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Patterning of the mesoderm in *Xenopus*: dose-dependent and synergistic effects of *Brachyury* and *Pintallavis*

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SUMMARY

Widespread expression of the DNA-binding protein Brachyury in *Xenopus* animal caps causes ectopic mesoderm formation. In this paper, we first show that two types of mesoderm are induced by different concentrations of Brachyury. Animal pole explants from embryos injected with low doses of *Xbra* RNA differentiate into vesicles containing mesothelial smooth muscle and mesenchyme. At higher concentrations somitic muscle is formed. The transition from smooth muscle formation to that of somitic muscle occurs over a two-fold increase in Brachyury concentration.

***Brachyury* is required for differentiation of notochord in mouse and fish embryos, but even the highest concentrations of *Brachyury* do not induce this tissue in *Xenopus* animal caps. Co-expression of *Brachyury* with the secreted glycoprotein noggin does cause notochord formation, but it is difficult to understand the molecular basis of this phenomenon without knowing more about the noggin signal transduction pathway. To overcome this difficulty, we have now tested mesoderm-specific transcription factors for the ability to synergize with *Brachyury*. We find that co-expression of *Pintallavis*, but not *goosecoid*, with *Brachyury* causes formation of dorsal mesoderm, including notochord. Furthermore, the effect of *Pintallavis*, like that of *Brachyury*, is dose-dependent: a two-fold increase in *Pintallavis* RNA causes a transition from ventral mesoderm formation to that of muscle, and a further two-fold increase induces notochord and neural tissue. These results suggest that *Pintallavis* cooperates with *Brachyury* to pattern the mesoderm in *Xenopus*.**

Key words: *Xenopus*, *Brachyury*, *Pintallavis*, *goosecoid*, mesoderm induction, thresholds

INTRODUCTION

Induction of the mesoderm in amphibian embryos is thought to be initiated by members of the FGF and TGF-β families of polypeptide growth factors (Kimelman and Kirschner, 1987; Slack et al., 1987; Asashima et al., 1990; Smith et al., 1990; Thomsen et al., 1990; van den Eijnden-van Raaij et al., 1990; Dale et al., 1992, 1993; Jones et al., 1992; Thomsen and Melton, 1993; reviewed by Smith, 1993; Slack, 1994). Whilst members of the FGF family induce mesoderm of predominantly ventral character in animal pole explants, TGF-β-related molecules such as activin A and the processed form of Vg1 can induce a range of dorsal mesodermal tissues. The type of dorsal mesoderm induced by activin is highly sensitive to the concentration of activin experienced by the responding cells, with increasing concentrations of activin-inducing mesoderm of progressively more anterior character (Green and Smith, 1990; Green et al., 1990, 1992, 1994; Symes et al., 1994; Wilson and Melton, 1994).

Activin induces the expression of a number of genes in an immediate-early fashion; that is, in the absence of protein synthesis (see Tadano et al., 1993). Genes of this class include *Xenopus Brachyury* (*Xbra*), which is expressed throughout the presumptive mesoderm (Smith et al., 1991), as well as several genes whose expression is restricted to the dorsal marginal zone. These include *goosecoid* (Cho et al., 1991), *Xnot* (von Dassow et al., 1993), *Xlim-1* (Taira et al., 1992) and fork head/HNF3-related genes such as *Pintallavis*/XFD-1 (Ruiz i Altaba and Jessell, 1992; Knöchel et al., 1992) and XFKH1/XFD-1′ (Dirksen and Jamrich, 1992; Knöchel et al., 1992). It is possible that these genes, all of which are putative transcription factors, act as determinants or modifiers of mesodermal fate and, indeed, we have previously shown that ectopic expression of *Xbra* in animal pole explants is sufficient to cause formation of ventral mesoderm (Cunliffe and Smith, 1992).

In this paper, we first examine in detail the responses of animal pole cells to different concentrations of Xbra. We find that increasing amounts of this factor cause the formation of two different types of mesoderm. At low doses, explants differentiate into vesicles containing mesothelium, mesenchyme and smooth muscle. At higher concentrations somitic muscle is formed. The transition from smooth muscle formation to that of somitic muscle occurs over a two-fold increase in Brachyury concentration.

Although *Brachyury* is expressed at high levels in the notochord, and is required for differentiation of notochord in mouse and fish embryos (Chesley, 1935; Herrmann et al., 1990;

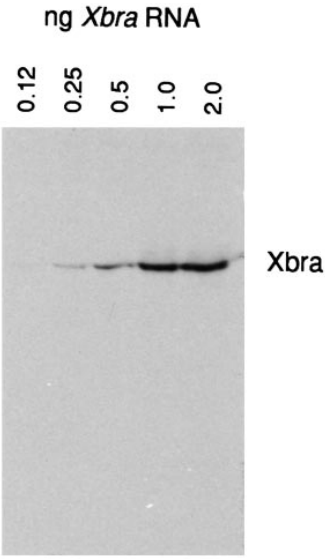

Fig. 1. Injection of increasing amounts of *Xbra* RNA does not saturate the translation machinery. The indicated quantities of *Xbra* RNA, together with 1 mCi/ml [^{35}S]methionine, were injected into *Xenopus* embryos at the 1-cell stage. Animal caps were dissected at the mid-blastula stage and cultured in 75% NAM to control stage 12, when cellular proteins were subjected to immunoprecipitation using an anti-*Xbra* antiserum. Immunoprecipitates were analyzed by polyacrylamide gel electrophoresis followed by fluorography.

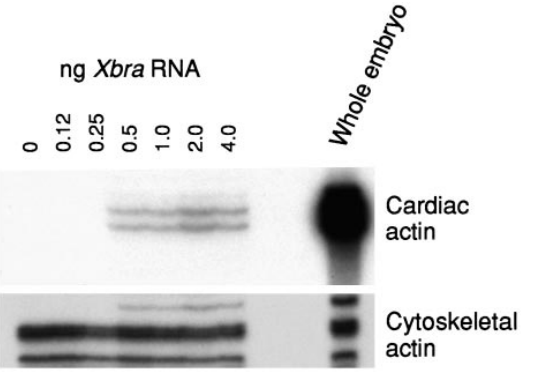

Fig. 2. Activation of muscle-specific cardiac actin genes requires a threshold quantity of injected *Xbra* RNA. Embryos at the 1-cell stage were injected with the indicated quantities of *Xbra* RNA. Animal caps were dissected from these embryos at the mid-blastula stage and cultured to stage 17/18. RNA was analyzed for expression of cardiac (muscle-specific) actin by RNAase protection.

Schulte-Merker et al., 1994a), even the highest concentrations of Xbra do not induce notochord to form in *Xenopus* animal caps. This suggests that notochord differentiation requires additional gene products, and we showed recently that co-expression of *Xbra* with the secreted glycoprotein noggin, which is expressed in presumptive notochord (Smith and Harland, 1992), indeed results in notochord formation (Cunliffe and Smith, 1994). Unfortunately, nothing is known about the molecular basis of this synergy, not least because nothing is known about the noggin signal transduction pathway. One possibility, however, is that synergy occurs through interactions between *Xbra* and one or more of the dorsally expressed immediate-early transcription factors described above. In this paper, we show that co-expression of *Pintallavis*, but not *goosecoid*, with *Xbra* causes formation of dorsal mesoderm, including notochord. The effect of *Pintallavis*, like that of *Xbra*, is dose-dependent: a two-fold increase in *Pintallavis* RNA causes a transition from ventral mesoderm formation to that of muscle, and a further two-fold increase induces notochord and neural tissue. As originally suggested by Ruiz i Altaba and Jessell (1992), these results indicate that *Pintallavis* cooperates with *Xbra* in a dose-dependent fashion to pattern the mesoderm in *Xenopus*.

MATERIALS AND METHODS

Xenopus embryos, microinjection and dissections

Xenopus embryos were obtained by artificial fertilisation as described by Smith and Slack (1983). They were dejellied with cysteine hydrochloride (pH 8.0-8.2) and staged according to Nieuwkoop and Faber (1967). Embryos were injected essentially as described previously (Cunliffe and Smith, 1992). During and after injection, embryos were cultured in 75% normal amphibian medium (NAM: Slack, 1984) containing 4% Ficoll 400. For animal cap assays, the embryos were transferred to 75% NAM at stage 8 (mid-blastula) and animal caps were dissected using fine watchmaker's forceps. Animal caps were cultured in 75% NAM. Some intact embryos were allowed to continue development to tailbud stages and, for these, the medium was adjusted to 10% NAM containing 4% Ficoll 400 by stage 7 and to 10% NAM alone by stage 12.

Table 1. Morphology of animal caps derived from embryos injected with increasing quantities of *Xbra* RNA

Xbra RNA (ng)	Uninduced	Ventral vesicles	Yolky masses	Total
0	18	–	–	18
0.12	10	19	–	29
0.25	1	31	–	32
0.5	–	16	16	32
1.0	–	10	20	30
2.0	–	1	17	18
4.0	–	4	27	31

In vitro transcription

DNA templates used for in vitro transcription were as follows:
(1) Zebrafish *no tail* (*ntl*: Schulte-Merker et al., 1994a) was inserted into the vector pSP64T (Krieg and Melton, 1984) to generate pSP64T-*ntl*.
(2) Zebrafish *goosecoid* (Schulte-Merker et al., 1994b) was inserted into the vector pSP64T to generate pSP64T-zf*gsc*.
(3) pSP64T-*Pintallavis* was the kind gift of Ariel Ruiz i Altaba (see Ruiz i Altaba and Jessell, 1992).
(4) pSP64T-*Xbra* is described by Cunliffe and Smith (1992).
(5) nogginΔ5′ was the kind gift of Richard Harland (see Smith and Harland, 1992).
In vitro transcription was according to Cunliffe and Smith (1992). RNA was precipitated and washed with 70% ethanol three times, then resuspended in DEPC-treated water.

Immunoprecipitation studies

Embryos were injected at the 1- to 2-cell stage with 8 nl of a solution

Table 2. Expression of α-SM actin and 12/101 antigens by animal caps injected with increasing quantities of *Xbra* RNA

Xbra RNA (ng)	Antibody staining	
	α-SM actin	12/101
0	–	–
0.12	+	–
0.25	+	–
0.5	+	–
1.0	+	–
2.0	–	+
4.0	–	+

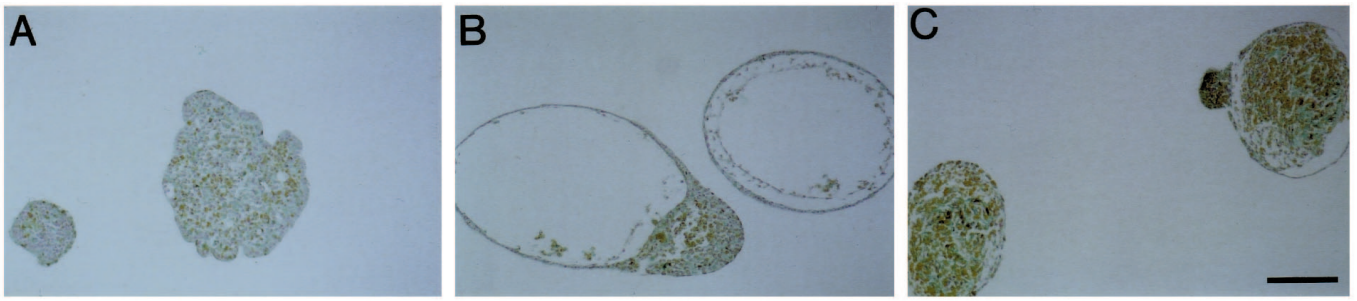

Fig. 3. Histological analysis of animal caps derived from embryos injected with increasing quantities of *Xbra* RNA. Embryos at the 1-cell stage were injected with increasing amounts of *Xbra* RNA. Animal caps were dissected from these embryos at the mid-blastula stage and cultured to stage 42 when they were fixed, sectioned at 7 μm and stained by the Feulgen technique and with Light Green and Orange G. (A) An uninjected animal cap forms atypical epidermis. (B) Animal caps from embryos injected with 0.5 ng *Xbra* RNA. This ventral tissue is typical of caps derived from embryos injected with 0.12 ng to 1 ng *Xbra* RNA. (C) Animal caps from embryos injected with 4 ng *Xbra* RNA. This muscle-like tissue is typical of caps from embryos injected with 1 ng to 4 ng *Xbra* RNA. Scale bar in C is 100 μm, and also applies to A and B.

containing *Xbra* RNA and [^{35}S]methionine (1 mCi/ml) in water. Animal caps were dissected at stage 8 and cultured to stage 12, when they were collected and subjected to immunoprecipitation using an antiserum directed against Xbra, as described by Cunliffe and Smith (1994).

RNAase protection assays

RNAase protections were carried out essentially as described by Cho and De Robertis (1990). The cardiac actin probe pSP21 is described by Mohun et al. (1984). The *Pintallavis* probe was made by subcloning an 830 bp *Xba*I/*Pst*I fragment of *Pintallavis* cDNA (Ruiz i Altaba and Jessell, 1992) into Bluescript KS II to generate pBS-*Pintallavis*. For RNAase protections the plasmid was linearised with *Bgl*II and transcribed with T3 RNA polymerase to give a probe size of 385 nucleotides and protected fragments of approximately 350 nucleotides.

Histological procedures

Specimens were fixed, sectioned and stained with a combination of Feulgen, Light Green and Orange G as described by Green et al. (1990).

Immunostaining

Immunostaining was carried out essentially as described by Cunliffe and Smith (1994). Primary antibodies were 12/101 (Kintner and Brockes, 1984), which is specific for *Xenopus* somitic muscle, and α-SM actin, an anti-α smooth muscle actin mouse monoclonal antibody, which recognises mesothelial cells of the ventral mesoderm and the smooth muscle of the gut (Saint-Jeannet et al., 1992). The second antibody in both cases was FITC-conjugated goat anti-mouse IgG.

In situ hybridisation and densitometric analysis

A *Pintallavis* in situ hybridization probe was prepared by linearising

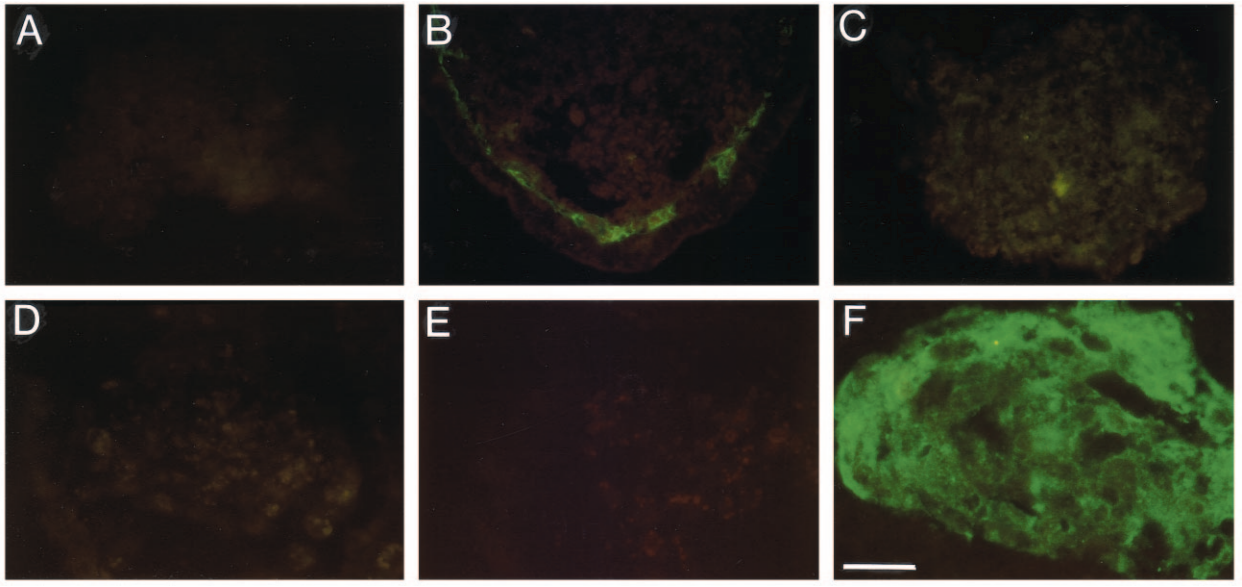

Fig. 4. Immunohistochemical analysis of animal caps injected with increasing quantities of *Xbra* RNA. α-smooth muscle actin and somitic muscle were defined with monoclonal antibodies α-SM actin (Saint-Jeannet et al., 1992) and 12/101 (Kintner and Brockes, 1984), respectively. Embryos at the 1-cell stage were injected with increasing amounts of *Xbra* RNA. Animal caps were dissected from these embryos at the mid-blastula stage and cultured to stage 47, when they were fixed and embedded in acrylamide prior to sectioning at 10 μm (see Cunliffe and Smith, 1994). Sections were immunostained with α-SM actin (A-C) and 12/101 (D-F), and a FITC-conjugated goat anti-mouse secondary antibody. (A,D) Uninjected animal caps. (B,E) Animal caps from embryos injected with 0.5 ng *Xbra* RNA. (C,F) Animal caps from embryos injected with 4 ng *Xbra* RNA. Results are summarised in Table 2. Scale bar in F is 50 μm, and also applies to A to E.

pBS-*Pintallavis* with *Xba*I. Digoxigenin-labelled antisense RNA was synthesized by transcribing with T3 RNA polymerase. Whole-mount in situ hybridization to albino *Xenopus* embryos followed the protocol of Harland (1991) except that the substrate used for the chromogenic reaction was Boehringer-Mannheim Purple AP Substrate. For densitometric analysis of the distribution of *Pintallavis* RNA, labelled embryos were embedded in paraffin wax and sectioned. Colour images of 10 μm sections were saved as Adobe Photoshop files using the ColourVision package (ImproVision UK). These were converted to greyscale images using Adobe Photoshop, and increasing densitometric values of grey were reassigned false colours.

RESULTS

Increasing quantities of injected *Xbra* RNA do not saturate the translational machinery of animal caps and reveal a threshold quantity of Xbra protein required for muscle-specific actin gene activation

Embryos were injected in the animal hemisphere at the 1-cell stage with serial two-fold dilutions of *Xbra* RNA and [^{35}S]methionine in order to discover whether there was a strict correspondence between the quantity of RNA injected and the amount of newly synthesised Xbra protein. Stage 8 animal caps were dissected from injected embryos and cultured to stage 12, whereupon proteins were extracted and Xbra polypeptides immunoprecipitated. Fig. 1 shows that injection of increasing quantities of injected *Xbra* RNA results in increasing amounts of newly synthesised Xbra protein, indicating that over the range of RNA concentrations used the RNA translation machinery is not limiting the rate of translation; it is instead the amount of injected RNA that determines the yield of newly synthesised protein.

Injected animal caps were dissected at blastula stages and cultured to control stage 17-18, when they were collected and analyzed for expression of actin genes by RNAase protection. Fig. 2 shows that, whilst injections of neither 0.12 ng nor 0.25 ng *Xbra* RNA elicited muscle-specific actin gene expression, quantities between 0.5 ng and 4 ng were equally potent in inducing muscle-specific actin RNA, revealing a sharp threshold for activation of muscle-specific actin between 0.25 ng and 0.5 ng. The location of the threshold in this experiment contrasts with previous results (Cunliffe and Smith, 1992) and with data shown in Tables 1 and 2, where the sharp threshold appears at slightly lower concentrations of injected RNA. This variability may be due to variation in the sizes of eggs, to variation in injection volume from experiment to experiment, and to differences in specific activities of RNA preparations. Nevertheless, in confirmation of our previous observations, the threshold for activation of muscle actin genes is sharp and the level of gene activation at the threshold RNA quantity is maximal (Cunliffe and Smith, 1992). We note, however, that this maximal level of muscle gene activation is low compared with levels of actin gene expression in whole embryos (see Fig. 2), in activin-treated animal caps (not shown) and animal caps co-expressing *Xbra* and *noggin* (Cunliffe and Smith, 1994).

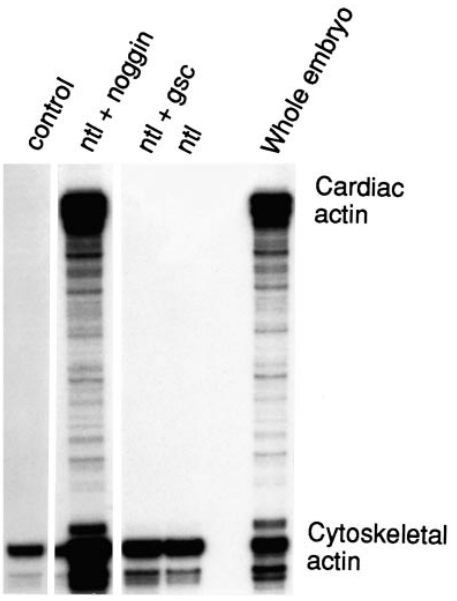

Fig. 5. Co-expression of zebrafish *goosecoid* and *ntl* RNAs does not induce cardiac (muscle-specific) actin expression in *Xenopus* animal caps. Embryos at the 1-cell stage were injected with RNA encoding zebrafish *goosecoid* (1 ng) and/or *ntl* (0.4 ng) and/or *Xenopus noggin* (100 pg) as indicated. Animal caps were dissected at the mid-blastula stage and cultured to stage 28, when they were analyzed for expression of actin genes by RNAase protection. 0.4 ng *ntl* did not induce expression of muscle-specific actin genes, but did cause formation of ventral mesoderm as judged by morphological and histological criteria (not shown). Co-expression of *noggin* and *ntl*, but not *goosecoid* and *ntl*, induces expression of muscle-specific actin RNA.

Mutually exclusive expression of mesothelial and somitic muscle markers in animal caps is caused by injection of low and high quantities of *Xbra* RNA

Xbra RNA-injected animal caps were cultured to stage 42 and subjected to histological analysis (Table 1). Injection of 0.12 to 0.5 ng *Xbra* RNA caused formation of ventral mesodermal vesicles containing mesothelium and mesenchyme, similar to those formed in response to FGF (see Green et al., 1990; Fig. 3B). When 1 ng and higher amounts were injected, most explants did not swell up but instead developed as a mass of

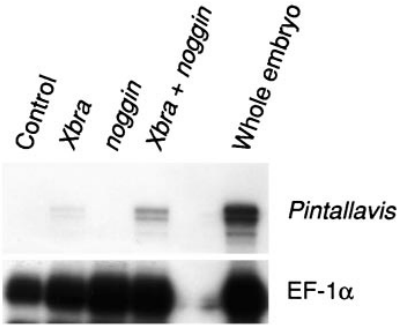

Fig. 6. *Xbra* and *noggin* act synergistically to induce expression of *Pintallavis*. Embryos at the 1-cell stage were injected with RNA encoding *Xbra* (1.0 ng) and/or *noggin* (200 pg) as indicated. Animal caps were dissected at the mid-blastula stage and cultured to stage 12, when they were analyzed for expression of *Pintallavis* by RNAase protection. *noggin* did not induce expression of *Pintallavis* and *Xbra* caused weak induction, but co-expression of the two genes elicited significant expression. This experiment was performed three times with similar results.

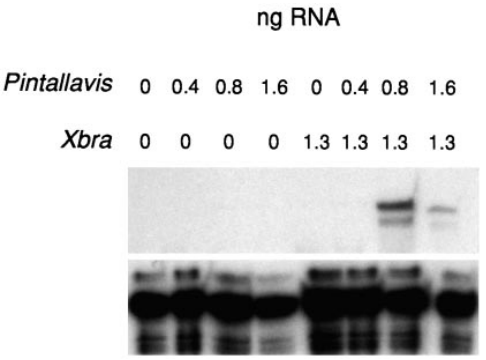

Fig. 7. Co-expression of *Pintallavis* and *Xbra* RNAs induces expression of muscle-specific actin RNA in *Xenopus* animal caps. Embryos were injected at the 1- to 2-cell stage with the indicated combinations of *Pintallavis* and *Xbra* RNAs. Animal caps were excised at the mid-blastula stage and cultured to stage 31 when they were analyzed by RNAase protection. Maximal expression of muscle-specific actin RNA is seen in explants co-injected with 1.3 ng *Xbra* RNA and 0.8 ng *Pintallavis* RNA. This experiment was performed four times with similar results.

tissue containing clumps of muscle-like cells (Fig. 3C). The identity of the mesothelial and somitic muscle cell types was confirmed using two monoclonal antibodies, one specific for smooth muscle and one for somitic muscle (see Materials and Methods; Fig. 4). Intriguingly, the expression of these two markers was mutually exclusive, such that the α-SM actin antibody only stained caps injected with 0.12, 0.25, 0.5, and 1 ng *Xbra* RNA, whereas 12/101 only stained animal caps injected with 2 ng and 4 ng (Table 2). Although the dorsal muscle marker 12/101 was induced by high concentrations of *Xbra* RNA, notochord was not induced at any concentration of injected *Xbra* RNA, as reported previously (Cunliffe and Smith, 1992).

The inability of *Xbra* to induce notochord differentiation in animal caps contrasts, at first sight, with the requirement for *Brachyury* in notochord formation in mouse and zebrafish embryos (see Introduction). We have previously shown, however, that co-expression of the secreted glycoprotein noggin with *Xbra* in *Xenopus* animal caps both causes notochord formation and increases the amount of somitic muscle formed (Cunliffe and Smith, 1994). It is difficult to study this synergy between Xbra and noggin at the molecular level, because nothing is known about the noggin signal transduction pathway. To overcome this problem, we have tested the abilities of *goosecoid* and *Pintallavis*, two transcription factor genes expressed in dorsal mesoderm, to cooperate with *Brachyury*.

Zebrafish *goosecoid* causes secondary axis formation in *Xenopus* embryos but does not synergize with *Brachyury*

Ectopic expression of *goosecoid* in the ventral marginal zone of *Xenopus* embryos causes formation of a partial secondary axis (Cho et al., 1991; Niehrs et al., 1993).We have investigated whether *goosecoid* synergizes with *Brachyury* by co-expressing the two gene products in animal caps. The first series of experiments used *Xenopus goosecoid* together with *Xbra*. A wide range of *goosecoid* concentrations was used and the results were assessed by observing elongation of animal caps (Symes and Smith, 1987; Howard and Smith, 1993), by RNAase protection using a muscle-specific actin probe, by histological analysis and by immunocytochemistry using a muscle-specific antibody. In no experiment did we observe an effect of *goosecoid*.

Another series of experiments made use of zebrafish *goosecoid* (Zf*gsc*: Schulte-Merker et al., 1994b), which in our hands is more efficient than *Xenopus goosecoid* in inducing secondary axes. In three experiments, injection of 0.4 to 1.6 ng Zf*gsc* RNA caused partial axis duplication in 35% of embryos (n=77). These experiments also used zebrafish *Brachyury* (or *no tail*; Schulte-Merker et al., 1994a), so that potential interactions between the two genes were not obscured by the species difference. To confirm that *no tail* (*ntl*) is biologically active, *Xenopus* embryos were injected with 0.1 ng to 1.6 ng *ntl* RNA. Animal caps were excised at the mid-blastula stage and cultured until stage 40. Histological and RNAase protection analyses showed that *ntl*, like *Xbra*, causes the differentiation of different types of mesoderm in a concentration-dependent fashion. No significant differences in the specific activities of the two gene products was noted (not shown).

To study interactions between the two genes, Zf*gsc* and *ntl* RNAs were co-injected into *Xenopus* embryos, and animal caps were excised at the mid-blastula stage and cultured until stage 28. In the experiment shown in Fig. 5, injection of 0.4 ng *ntl* RNA alone was sufficient to cause formation of ventral mesodermal vesicles but not muscle. Co-injection of 1.0 ng Zf*gsc*, which was sufficient to cause partial axis duplication in

Table 3. Histological analysis of animal caps derived from embryos injected with *ntl* and increasing quantities of *Pintallavis* RNA

RNA (ng)		Number of explants showing				Total explants
ntl	*Pintallavis*	No induction	Ventral vesicles	Muscle	Notochord	
–	–	11	1	–	–	12
0.4	–	6	8	–	–	14
–	1.6	7	7*	–	–	14
0.4	0.05	5	4	1	–	9
0.4	0.2	3	11	2	–	14
0.4	0.4	–	3	13	–	16
0.4	0.8	3	12	9	4	15
0.4	1.6	4	9	4	–	13

*These seven explants showed very weak ventral inductions.

intact embryos, did not induce transcription of muscle-specific actin. This contrasted with the effect of co-injecting *noggin* and *ntl* RNAs, which caused strong activation of muscle-specific actin (see Cunliffe and Smith, 1994). Additional experiments used a wide range of Zf*gsc* and *ntl* RNA concentrations, and in no case was synergy between the two gene products observed.

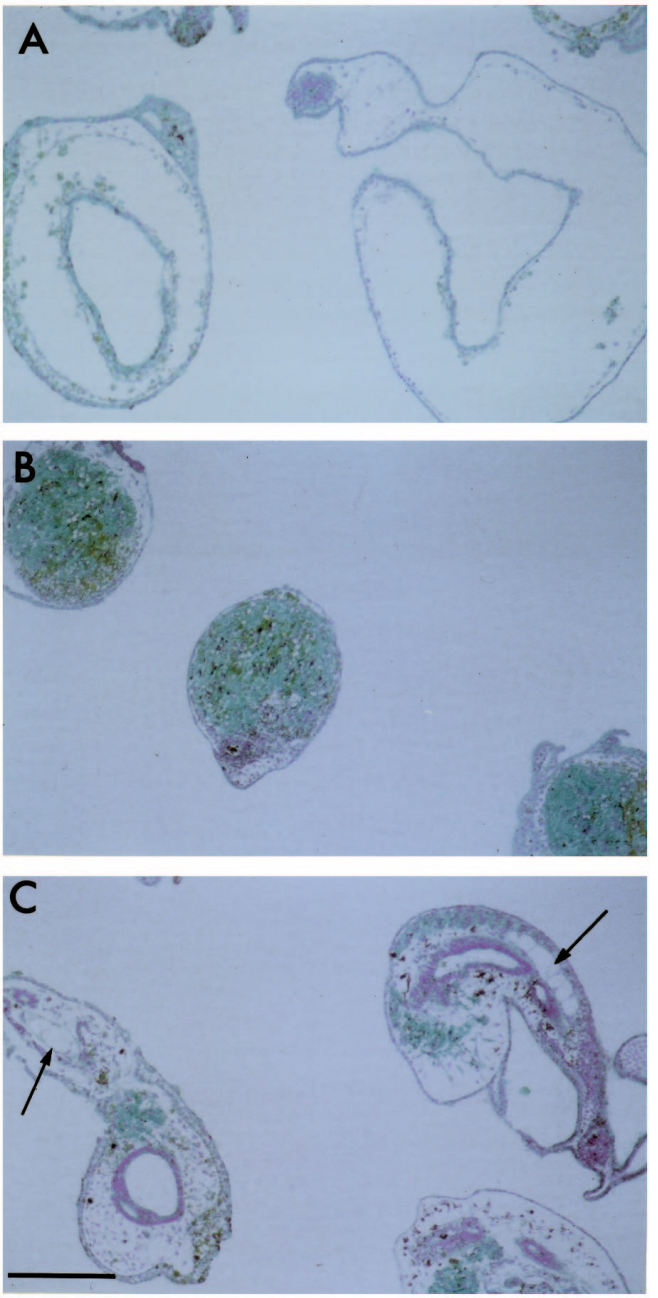

Fig. 8. Histological analysis of animal caps derived from embryos co-injected with *ntl* RNA and increasing quantities of *Pintallavis* RNA. (A) Ventral mesoderm resulting from injection of 0.4 ng *ntl* RNA and 0.2 ng *Pintallavis* RNA. (B) Muscle masses resulting from injection of 0.4 ng *ntl* RNA and 0.4 ng *Pintallavis* RNA. (C) Notochord, muscle and neural tissue resulting from injection of 0.4 ng *ntl* RNA and 0.8 ng *Pintallavis* RNA. Notochord is marked with arrows. Scale bar in C is 100 μm and also applies to A and B.

The inability of *goosecoid* to act synergistically with *Xbra* is consistent with the observation that coexpression of *Xbra* and *noggin* does not induce transcription of *goosecoid* (Cunliffe and Smith, 1994). *goosecoid* is therefore unlikely to act downstream of these two genes.

Pintallavis synergizes with *Xbra* in a dose-dependent manner to cause formation of dorsal mesoderm

We next investigated whether *Pintallavis* could synergize with *Brachyury* to cause dorsal mesoderm formation. *Pintallavis* is more likely than *goosecoid* to interact with *Brachyury* in this way, because the two genes are both expressed in notochord (Smith et al., 1991; Ruiz i Altaba and Jessell, 1992), and *Xbra* and *noggin* act synergistically to induce transcription of *Pintallavis* (Fig. 6). This suggests that *Pintallavis*, unlike *goosecoid*, acts downstream of these two genes.

Two-fold serial dilutions of *Pintallavis* RNA were co-injected with 1.3 ng *Xbra* RNA into *Xenopus* embryos. Animal caps were excised at the mid-blastula stage and cultured. An early indication of dorsal mesoderm induction is a substantial elongation of animal caps beginning during gastrula stages (Symes and Smith 1987; Howard and Smith, 1993). Animal caps derived from embryos that had been injected with both *Pintallavis* and *Xbra* carried out such movements whilst caps from embryos injected with either gene alone did not (data not shown). To confirm this initial impression that *Pintallavis* and *Xbra* act synergistically, caps were cultured until stage 31 and analyzed by RNAase protection (Fig. 7). Whilst injection of

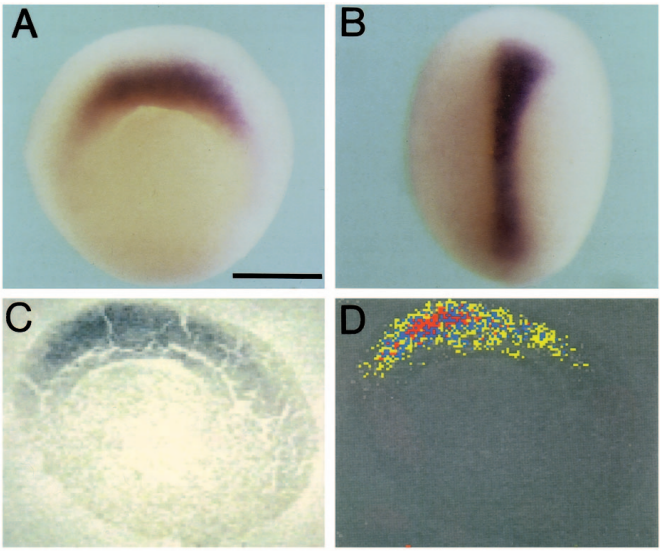

Fig. 9. Distribution of *Pintallavis* RNA in *Xenopus* embryos visualised by whole-mount in situ hybridisation. (A) Vegetal view of early gastrula stage showing expression of *Pintallavis* in prospective dorsal mesoderm. (B) Dorsal view of early neurula showing *Pintallavis* expression in midline structures including the notochord. (C) Horizontal section through the marginal zone of an early gastrula as in A, showing graded distribution of *Pintallavis* RNA in dorsal mesoderm. (D) Computer densitometric analysis of the section shown in C. The image was converted to greyscale and after background subtraction different degrees of grey were assigned false colours, with 1-7 units of grey as yellow, 8-15 units as green, and 16-30 units as red. Scale bar in A is 0.5 mm and also applies to B-D.

0.4 ng *Pintallavis* RNA did not synergize with 1.3 ng of *Xbra* RNA to induce muscle-specific actin gene expression, injection of 0.8 ng *Pintallavis* RNA elicited significant expression. Injection of 1.6 ng *Pintallavis* RNA also induced expression of muscle-specific actin in the presence of *Xbra* RNA, but at a lower level than that observed with 0.8 ng.

Histological analysis revealed that *Pintallavis* is able to induce dorsal mesoderm in a concentration-dependent manner when co-expressed with *Brachyury*. These experiments were carried out with both *Xbra* and with *ntl*. The results in Table 3 and Fig. 8 are based on experiments using *ntl*, but similar data were obtained with *Xbra* (not shown). Whilst low quantities of *Pintallavis* did not modify the ventral character of the mesoderm induced by *ntl* (Fig. 8A), injection of quantities of *Pintallavis* that elicited muscle-specific actin expression (Fig. 7) also caused differentiation of large muscle masses (Fig. 8B; see Table 3). The muscle observed in these experiments appeared better differentiated than that observed in response to high concentrations of *Xbra* alone (compare Fig. 3C with Fig. 8B). A further two-fold increase in *Pintallavis* RNA caused animal caps to differentiate into notochord and neural tissue, with reduced quantities of muscle (Fig. 8C; see Table 3).

In the experiment described above, half the explants injected with 1.6 ng *Pintallavis* RNA alone showed evidence of weak ventral induction. This was not observed in other experiments.

Whole-mount in situ hybridisation studies by Ruiz i Altaba and Jessell (1992) indicate that the distribution of *Pintallavis* RNA in the dorsal mesoderm of the early gastrula is graded, with highest levels located in cells of the future dorsal midline and lower levels in paraxial mesoderm. This was confirmed by sectioning embryos prepared for whole-mount in situ hybridisation (Fig. 9). Densitometric analysis revealed that *Pintallavis* RNA is distributed in at least a three- to four-fold concentration gradient across the dorsal marginal zone with highest levels dorsally, in the prospective notochord (Fig. 9D). This finding, together with our observation that a four-fold increase in *Pintallavis* RNA causes successive formation of ventral mesoderm, followed by muscle, and finally notochord and neural tissue, is consistent with the idea that graded expression of *Pintallavis* is involved in patterning the mesoderm of *Xenopus*.

DISCUSSION

Brachyury specifies two types of mesoderm

In this paper, we first demonstrate the novel observation that different concentrations of Xbra induce two different types of mesoderm in isolated *Xenopus* animal caps. At low doses, animal pole cells form mesenchymal cells and mesothelial cells some of which express α-smooth muscle actin. Increasing the concentration of Xbra by a factor of two then extinguishes expression of smooth muscle actin and induces the differentiation of muscle masses that react with monoclonal antibody 12/101. This concentration-dependent effect of *Xbra* is consistent with evidence suggesting that greater *Brachyury* activity is required posteriorly in the mouse embryo than anteriorly (MacMurray and Shin, 1988; Yanagisawa, 1990; Stott et al., 1993). In the tails of *T*/+ heterozygous mice, for example, the notochord is absent or greatly reduced (see Dietrich et al., 1993; Kispert and Herrmann, 1994). Together, these results suggest that cells are extremely sensitive to the concentration of Brachyury protein they experience.

We do not know how Brachyury exerts its concentration-dependent effects, but the mechanism may resemble the situation in *Drosophila* development, where gradients of bicoid and dorsal, which are also sequence-specific DNA-binding proteins, specify position along the anteroposterior and dorsoventral axes of the embryo, respectively (see Driever and Nüsslein-Volhard, 1989; Struhl et al., 1989; Ip and Levine, 1992; Ip et al., 1992). Thus, Brachyury-responsive genes may possess low- or high-affinity Brachyury-binding sites in their *cis*-acting regulatory regions. At low concentrations, only high affinity sites would interact with Brachyury and this would lead to ventral mesodermal differentiation. At higher concentrations of Brachyury, both high and low affinity sites would interact and muscle differentiation would then occur. An alternative mechanism is based on the observation that Brachyury can bind to a palindromic DNA sequence (Kispert and Herrmann, 1993). At low concentrations of Brachyury only half-site sequences might be contacted, leading to ventral mesodermal differentiation. At higher concentrations, however, Brachyury might form homodimers and thus contact full palindromic sequences in *cis*-acting regulatory regions of muscle-specific genes. Such a mechanism would resemble that of the λ repressor in its interaction with DNA (see Ptashne, 1986).

Pintallavis, but not *goosecoid*, synergizes with *Brachyury*

Expression of *Xbra* alone is not sufficient to cause animal caps to form notochord, and this suggests that other genes cooperate with *Brachyury* to cause the formation of dorsal mesoderm. Our results show that *Pintallavis*, but not *goosecoid*, is able to synergize with *Brachyury* in this way.

The inability of *goosecoid* to cooperate with *Brachyury* contrasts with the observation that *goosecoid* can dorsalize mesoderm of the ventral marginal zone in a dose-dependent fashion (Niehrs et al., 1994). It suggests that ventral marginal zone tissue possesses a broader competence to respond to the effects of dorsalizing agents than the ventral mesoderm induced by ectopic expression of Brachyury (see also Cunliffe and Smith, 1994). This is under investigation.

Pintallavis (or XFD-1) is a member of the forkhead/HNF3β family of transcription factors (Ruiz i Altaba and Jessell, 1992; Knöchel et al., 1992). *Pintallavis* transcripts are first detected in the dorsal blastopore lip of the early gastrula and expression is subsequently maintained in the notochord, the floor plate and midline endoderm. Ectopic expression of *Pintallavis* in whole embryos affects patterning of the neural tube but has no discernable effect on mesodermal derivatives, and animal caps from injected embryos generally differentiate only into atypical epidermis (Ruiz i Altaba and Jessell, 1992; Ruiz i Altaba et al., 1993). Nevertheless, the expression pattern of *Pintallavis* suggested that it might interact with *Brachyury*, and our experiments demonstrate that it does so in a dose-dependent fashion. Thus, when co-expressed with *Brachyury*, a small increase in *Pintallavis* RNA causes formation of progressively more dorsal mesoderm, from mesenchyme and mesothelium, to muscle, and finally to notochord and neural tissue.

It is interesting that both *Brachyury* and *Pintallavis* show

dose-dependent effects, and it may be significant that mice heterozygous for a null mutation in the HNF-3β gene, like *T*/+ mice, show an obvious mutant phenotype. This phenotype is characterized by malocclusion of the jaws, overgrowth of the upper and lower incisors, a circling defect and a small and hunched appearance (Ang and Rossant, 1994). It serves to emphasize that embryonic cells are sensitive to the concentration of HNF-3β protein that they experience as well as the concentration of Brachyury. The importance of the HNF-3β gene family in axis formation is further demonstrated by the fact that mouse embryos lacking HNF-3β gene function completely do not form a node or notochord and the primitive streak is truncated (Ang and Rossant, 1994; Weinstein et al., 1994).

We do not know how the synergy between *Brachyury* and *Pintallavis* occurs. In an effort to investigate this question, we are searching for direct targets of the two gene products, and asking whether they interact directly, perhaps by forming heterodimers, or indirectly.

Concentration-dependent effects of *Brachyury* and *Pintallavis* in mesodermal patterning

Ruiz i Altaba and Jessell (1992) have proposed that patterning of the mesoderm in *Xenopus* may involve concentration-dependent effects of *Xbra* and *Pintallavis*, and our experiments, in which we ectopically express different quantities of each gene product, are consistent with this suggestion. The expression patterns of the two genes also fit with the idea. *Pintallavis* is indeed expressed in a graded fashion at the early gastrula stage (see Fig. 9) while the expression pattern of *Xbra* is graded in both a spatial and a temporal sense. First, dorsal expression of *Xbra* precedes expression in lateral and ventral regions of the early gastrula (Ruiz i Altaba and Jessell, 1992). Then, even though expression appears uniform throughout the marginal zone (Smith et al., 1991; Green et al., 1992), prospective anterior mesodermal cells express *Xbra* for only a short time because they down-regulate expression of the gene as they undergo involution. Posterior mesodermal cells, which are the last to involute, express *Xbra* for the longest time. This anteroposterior temporal gradient of expression is consistent with work suggesting that greater *Brachyury* activity is required posteriorly in the mouse embryo than anteriorly (MacMurray and Shin, 1988; Yanagisawa, 1990; Stott et al., 1993).

Expression of both *Xbra* and *Pintallavis* persists in the notochord (Smith et al., 1991; Green et al., 1992; Ruiz i Altaba and Jessell, 1992; see Fig. 9), and this is consistent with the requirement for high concentrations of both factors for dorsal mesoderm formation.

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