. Later, the Xtwi gene is activated, in response to a second induction, in the developing neural crest.

Key words: helix-loop-helix proteins, mesoderm induction, myogenesis, neural crest, neural induction, *Xenopus laevis*.

Introduction

Amphibian embryos are big, develop in large numbers outside the mother, and can readily survive surgery. These properties have made them favoured experimental material for embryologists for over a century. Removing pieces of embryonic tissue for culture in isolation, or transplantation to another region of an embryo, was used to show that cell potency is progressively restricted in early development by a series of cellular interactions called inductions (Spemann, 1938; Holtfreter and Hamburger, 1955; Gurdon, 1987). The most celebrated induction is the direction by the mesoderm of neural differentiation from ectodermal cells that would otherwise have formed skin (Spemann and Mangold, 1924). Only comparatively recently was it discovered that the mesoderm itself develops in response to a previous induction (Nieuwkoop, 1969; Sudarwati and Nieuwkoop, 1971). Following the work of Nieuwkoop, we have gained in amphibia over the last two decades unparalleled knowledge of the origin and early development of the mesoderm (reviewed by Gerhart and Keller, 1986; Smith, 1989; Hopwood, 1990).

The mesoderm arises in the blastula, the hollow ball of cells that results from rapid cleavage of the egg cytoplasm following fertilization. When isolated and cultured, tissue from around the equator of the blastula differentiates into mesodermal derivatives in accordance with its normal fate. Explants of tissue from the pigmented animal cap form (atypical) epidermis, and explants of the yolky vegetal tissue from the opposite pole of the embryo form (poorly differentiated) endoderm. However, when animal and vegetal pieces are cultured in combination, mesoderm forms from the animal tissue (Nieuwkoop, 1969; Sudarwati and Nieuwkoop, 1971). This suggests that at least part of the mesoderm normally develops from animal cells at the equator of the blastula in response to induction by vegetal cells (Fig. 1).

A major obstacle to the study of amphibian development is that the long generation time (at least several months) makes impractical the extensive screens for mutants that have been so fruitfully applied to *Drosophila melanogaster* (Nüsslein-Volhard and Wieschaus, 1980; Nüsslein-Volhard *et al.* 1987; Anderson, 1987). However, since the discovery of the conservation of the homeobox between flies and frogs (Carrasco *et al.* 1984), the hybridization of *Drosophila* probes to *Xenopus* libraries under conditions of

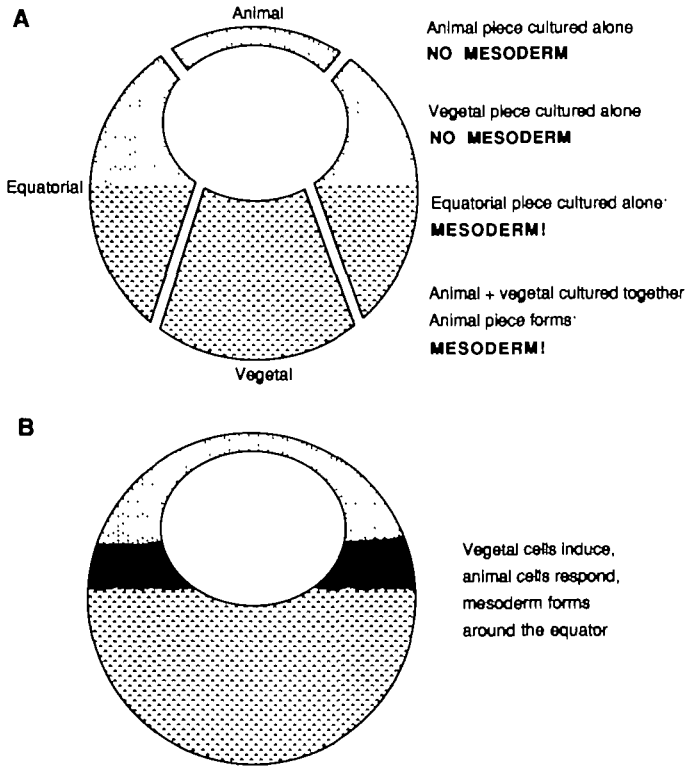


Fig. 1. The primary evidence for the origin of the mesoderm by induction. Diagrams of blastulae depicting the experiment of Nieuwkoop (1969; Sudarwati and Nieuwkoop, 1971). (A) The embryo is divided into animal, vegetal, and equatorial parts, each of which is cultured separately until the time of mesodermal differentiation. Mesoderm develops in the equatorial explant, which contains the cells normally fated to form it, but not in the animal or vegetal pieces. When animal and vegetal tissue are combined and cultured for the same period, mesoderm forms from the animal cells. (B) It is deduced that vegetal cells normally induce overlying animal hemisphere cells to form the mesoderm in the equator of the embryo.

reduced stringency has allowed vertebrate embryologists into the well-stocked larder of the *Drosophila* geneticists. Several close and distant relatives of genes that regulate development in *Drosophila* have now been cloned from the genome of the frog, *Xenopus laevis*. Such genes can provide useful molecular markers for the study of a vertebrate embryo that is particularly accessible to analysis and consequently well understood at the cellular and tissue levels of organization. *Xenopus* embryos also allow relatively straightforward testing of the function of cloned genes. Examples of this approach are the cloning and tests of function of the homeobox-containing genes, Xhox-1A and Xhox3 (Harvey and Melton, 1988; Ruiz i Altaba and Melton, 1989).

We have used low stringency library screening to isolate two *Xenopus* genes, belonging to the helix-loop-helix family of DNA-binding proteins (Murre *et al.* 1989), that are expressed early in mesodermal differentiation. One is a frog homologue of MyoD (Davis *et al.* 1987). We find that the early embryonic expression of *Xenopus* MyoD (XMyoD) is consistent with a role in initiating muscle differentiation in normal development, and go on to show that it can indeed activate muscle-specific gene expression in embryonic cells. The other is a relative of the *twist* gene of *Drosophila*, which is required for mesodermal differentiation in flies (Simpson, 1983; Nüsslein-Volhard *et al.* 1984). We show that this gene (Xtwi) is expressed widely in the early frog mesoderm, but not in the myotomes, where XMyoD is expressed.

XMyoD is expressed in the developing somites before contractile protein mRNAs first appear

MyoD is a mouse cDNA that, when transfected into cultured fibroblasts, can convert them into myoblasts (Davis *et al.* 1987). It encodes a helix-loop-helix DNA-binding protein that can interact with promoter elements required for the transcription of several muscle genes (Lassar *et al.* 1989). A *Xenopus* cDNA was cloned which encodes a protein that shares 73 % of its amino acid residues with MyoD (Hopwood *et al.* 1989*a*; Harvey, 1990; Scales *et al.* 1990). We have used probes made from this XMyoD cDNA to study the pattern of transcription of XMyoD in early embryos. This has been compared to that of the cardiac actin gene, until now the earliest marker of muscle differentiation in *Xenopus* (Mohun *et al.* 1984). We have asked, in particular, if XMyoD shows a pattern of expression consistent with a role in the normal activation of such genes as the cardiac actin gene.

The location of XMyoD transcripts has been determined by *in situ* hybridization, and by Northern blotting of RNA from dissected embryo parts. A cardiac actin probe labels the myotomal cells of the somites, from which the axial musculature will develop, and a XMyoD probe labels the same region (Fig. 2A,B). A stronger case for the specificity of XMyoD expression was made by Northern analysis of tissues dissected from a post-neurula embryo: XMyoD transcripts, like cardiac actin RNA, are restricted to the somite fraction (Fig. 2C). Later in development the cardiac actin gene is also expressed in the developing heart, but we have not detected XMyoD transcripts in this tissue (Hopwood *et al.* 1989*a*). (The eventual specificities of cardiac and skeletal isoforms of α-actin for heart and skeletal muscle, respectively, are not established until adulthood [Mohun *et al.* 1984].) Thus, whilst the location of XMyoD transcripts is consistent with a role in muscle gene activation in the somites, another factor(s) must activate the same contractile protein genes in the early heart. All of the mammalian members of the family of myogenic factors related to MyoD are also skeletal muscle-specific (Sassoon *et al.* 1989; Braun *et al.* 1989; Rhodes and Konieczny, 1989; Braun *et al.* 1990).

Cardiac actin transcripts first accumulate in mid-gastrulae (st11 of Nieuwkoop and Faber, 1967) (Cascio and Gurdon, 1986). XMyoD RNA is detectable at a very low concentration in eggs and early embryos, the number of transcripts first rising in early (st10) gastrulae

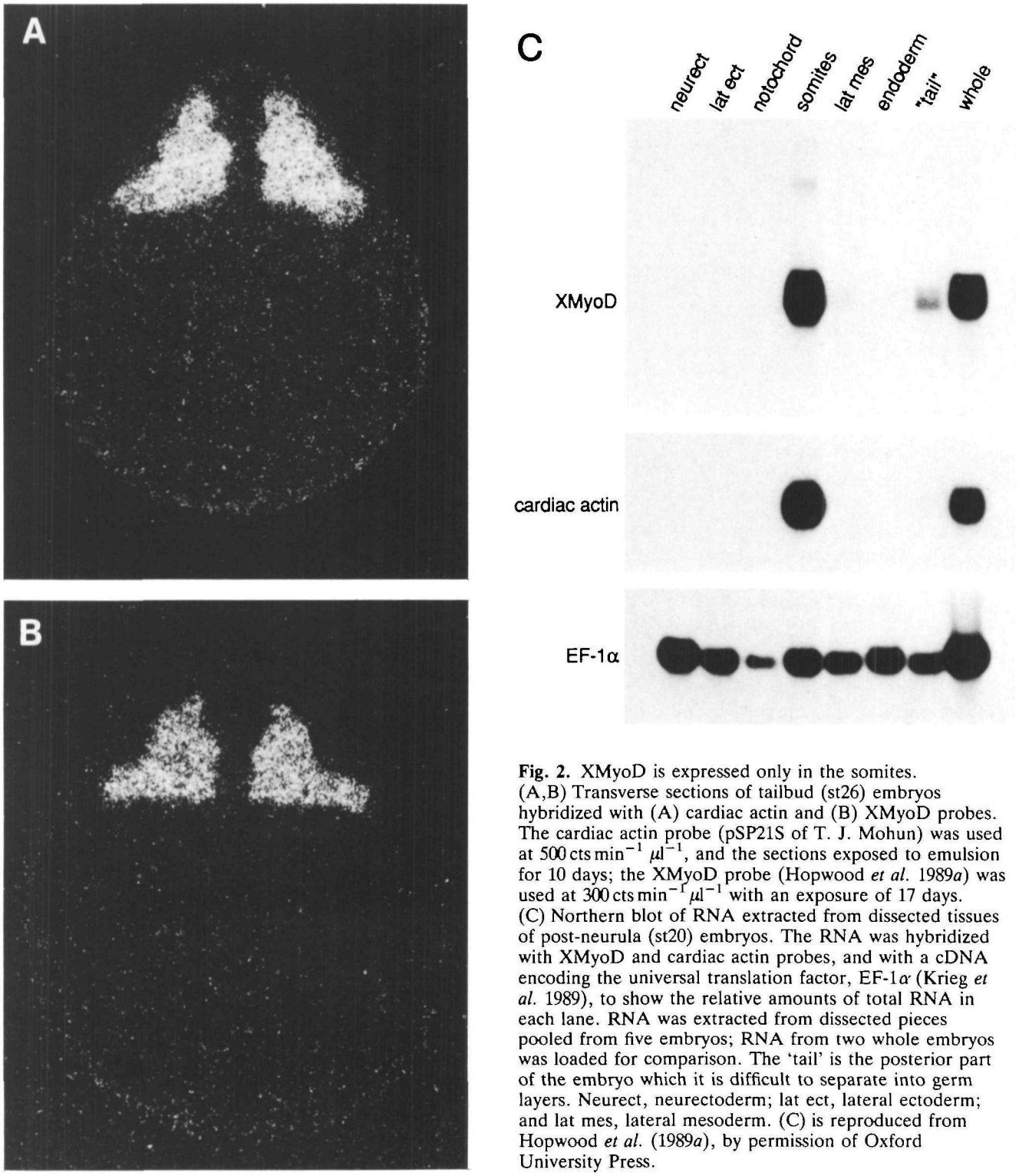


Fig. 2. XMyoD is expressed only in the somites. (A,B) Transverse sections of tailbud (st26) embryos hybridized with (A) cardiac actin and (B) XMyoD probes. The cardiac actin probe (pSP21S of T. J. Mohun) was used at 500 cts min^{-1} μl^{-1}, and the sections exposed to emulsion for 10 days; the XMyoD probe (Hopwood *et al.* 1989*a*) was used at 300 cts min^{-1} μl^{-1} with an exposure of 17 days. (C) Northern blot of RNA extracted from dissected tissues of post-neurula (st20) embryos. The RNA was hybridized with XMyoD and cardiac actin probes, and with a cDNA encoding the universal translation factor, EF-1α (Krieg *et al.* 1989), to show the relative amounts of total RNA in each lane. RNA was extracted from dissected pieces pooled from five embryos; RNA from two whole embryos was loaded for comparison. The 'tail' is the posterior part of the embryo which it is difficult to separate into germ layers. Neurect, neurectoderm; lat ect, lateral ectoderm; and lat mes, lateral mesoderm. (C) is reproduced from Hopwood *et al.* (1989*a*), by permission of Oxford University Press.

to reach a peak in late neurulae, before declining in later development. By measuring absolute numbers of transcripts from the two genes, we found that the rise in the XMyoD transcript level genuinely preceded the appearance of cardiac actin RNA by one to two hours (Fig. 3). This would be enough time for XMyoD protein to be made, enter nuclei, and activate cardiac actin transcription, and for mature cardiac actin transcripts to accumulate. In contrast, in mouse embryos, myogenin and cardiac actin RNAs were first detected by *in situ* hybridization at 8.5 days *post coitum*, but MyoD transcripts were not detectable until two days later (Sassoon *et al.* 1989). It therefore seems unlikely that MyoD could activate the cardiac actin gene in the somites of mouse embryos. However, transcripts encoding a quail relative of MyoD, qmf1,

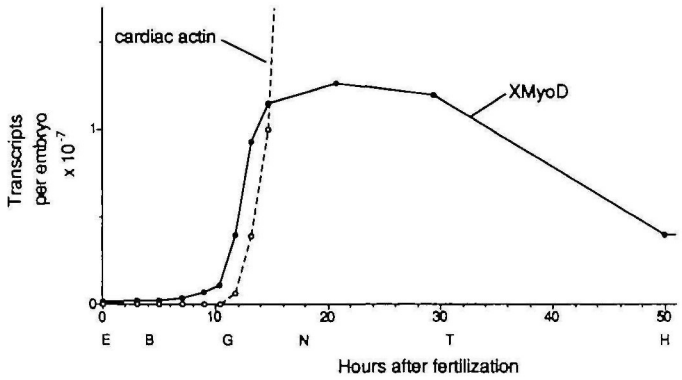


Fig. 3. XMyoD RNA accumulates before the onset of transcription of the cardiac actin gene. Graph of numbers of XMyoD and cardiac actin transcripts per embryo through development based on densitometry of appropriately exposed autoradiographs of a Northern blot. E, egg; B, blastula; G, gastrula; N, neurula; T, tailbud; and H, heartbeat. For further details see Hopwood *et al.* (1989*a*), from which this figure is reproduced by permission of Oxford University Press.

first appear at about the same time as cardiac actin RNA (de la Brousse and Emerson, 1990). In the amniotes, therefore, the necessary temporal sequence of myogenic factor expression followed by the appearance of contractile protein mRNAs has yet to be established.

XMyoD can activate muscle-specific gene expression in embryo cells

The foregoing description of XMyoD expression showed that XMyoD RNA is present in the right place at the right time to play a role in activating muscle-specific gene expression in the somites. MyoD can activate muscle genes in a wide variety of cells in culture, although even in the successful cases the frequency of activation has often been low (Davis *et al.* 1987; Weintraub *et al.* 1989; Schäfer *et al.* 1990). We have asked if XMyoD can activate muscle genes in embryonic cells at the time when they are normally being allocated to the muscle lineage or to other pathways of differentiation. We expressed XMyoD ectopically by microinjection of synthetic mRNA into early embryos (Gurdon *et al.* 1974; Woodland *et al.* 1974; Andrews and Brown, 1987), a technique that has been used to create widespread high levels of several mRNAs which are normally spatially restricted (Harvey and Melton, 1988; Kintner, 1988; McMahon and Moon, 1989; Ruiz i Altaba and Melton, 1989; Detrick *et al.* 1990). A major concern in interpreting the results of experiments of this kind is to distinguish between specific and non-specific effects, the latter often including reproducible syndromes such as abnormal gastrulation leading to microcephaly. A stringent negative control is the injection of transcripts identical to the test RNA but containing a single point mutation that alters an amino acid known or predicted to be

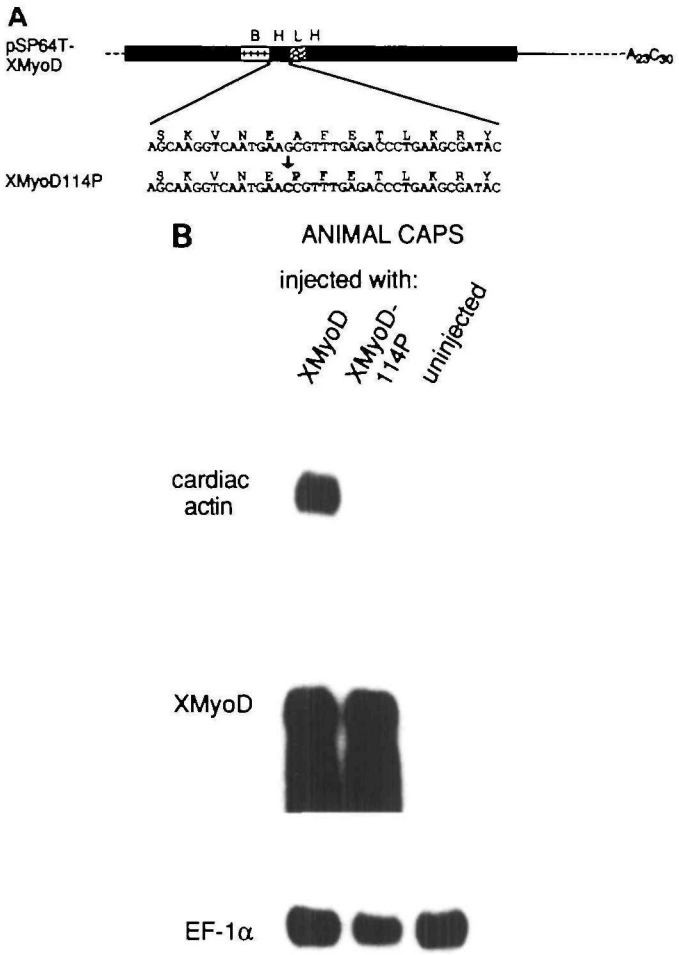


Fig. 4. XMyoD can activate cardiac actin transcription. (A) Diagram of the pSP64T-XMyoD clone used for production of synthetic XMyoD mRNA and the XMyoD114P point mutant, which was used as a control for the non-specific effects of RNA injection. (B) Animal caps were removed from late blastulae (st9) and cultured until beyond the time of normal activation of the cardiac actin gene in the myotomes (st18). Northern blotting shows that animal caps from embryos injected with the XMyoD114P mutant RNA, like uninjected animal caps, contain no cardiac actin RNA, but that the gene has been activated strongly in XMyoD-injected animal caps. Re-probing the blot for XMyoD shows that XMyoD- and XMyoD114P-injected animal cap samples contain similar amounts of surviving injected RNA; re-hybridizing with an EF-1α probe shows that the samples contain similar amounts of total RNA. For further details see Hopwood and Gurdon (1990). BHLH, basic-helix-loop-helix region; $A_{23}C_{30}$, poly(dA)–poly(dC) tracts; dotted line, *Xenopus* β-globin untranslated regions.

essential for function of the protein. A control XMyoD RNA was made by mutating a G residue to a C, which changes alanine 114 of the XMyoD protein to a proline (XMyoD114P RNA) (Fig. 4A). While minimally changing the primary structure of the RNA, this would be expected to disrupt the first helix of the helix-loop-

helix domain, preventing dimerization and thus DNA binding (Davis *et al.* 1990).

We tested the effect of XMyoD RNA injection in animal cap cells (Hopwood and Gurdon, 1990), which normally give rise only to ectodermal derivatives. XMyoD or XMyoD114P RNA was injected into two-cell embryos; the animal caps were separated at the late blastula stage (st9) and then cultured until after the time of normal activation of the cardiac actin gene in the somites. Northern blotting showed that this gene was activated in the animal caps from XMyoD-injected embryos, but not in controls injected with the mutant RNA (Fig. 4B). XMyoD could also activate the skeletal actin gene (data not shown) and desmin synthesis (N. D. Hopwood, A. Pluck and J. B. Gurdon, in preparation). It can thus bypass mesoderm-inducing factors and activate muscle-specific gene expression in animal cap cells. XMyoD is likely to act directly on the cardiac actin promoter. It has been shown that MyoD can bind to sites in the promoters of muscle genes (Lassar *et al.* 1989), and we note that, in addition to a CArG sequence that is essential for transcription (Mohun *et al.* 1989), there are potential XMyoD-binding sites located further upstream in the *Xenopus* cardiac actin promoter (M. V. Taylor, N. D. Hopwood, and T. J. Mohun, unpublished data).

We have shown that the cardiac actin gene was activated in animal caps of XMyoD-injected embryos to an extent similar to its normal activation in the myotomes of early neurulae (Hopwood and Gurdon, 1990). However, transcription of the cardiac actin gene was not sustained, the 12/101 muscle-specific antigen that is normally activated later in myogenesis (Kintner and Brockes, 1984) did not accumulate, the animal caps did not form muscle, and whole XMyoD-injected embryos also developed substantially normally. This could be because a high enough concentration of XMyoD protein was not maintained, as the injected RNA was progressively degraded. However, we did create a concentration of XMyoD RNA in the XMyoD-injected animal caps several-fold greater than that in normal myotomal cells of late neurulae, and note that the XMyoD RNA concentration *normally* falls significantly once somites have formed (Hopwood *et al.* 1989*a*).

A more likely explanation for the failure to differentiate muscle is that other specific factors are required to co-operate with XMyoD to activate the full myogenic programme. Other members of the MyoD family are potential co-operators, but since these myogenic factors can activate each other's synthesis, it is possible that their expression would in fact be activated in XMyoD-injected animal caps. If so, they are clearly insufficient to cause muscle differentiation. Two other factors have been shown to be able to convert cultured fibroblasts into myoblasts, but it is not yet 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 specific factor(s) 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 XMyoD itself, or a necessary partner of XMyoD, and so be a buffer against the effects of XMyoD in animal cap cells. Myogenesis might require the inactivation of negative regulators like Id.

We conclude that XMyoD is sufficient, together with components present in animal cap cells, to activate expression of several muscle genes, but not to cause complete myogenesis. The significance of this experiment is that it shows the ability of XMyoD to activate muscle genes in normal embryonic cells, at the time when muscle differentiation normally begins. The expression of cDNA clones in animal cap cells by microinjection of synthetic mRNAs, alone or in combination with XMyoD, should provide an effective assay for additional factors involved in myogenesis.

A *Xenopus* relative of the *twist* gene of *Drosophila*

The *twist* gene of *Drosophila* is required for mesodermal differentiation: *twist* mutant embryos lack mesoderm and internal organs (Simpson, 1983; Nüsslein-Volhard *et al.* 1984). *Twist* gene products accumulate in mesodermal (and some endodermal) cells as an early zygotic response to the activity of the *dorsal* group of maternal effect genes (Thisse *et al.* 1987, 1988). The *twist* protein is a member, like MyoD, of the helix-loop-helix family of DNA-binding proteins (Thisse *et al.* 1988; Murre *et al.* 1989).

We have used a probe made from the *twist* gene to isolate a related *Xenopus* cDNA, called Xtwi (Hopwood *et al.* 1989*b*). It encodes a protein of predicted M_r 18000, about one-third the size of *twist*, and related to its C-terminal region. The *twist* and Xtwi proteins are very similar in the putative DNA binding and dimerization domain and also share 11 out of a stretch of 14 amino acids near their C-termini; the rest of the proteins are substantially different. In view of the specific expression of Xtwi in the early mesoderm (see below), we believe that *twist* and Xtwi are probably homologues, although the possibilities cannot be excluded either that the two genes evolved independently, or that there is another *Xenopus* gene more similar to *twist* than Xtwi.

The expression of a gene related to *twist* in the mesoderm of a vertebrate encourages us to seek further similarities in the mesodermal differentiation of the two groups. It is already known that the *dorsal* protein, which is believed to activate the *twist* gene, shares a large domain with the avian proto-oncogene, c-*rel* (Steward, 1987). Transcripts that encode proteins related to *dorsal* are present in *Xenopus* embryos at the time of Xtwi gene activation (K. R. Kao and N. D. Hopwood, unpublished data). A putative *Xenopus* homologue of the *snail* gene (Boulay *et al.* 1987), which, like *twist*, is activated in the *Drosophila* zygote by *dorsal*

and required for development of ventral structures, is also expressed in early frog embryos (Sargent and Bennett, 1990).

Embryonic expression of Xtwi

Xtwi transcripts first accumulate in early gastrulae (st10½), about an hour later than zygotically transcribed XMyoD RNA appears (Hopwood *et al.* 1989*b*). We have used *in situ* hybridization and Northern blotting of dissected tissues to show that in early embryos Xtwi transcripts are present only in the mesoderm. Later in development, the Xtwi gene is also expressed in the neural crest, a transient structure in vertebrate embryos that gives rise to a wide variety of tissues, including the peripheral nervous system, adrenal medulla, pigment cells, and much of the muscle and connective tissue of the face and neck (reviewed by Le Douarin, 1982). Xtwi transcripts are restricted to the anterior mesoderm of early neurulae, presumably reflecting the earlier differentiation of anterior structures. Only by the tailbud stage are they found in the most posterior regions.

The three major divisions of the early mesoderm are the notochord, or embryonic backbone, the somites, and the lateral plate, the last of which gives rise, among a variety of other structures, to the heart, and smooth muscle of the gut. The somites consist of three parts: the myotomes, from which axial muscles develop; the sclerotome, which gives rise to skeleton; and the dermatome, which contributes the connective tissue of the skin. The Xtwi gene is expressed in the notochord (anterior half of the embryo) and lateral plate mesoderm (anterior quarter) of early (st14) neurulae (Fig. 5). There is also Xtwi RNA anterior to the somites in what may be prechordal mesoderm. However, we have detected no Xtwi transcripts in the somitic cells, which express muscle markers such as cardiac actin and XMyoD. This lack of Xtwi expression in the myotomes contrasts to its otherwise widespread expression in the differentiating mesoderm and is the most striking feature of its pattern of transcript accumulation. Although we do not yet know the location of Xtwi transcripts in gastrulae, it is unlikely that they are present in the prospective somites of gastrulating embryos. This is because the anterior–posterior progression in the onset of Xtwi expression allows us to examine the more posterior parts of the embryo as they *begin* to express Xtwi; in doing so we have never seen any Xtwi transcripts in the myotomes. Indeed, myotomal differentiation, as indicated by transcription of the cardiac actin gene, has been established throughout the somites of the trunk many hours before Xtwi RNA is first detectable in the posterior half of the embryo (Hopwood *et al.* 1989*a*). This suggests that Xtwi is unlikely to function primarily to define mesodermal cells as non-myotomal, since myotomal differentiation appears, as judged by the expression of myogenic factors and contractile proteins, to be specified positively at an earlier stage in the development of the mesoderm.

By the late tailbud stage (st28–30), Xtwi transcripts are still detectable in the notochord, particularly in posterior regions, and in anterior and posterior lateral plate mesoderm (Fig. 7). We have not seen, at the stages examined (up to st32), any Xtwi expression in lateral plate cells in the middle of the embryo. Instead, there is strong expression around the notochord, which probably corresponds to mesenchymal cells derived from the sclerotomes. Expression becomes more ventral and lateral posteriorly. Since Xtwi is itself the most general marker of non-muscle mesoderm in *Xenopus*, and no specific, early markers are available for mesoderm outside the myotomes and notochord, it is difficult to assess either the degree of generality or the meaning of the specific and evolving pattern of Xtwi expression. However, it is possible that all non-myotomal mesodermal cells express the gene at some

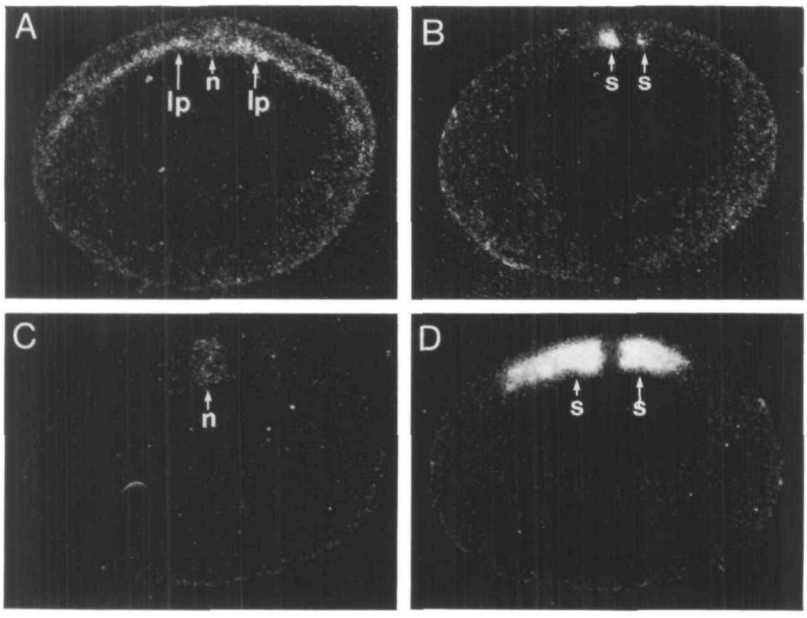
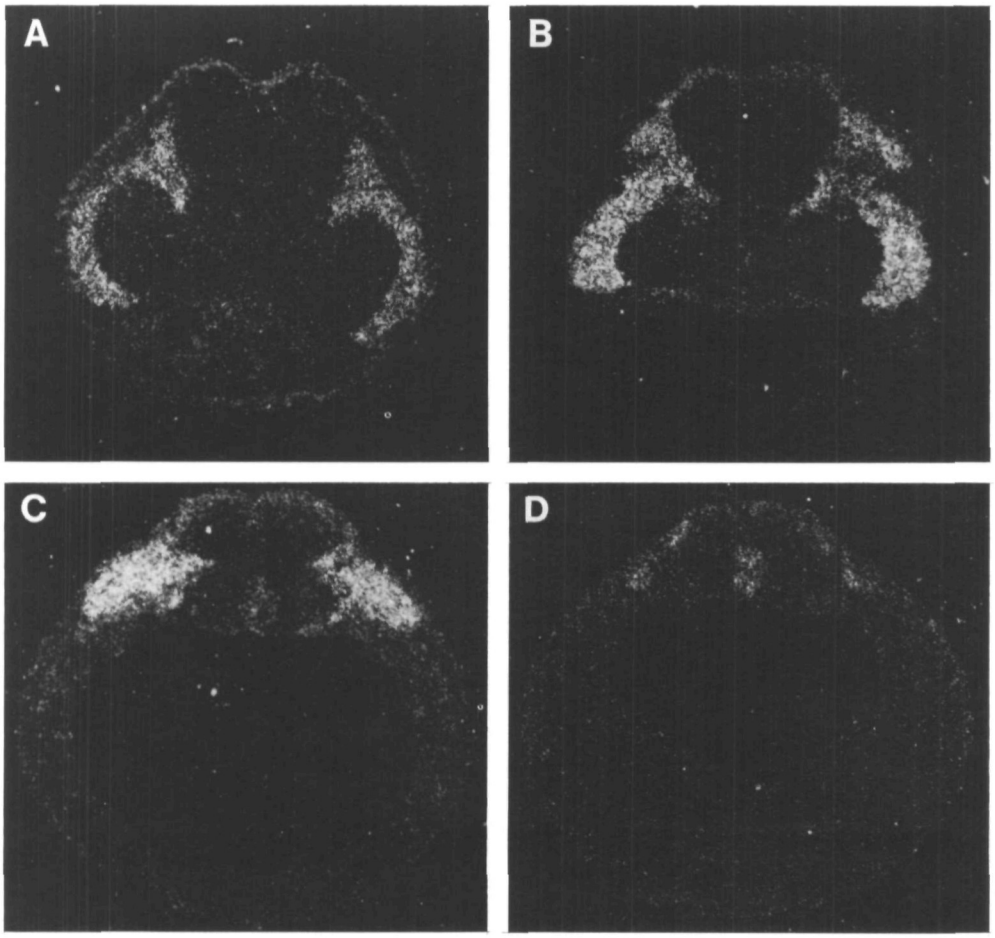


Fig. 5. Xtwi expression in midneurulae. Transverse sections of st14–16 embryos hybridized with Xtwi (A and C) and cardiac actin (B and D) probes. A and B are sections about a quarter of the way through the embryo from the anterior end, at the level of the most anterior somites. In this part of the embryo, Xtwi is expressed in the lateral plate (lp) and in the notochord (n), in contrast to cardiac actin, which is expressed only in the somites (s). Sections cut about halfway through the embryo show Xtwi expression restricted to the notochord (C) and cardiac actin in only the somites (D). Reprinted with the permission of Cell Press from Hopwood *et al.* (1989*b*).



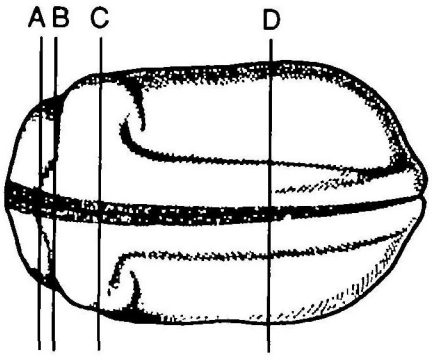


Fig. 6. Expression of Xtwi in post-neurulae. (A–D) Transverse sections cut at the positions shown in the diagram through a st20–21 embryo. There is strong labelling of what we believe to be migrating cephalic neural crest (A–C). The sections in C and D show notochordal expression of the Xtwi gene. The *in situ* hybridizations of Figs 6 and 7 were carried out as described by Kintner and Melton (1987) and Hopwood *et al.* (1989*a*,*b*), using the probe at 400 cts min^{-1} μl^{-1} and exposing for three weeks.

time, the pattern of expression reflecting the order of mesodermal cell differentiation.

We have previously described Xtwi expression in the anterior (cephalic) neural crest from mid-neurula stages onwards (Hopwood *et al.* 1989*b*). Fig. 6 shows strong labelling of anterior neural crest cells in a post-neurula embryo (st21). Having now examined slightly later embryos, we can report that it is also expressed in the neural crest along the length of the trunk (Fig. 7). It is difficult, in the absence of other early neural crest markers in the frog, to know what derivatives these Xtwi-expressing cells will form. However, we have not seen Xtwi transcripts in the cephalic ganglia, some of which are derived from the neural crest.

XMyoD and Xtwi: early responses to embryonic induction

As expected from the mesodermal location of XMyoD and Xtwi transcripts, these genes are activated following mesoderm induction. We carried out an experiment based on Nieuwkoop's (see Introduction), in which blastulae were dissected into animal, equatorial, and vegetal parts, which were cultured separately until sibling embryos became late (st12½) gastrulae. Animal and vegetal pieces were also cultured in combination for the same period. No XMyoD or Xtwi transcripts were present in the animal or vegetal pieces cultured alone (the maternal contribution of XMyoD RNA

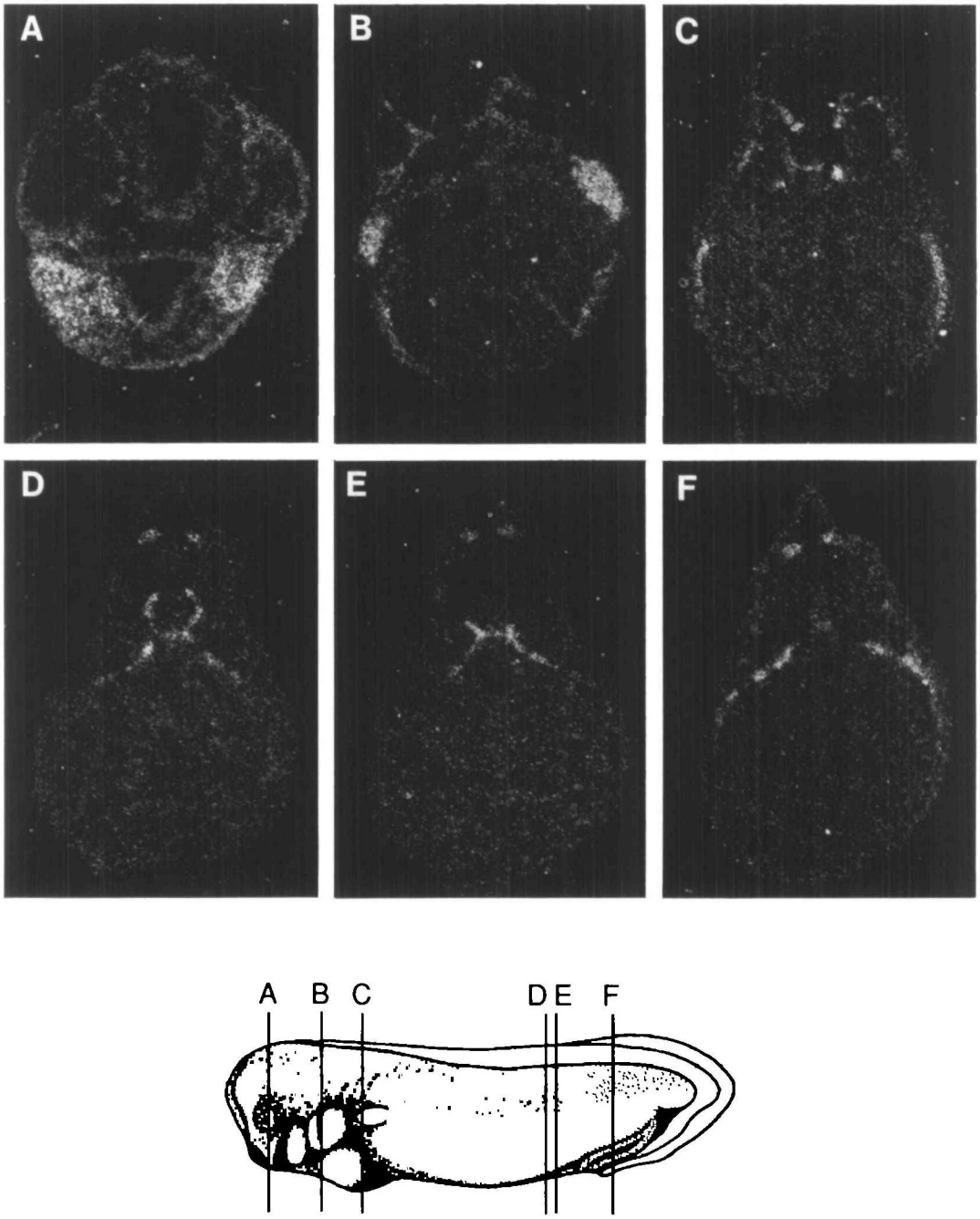


Fig. 7. Expression of Xtwi in a st29–30 embryo. (A–F) Transverse sections cut at the positions shown in the diagram through a st29–30 embryo. (A) shows labelling of cells in the head that are probably largely derived from the neural crest cells seen in Fig. 6. B–E show different patterns of labelling of tissue around the notochord and somites. The labelled cells are probably derived mostly from the sclerotome of the somites. More lateral mesodermal cells are labelled in the anterior trunk and in the posterior part of the embryo (C, F). C–F show labelling of two patches of cells at the dorsal aspect of the neural tube, which are likely to be neural crest cells beginning to migrate laterally away from the neural tube.

having been degraded), but transcription of XMyoD and Xtwi genes was activated in the animal:vegetal conjugates (Hopwood *et al.* 1989*a*; Fig. 8A). Taken together with knowledge of the location of XMyoD and Xtwi transcripts in normal development, this shows that the genes are activated following mesoderm induction.

The neural crest arises at the lateral margins of the neural plate as a consequence of its induction by the mesoderm. In order to test the responsiveness of the Xtwi gene to neural induction, tissue recombinates were made between inducing gastrula mesoderm and responsive gastrula ectoderm. After culture until sibling embryos reached the tailbud stage, the mesoderm was removed from the conjugates. The induced ectoderm, but not control uninduced ectoderm, contained Xtwi transcripts (Fig. 8B), which indicates that Xtwi ex-

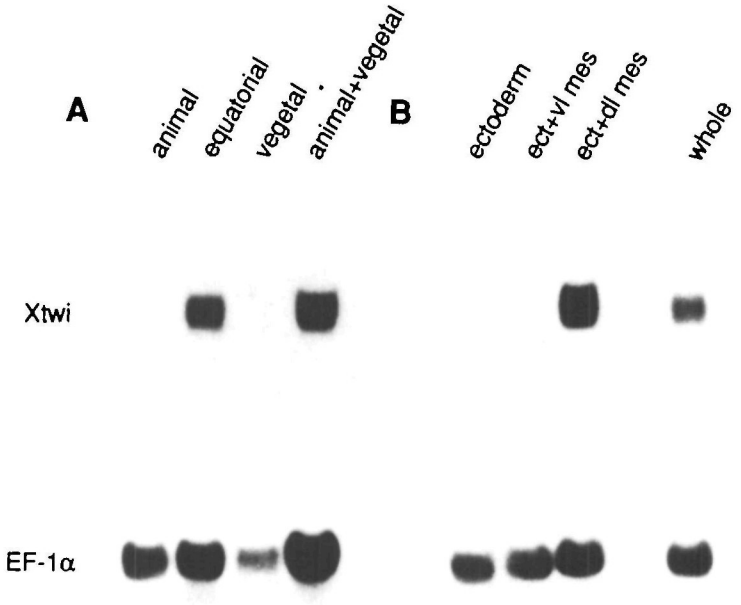


Fig. 8. Xtwi expression is activated in response to two successive inductions. Northern blots probed with Xtwi and EF-1α probes. (A) St8½ blastulae were dissected into animal, equatorial and vegetal pieces and these cultured to control st12½. Animal and vegetal pieces were also cultured in combination. (B) Early gastrula ectoderm (ect) was cultured alone or in combination with mesoderm (mes) from the dorsal (dl) or ventral (vl) sides of mid-gastrulae until control embryos reached the tailbud stage. The mesoderm was then removed before analysis of the ectoderm fragments. Xtwi expression, like that of genes expressed in the neural plate, is induced by dorsal, but not by ventral mesoderm. In other experiments, pieces of induced ectoderm similar to those assayed here were found to be free of cardiac actin transcripts (C. R. Sharpe and J. B. Gurdon, unpublished data). RNA from anterior ectoderm dissected from five embryos was loaded in each lane; a sample of RNA from one whole embryo was loaded for comparison. Reproduced, with the permission of Cell Press, from Hopwood *et al.* (1989*b*).

pression is activated in the neural crest following neural induction. First commencing in mid-neurulae (st14–15), transcription of the Xtwi gene is the earliest specific activity of the amphibian neural crest so far described. We conclude that Xtwi is activated in response to two successive early inductions.

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