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Xbra3 Induces Mesoderm and Neural Tissue in *Xenopus laevis*

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Homologues of the murine *Brachyury* gene have been shown to be involved in mesoderm formation in several vertebrate species. In frogs, the *Xenopus Brachyury* homologue, *Xbra,* is required for normal formation of posterior mesoderm. We report the characterisation of a second *Brachyury* homologue from *Xenopus, Xbra3,* which has levels of identity with mouse *Brachyury* similar to those of *Xbra.* X*bra3* encodes a nuclear protein expressed in mesoderm in a temporal and spatial manner distinct from that observed for Xbra. Xbra3 expression is induced by mesoderm-inducing factors and overexpression of Xbra3 can induce mesoderm formation in animal caps. In contrast to Xbra, Xbra3 is also able to cause the formation of neural tissue in animal caps. Xbra3 overexpression induces both *geminin* and *Xngnr-1,* suggesting that Xbra3 can play a role in the earliest stages of neural induction. Xbra3 induces posterior nervous tissue by an FGF-dependent pathway; a complete switch to anterior neural tissue can be effected by the inhibition of FGF signalling. Neither *noggin, chordin, follistatin,* nor *Xnr3* is induced by Xbra3 to an extent different from their induction by Xbra nor is *BMP4* expression differentially affected.

Key Words: **Xbra; Xbra3; mesoderm; neural induction; *Xenopus*.**

INTRODUCTION

During the development of vertebrates, the nervous system arises from the ectoderm by a process of induction which requires signals from the dorsal mesoderm. This was first demonstrated through transplants of dorsal mesoderm-containing explants to the ventral region of amphibian gastrulae, causing induction of host cells to form a second body axis including nervous tissue (Spemann and Mangold, 1924). The inducing tissue, referred to as the Spemann organiser, has been shown to be the source of signals which elicit neural differentiation. Dissection of the molecular events which instruct this process has revealed that an active signalling event, mediated by bone morphogenetic proteins (BMPs), is involved in the development of epidermis from the ectoderm and that neural induction is achieved through the local inhibition of BMP signalling by secreted factors including noggin, chordin, and follistatin (Hemmati-Brivanlou *et al.*, 1994; Lamb *et al.*, 1993; Sasai *et al.*, 1995). Thus, at least in terms of BMP signalling, neural fate is achieved through adoption of a default pathway (Hemmati-Brivanlou and Melton, 1997). This finding agrees with earlier observations which indicated that ectodermal cells would adopt a neural fate when intercellular signalling was disrupted through disaggregation (Godsave and Slack, 1989; Grunz and Tacke, 1989), while differentiation to epidermis could be restored by addition of BMP (Wilson and Hemmati-Brivanlou, 1995).

Neural-inducing molecules such as chordin, noggin, and follistatin which act in this fashion are secreted by cells of the dorsal mesoderm and bind directly to and prevent receptor binding by BMPs (Fainsod *et al.*, 1997; Piccolo *et al.*, 1996; Zimmerman *et al.*, 1996). Xnr3, a member of the TGFβ family, also inhibits BMP signalling, but the precise mechanism by which it does so is as yet unclear (Hansen *et al.*, 1997). This route to neural determination is also conserved in invertebrates. In *Drosophila,* the nervous system forms in the ventral region of the embryo, through inhibition of the activity of the BMP4 homologue, decapentaplegic, by the chordin homologue, short gastrulation (sog). sog will induce a secondary axis in *Xenopus* embryos (Schmidt *et al.*, 1995), while both chordin and noggin will substitute for sog in *Drosophila* (Holley *et al.*, 1995, 1996).

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In addition to the initial induction of the nervous system, both its anteroposterior and its dorsoventral patterning depend on secreted factors from tissues including the dorsal mesoderm. Dorsoventral patterning is influenced by molecules including sonic hedgehog, produced by both dorsal mesoderm and floorplate (Echelard *et al.*, 1993; Marti *et al.*, 1995) and BMPs produced by the ectoderm (Dickinson *et al.*, 1995; Liem *et al.*, 1995). Similarly, anteroposterior patterning appears to be influenced by different signalling centres for different regions of the axis (see Gilbert and Saxen, 1993, for review). Dorsal mesoderm induces neural tissue of anterior character which is subsequently converted, or transformed, to more posterior character by posteriorising signals such as FGF (Cox and Hemmati-Brivanlou, 1995; Kengaku and Okamoto, 1995; Barnett *et al.*, 1998). FGF also appears to operate in vertebrates to induce neural tissue of a posterior character directly (Lamb and Harland, 1995; Storey *et al.*, 1998).

Xbra is a homologue of the mouse *Brachyury* gene, a member of the T-box family of transcription factors which are expressed early in the process of mesoderm formation in vertebrates in response to mesoderm-inducing factors (Smith, 1997). Xbra is expressed throughout the mesoderm and has been shown to act to direct the differentiation of mesoderm cells of different dorsoventral characters dependent on the level of Xbra expression (Cunliffe and Smith, 1992, 1994). Whilst Xbra has been shown to transcriptionally induce genes expressed in mesoderm (Conlon *et al.*, 1996) it appears incapable of inducing neural tissue, although a mutated form of *Xbra* has been shown to elicit anterior neural inductions (Rao, 1994). Although Xbra alone is unable to induce neural tissue, its expression has been shown to modify the character of induced nervous tissue. Thus, Xbra has been shown capable of posteriorising anterior nervous tissue induced by Xlim-1, through an FGF-dependent mechanism (Taira *et al.*, 1997).

In this work, we report experiments to characterise the activity of a second *Brachyury* homologue in *Xenopus*, named *Xbra3* (Hayata *et al.*, 1999). The product of this gene is expressed in the dorsal mesoderm, persisting to much later developmental stages than Xbra. In contrast with Xbra, Xbra3 is capable both of inducing anterior neural tissue in the absence of a functional FGF signalling pathway and of inducing posterior neural tissue via an FGF-dependent mechanism.

MATERIALS AND METHODS

Library Screening and Sequencing

Libraries were plated at a density of 2×10^5 plaques on 22-cm^2 plates and duplicate plaque lifts taken on Hybond N+ (Amersham) nylon filters. Probes were made by random-primed synthesis (Feinberg and Vogelstein, 1984), using template DNA fragments indicated in the text, and hybridised in 7% SDS, 0.5 M NaP_i (pH 6.8), at 65°C (Church and Gilbert, 1984). All washes were at high stringency, i.e., 1% SDS, 50 mM NaP_i (pH 6.8), 65°C. Sequencing was carried out on both strands by a combination of manual sequencing using a Sequenase II kit (USB) according to the manufacturer's instructions and automated sequencing using an Applied Biosystems 373A instrument.

Embryo Culture and Dissection

All embryos used in this study were obtained by *in vitro* fertilisation of hormonally stimulated *Xenopus laevis* and staged according to published tables (Nieuwkoop and Faber, 1967). Standard embryological procedures were as described (Jones and Woodland, 1986). Whole control, or mRNA-injected, embryos were cultured in 1/10 Barth X saline after dejellying in 2% cysteine hydrochloride, pH 8. Dissected caps were taken from an approximately 60° arc on the animal pole. Explants were cultured in full-strength Barth X saline. Whole embryo stage controls were also cultured to allow staging of the caps.

Sandwiches of *X. borealis* and *X. laevis* caps were generated by placing animal caps from the two species together with their dissected sides touching. The caps then annealed to each other and remained together until harvested.

Growth Factor Treatments

In order to assess the ability of activin and FGF to induce the expression of *Xbra* and *Xbra3*, animal caps from stage 9 embryos were incubated in Barth X saline containing 0.1 μg/ml bovine serum albumin with the addition of either human recombinant bFGF (Promega) or activin (A. F. Schuetzdeller) at concentrations from 50 to 3·125 ng/ml. Animal caps were harvested for analysis at stage 12, stage 20, or stage 25 equivalent.

mRNA Synthesis and Microinjection

RNA was synthesised from plasmid templates using SP6 or T7 polymerase. All microinjections were carried out with RNA diluted to approximately 50 μg/ml. Approximately 20 nl was injected into one blastomere of dejellied two-cell embryos under 5% Ficoll in Barth X. Embryos were removed from Ficoll after an overnight incubation and transferred into Barth X for dissection.

RT-PCR Analysis and Primers

Each group of five animal caps was homogenised in 150 μl XT buffer (300 mM NaCl, 20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1% SDS) and digested at 37°C with proteinase K. Following phenol extraction and addition of 5 μg glycogen (as carrier), samples were ethanol precipitated at −20°C. Dried pellets were resuspended in 30 μl transcription buffer containing DNase I and placental RNase inhibitor and incubated for 15 min at 37°C. One hundred twenty microliters of XT buffer was added and samples were digested at 37°C with proteinase K. After phenol and phenol/chloroform extraction, the RNA sample was ethanol precipitated at room temperature and resuspended in 10 μl water. One microgram of RNA was used to generate first-strand cDNA using random hexamers as primers. Synthesis was carried out in the presence and absence of Superscript reverse transcriptase (Gibco) and RNA. The cDNA was then used for PCR analysis using the primers shown in Table 1. The conditions were as follows: first, denaturation step at 94°C for 3 min; second, annealing step at 55°C for 1 min; third, extension step at 72°C for 1 min; and fourth back to the first step for 24 cycles, with 1 min for the 94°C denaturation step. The final

TABLE 1
Sequences of Primers Used for RT-PCR Analysis of RNA

Markers	Sequence (5′ to 3′)	Reference
Xbra3	U—CAAACCCTGTTGGAGTTG	This work
	D—CCTTCTCACTTCCAACTTGC	
Xbra	U—GGATCGTTATCACCTCTG	Smith *et al.*, 1991
	D—GTGTAGTCTGTAGCAGCA	
eFGF	U—TTACCGGACGGAAGGATA	Isaacs *et al.*, 1992
	D—CCTCGATTCGTAAGCGTT	
Cardiac actin	U—TCCCTGTACGCTTCTGGTCGTA	Mohun *et al.*, 1986
	D—TCTCAAAGTCCAAAGCCACATA	
NCAM	U—CACAGTTCCACCAAATGC	Kintner and Melton, 1987
	D—GGAATCAAGCGGTACAGA	
Xnot	U—ATACATGGTTGGCACTGA	Von Dassow *et al.*, 1993
	D—CTCCTACAGTTCCACATC	
Hoxb9	U—TACTTACGGGCTTGGCTGGA	Wright *et al.*, 1990
	D—AGCGTGTAACCAGTTGGCTG	
EF1-α	U—CAGATTGGTGCTGGATATGC	Mohun *et al.*, 1989
	D—CACTGCCTTGATGACTCCTA	
Geminin	U—GCTGGACATGTACAGTACA	Kroll *et al.*, 1998
	D—TCACCTCACATAAAGGCTGG	
Xngnr-1	U—TACATCTGGGCTCTTAGCGA	Kroll *et al.*, 1998
	D—CAAATGAAAGCGCTGCTGGC	
Chordin	U—GGAGCAGGATCATGCAGATT	Sasai *et al.*, 1994
	D—CCTGGCAGTTGTCTCAGCTT	
Follistatin	U—CAGTGCAGCGCTGGAAAG	Hemmati-Brivanlou *et al.*, 1994
	D—TGCGTTGCGGTAATTCAC	
Noggin	U—CCAGACCTTCTGTCCTGT	Smith and Harland, 1992
	D—AGTCCAAGAGTCTCAGCA	
Xnr-3	U—GTGCAGTTCCACAGAATGAG	Smith *et al.*, 1995
	D—CCATGGATCGGCACAACAGA	
BMP4	U—ACAGGCTTCAGTCAGCGG	Nishimatsu *et al.*, 1992
	D—GGTGAATGACCTCAATGG	
Hoxb-9 (*X. laevis*)	U—TTCCGCTCTGCGCAATTC	This work
	D—GAAGTTGCCGCTGTGAGT	
Hoxb-9 (*X. borealis*)	U—CTGGAGCCTCTGCAAAGA	This work
	D—CGAGCGTGTAACCAGTTG	

Note. U and D refer to upstream and downstream primers.

step was at 72°C for 5 min. These conditions were followed for all primers except that the annealing step was at 62°C for muscle actin and the total number of cycles for EF1-α was 20. PCR products were analysed on 6% nondenaturing polyacrylamide gels and exposed to X-ray film.

Synthesis of cDNA from RNA extracted from sandwiches of *X. borealis* and *X. laevis* caps was carried out as above but with the addition of 1 μCi [α-^{32}P]dGTP. cDNA was then precipitated with 20 μg SSS DNA and 10% TCA followed by resuspension in 50 μl TE and addition of 500 μl Optiphase scintillation fluid (Wallac). cDNA samples were then counted on a scintillation counter and equalised by the number of counts.

In Situ Hybridisation and Immunohistochemistry

In situ hybridisation to albino embryos was carried out according to the method of Harland (1991). Embryos were fixed in MEMFA (0.5 M Mops, pH 7.4, 100 mM EGTA, 1 mM $MgSO_4$, 4% formaldehyde) and hybridised with RNA probes corresponding to the specified regions of *Xbra* and *Xbra3* cDNA clones. For detection of *Xbra*, a 682-nt probe representing bases 1528 to 2210 of the *Xbra* cDNA was used. This probe contains a 37-bp region with 75% identity with *Xbra3* and overall is 44% identical. For detection of *Xbra3*, a 681-nt probe representing bases 530 to 1210 of the *Xbra3* cDNA was used. This sequence is 70% identical with corresponding sequences of *Xbra*. Probes were synthesised and labelled using a DIG labelling kit (Boehringer). Whole-mount immunohistochemistry was performed according to published procedures (Dent *et al.*, 1989).

RESULTS

A Second Brachyury Homologue in Xenopus

We identified two genomic clones from a λ genomic library encoding distinct homologues of the murine

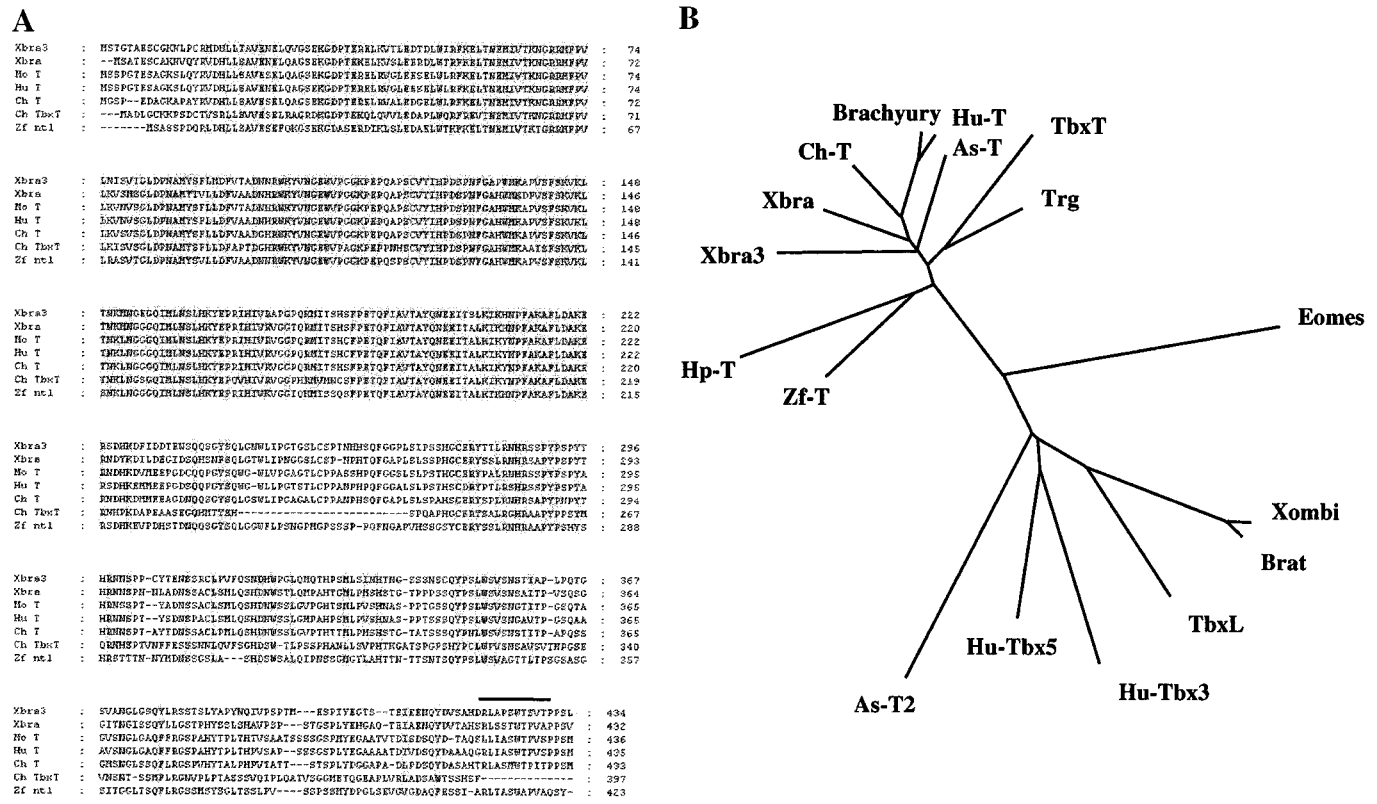

FIG. 1. Comparison of Xbra3 with other T-domain proteins. (A) Alignment of the predicted polypeptide sequence of Xbra3 with Xbra (Smith *et al.*, 1991), mouse *Brachyury* (Mo T; Herrmann *et al.*, 1990), human T (Hu T; Edwards *et al.*, 1996), chick T (Ch T; Kispert *et al.*, 1995), chick T-box T (Ch TbxT; Knezevic *et al.*, 1997) and zebrafish no-tail (Zf ntl; Schulte-Merker *et al.*, 1994). Dashes have been inserted to introduce gaps to maximise the alignment. An antiserum specific for Xbra3 was raised against a peptide representing residues 420–431 (overlined). (B) Dendrogram of T-box genes produced by the PHYLIP program. Sequences and SwissProt accession numbers (except where indicated) are *Brachyury* (P20293), human T (Hu-T; translated from GenBank; AJ001699), chicken T-box T (TbxT; P79778), *Halocynthia* T (As-T; P56158), chicken T (Ch-T; P79777), *Drosophila* T-related gene (Trg; P55965), *Xenopus Brachyury* (Xbra; P24781), *Xenopus Brachyury* 3 (Xbra3; this work), *Hemicentrotus* T (Hp-T; Q25113), zebrafish T (Zf-T; Q07998), *Xenopus* Eomesodermin (Eomes; P79944), *Xenopus* Xombi (Xombi; translated from GenBank; S83518), *Xenopus* Brat (Brat; P87377), chicken T-box L (TbxL; P79779), human T-box 5 (Hu-Tbx5; Q99593), human T-box 3 (Hu-Tbx3; translated from GenBank; AF002228) and Ascidian T (As-T2; O01409; also from *Hemicentrotus*).

Brachyury gene. Partial sequence analysis of λXbc1 revealed 100% identity to a 51-base sequence of the *Xbra* cDNA, suggesting that this clone represented a genomic locus allelic with *Xbra*. However, the second clone isolated, λXbc2, revealed only 71% homology with bases 148–337 of the published *Xbra* sequence. Furthermore, analysis of predicted intron sequences from the two clones showed no significant homology, demonstrating them to be nonallelic (data not shown).

In order to determine whether λXbc2 represented a novel functional *Brachyury* homologue, we cloned a corresponding cDNA by screening a stage 17 λgt11 library using a 310-bp fragment from λXbc2 including 203 bp of the potential 5′ untranslated sequences and 107 bp of coding sequence. This probe showed 72% identity to *Xbra* over 90 bases. Four clones were isolated from 3×10^5 plaques and the longest, λXbra3, was sequenced in its entirety. This sequence contained a 404-bp region identical with the sequence downstream of the predicted initiation codon of the genomic clone λXbc2 and shares 99% identity with the *Xbra3* cDNA sequence reported by Hayata *et al.* (1999).

The *Xbra3* cDNA sequence contains an open reading frame of 1302 bp, conceptual translation of which yields a 434-amino-acid polypeptide which was compared with other published Brachyury homologues (Fig. 1A). As with other members of the family, the highest level of identity is found in the N-terminal region, the T domain, which encodes a highly conserved DNA binding domain (Kispert and Herrmann, 1993). While the T domain exhibits a high level of identity with Xbra (88% over residues 17 to 221), overall the sequence is only 73% identical to Xbra, suggesting that the two genes are neither allelic nor pseudoallelic;

genes duplicated through tetraploidy in *Xenopus laevis* generally differ by less than 10% (Graf and Kobel, 1991). Comparison of the Xbra3 peptide sequence with other Brachyury homologues shows that Xbra3 is almost as divergent from mouse (Herrmann *et al.*, 1990) or human T (Edwards *et al.*, 1996) or from Ch-T, the chick orthologue of Brachyury (Kispert *et al.*, 1995), or Ch-TbxT (Knezevic *et al.*, 1997) as it is from Xbra (Fig. 1B). *Xbra3* is, however, clearly a homologue of *Brachyury* rather than a more divergent member of the T-box gene family. Sequence comparison of known Brachyury homologues and other T-box polypeptides shows that Xbra3 is overall more related to Brachyury homologues from other species than it is to other known T box proteins (Fig. 1B; see Discussion). This is reflected by comparisons of sequences C terminal to the T box, in which Xbra3 exhibits 59.9% identity with Xbra, 56.2% identity with mouse Brachyury, and 56.8% identity with Ch-T, but only 22% identity with Xombi (Lustig *et al.*, 1996), 20% identity with eomesodermin (Ryan *et al.*, 1996), and 31.3% identity with chicken T-box L (Knezevic *et al.*, 1997). We therefore believe that Xbra3 represents a distinct homologue of Brachyury in *X. laevis*.

Xbra3 Is Expressed in a Pattern Distinct from That of Xbra

In order to determine the temporal expression profile of *Xbra3*, and to compare it to that of *Xbra*, we used quantitative RT-PCR to determine the abundance of messenger RNA transcribed from the two genes during development. As reported previously (Smith *et al.*, 1991) *Xbra* mRNA is first clearly detectable in stage 9.5 embryos at the onset of gastrulation, with levels rising between stages 10 and 10.5 as gastrulation proceeds (Fig. 2A). Between stages 12 and 15, during neurulation, levels of *Xbra* message decline markedly, the signal becoming barely detectable at stage 24 and being undetectable by stage 35 in the hatched tadpole. In contrast, *Xbra3* message is only weakly detectable by RT-PCR before stage 10.5, the decrease in signal between stages 12 and 15 is not apparent, and the message remains readily detectable at stage 35 (Fig. 2A). Thus, the timing of onset and extinction of the expression of the two genes are distinct. Overall, *Xbra3* is expressed later than *Xbra* and over an extended developmental period.

To examine the spatial regulation of *Xbra3, in situ* hybridisation was performed with short regions of both *Xbra* and *Xbra3* messages, chosen to maximise the specificity of the probes (see Materials and Methods). Although we were unable to test these probes directly for specificity in the *in situ* procedure, the distinct natures of the expression patterns revealed by the analysis argue that they are specific for two distinct gene products. Furthermore, the *in situ* patterns we detect mirror precisely the temporal expression identified by RT-PCR. These probes are relatively short (680 nt) and give weaker hybridisation signals in our procedure than do full-length cDNA probes for *Xbra* and *Xbra3*. However, the latter fail to show differences in

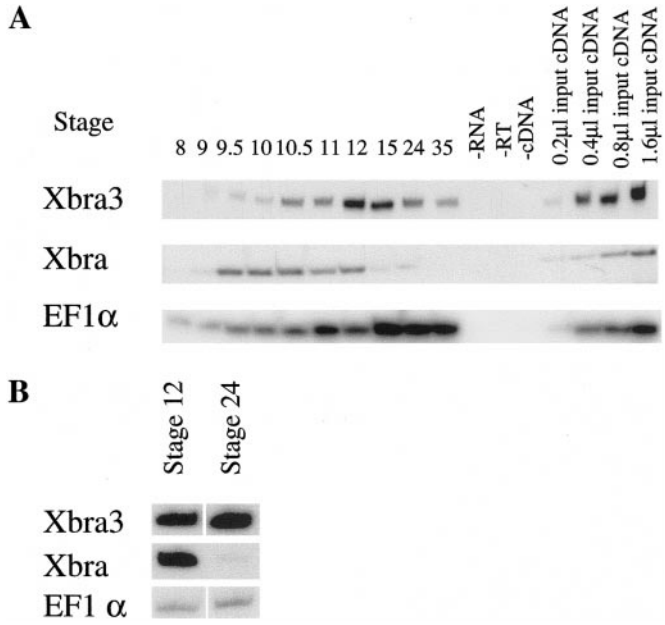

FIG. 2. RT-PCR analysis of the temporal expression patterns of *Xbra* and *Xbra3*. (A) RNA was extracted from embryos at the stages shown and analysed for the abundance of *Xbra* and *Xbra3* mRNA. (B) Analysis of *Xbra* and *Xbra3* expression in RNA extracted from isolated notochords at stages 12 and 24. All assays were controlled for linearity of the amplification (data not shown).

expression between the two genes, suggesting that they hybridise to both messages (not shown). As reported previously (Smith *et al.*, 1991), hybridisation with the *Xbra* probe was first detected weakly at stage 10, in the marginal zone, with higher levels dorsally (not shown). By stage 12, expression could be seen clearly in the presumptive notochord and in the marginal zone (Fig. 3B). *Xbra* expression was barely detectable in tailbud regions by stage 25 (Fig. 3C) and undetectable by stage 35 (Fig. 3D). In contrast, *Xbra3* was not detected at stage 10, first appearing at stage 11 in a ring around the blastopore, in a pattern similar to that shown by *Xbra* at the same stage (not shown). At stage 12, expression remained weak in the marginal zone (Fig. 3A). By stage 25, *Xbra3* was expressed strongly in the notochord where it was maintained at least until stage 35 (Figs. 3C and 3D). Expression of *Xbra3* was also seen in the tail tip at later tadpole stages (not shown). These patterns of expression are essentially identical to those reported by Hayata and coworkers (1999).

In order to verify the later persistence of *Xbra3* expression relative to that of *Xbra* in the notochord of later stage embryos, as detected by *in situ* hybridisation, RT-PCR detection of both transcripts was performed on RNA extracted from notochords dissected from stage 12 and 24 embryos. Whereas an increase in *Xbra3* transcript levels is observed in isolated notochords between these two stages, the levels of *Xbra* transcripts drop dramatically over this

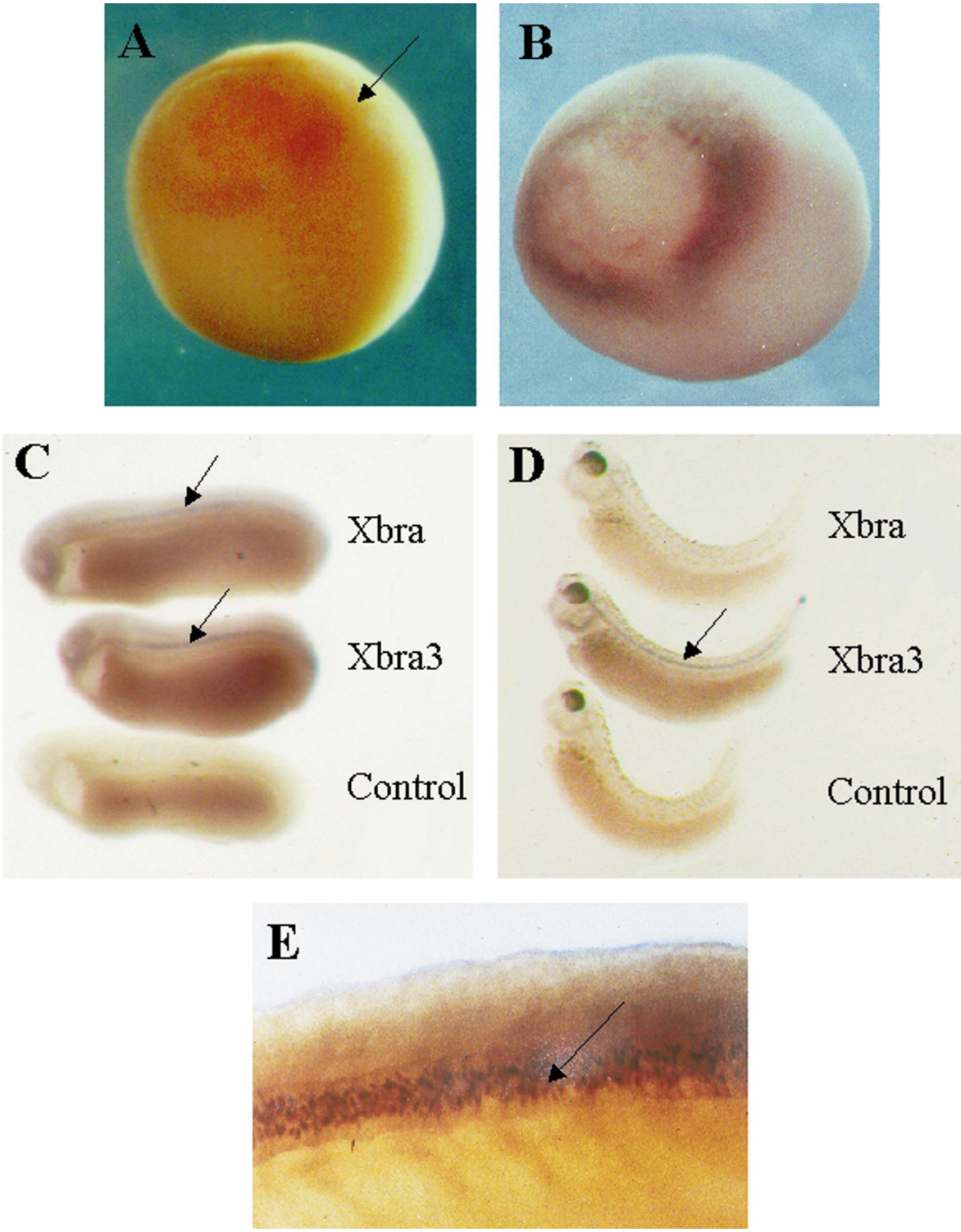

FIG. 3. Spatial distribution of *Xbra* and *Xbra3* mRNA. Embryos were hybridised with antisense RNA probes representing regions of the two transcripts which differed sufficiently to ensure specificity for the two gene products (see text). At stage 12, *Xbra3* (A, arrowed) is detected weakly around the blastopore in a pattern similar to that seen with *Xbra* (B). While *Xbra* message levels decline at stages 25 (C, arrowed) and 35 (D), *Xbra3* is maintained at these stages in notochord and tail tip (C, D, arrowed). Hybridisation with an *Xbra3* sense transcript gave no signal (C, D, control). A specific antiserum which recognises Xbra3 and not Xbra protein stains the nuclei of notochord cells at stage 35 (E, arrowed). No staining was seen with preimmune serum (not shown).

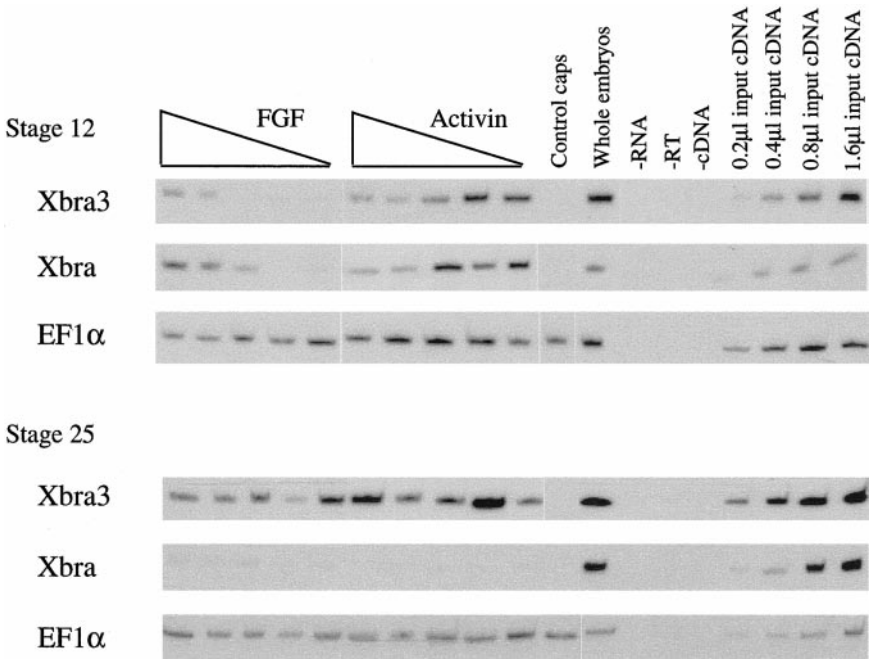

FIG. 4. Induction of *Xbra* and *Xbra3* in isolated animal caps by mesoderm-inducing factors. Caps were isolated at stage 9 and treated with either activin or FGF at concentrations from 50 to 3·125 ng/ml. RNA was extracted and analysed by RT-PCR at the stages shown. All assays were controlled for linearity of the amplification. Analysis at stage 12 shows that both *Xbra* and *Xbra3* are induced by activin and FGF, with *Xbra* showing a lower threshold for induction by FGF. At stage 25, a clear difference is seen between *Xbra* and *Xbra3* in the response to both growth factors. Expression of *Xbra3* is clearly maintained at high levels, whereas *Xbra* transcripts are barely detectable.

period (Fig. 2B). The results of *in situ* hybridisation analysis therefore agree closely with the temporal expression pattern detected by RT-PCR revealing clear differences in spatial expression between *Xbra* and *Xbra3* at different stages of development, particularly with respect to expression in the notochord. Together, these results support the conclusion that two distinct *Brachyury* homologues are expressed with different spatiotemporal profiles during the development of the mesoderm in *Xenopus,* raising the possibility that they play different functional roles.

Xbra3 Encodes a Nuclear Protein

The mouse *Brachyury* gene encodes a nuclear protein which has both DNA binding and transcriptional regulatory activities (Kispert and Herrmann, 1993; Kispert *et al.,* 1995a) and similar properties have been reported for Xbra (Conlon *et al.,* 1996). In order to determine the subcellular localisation of the Xbra3 polypeptide, we raised a polyclonal antiserum against a peptide representing residues 420 to 431 of the predicted Xbra3 protein, which differs from the equivalent region of Xbra at 6 of the 12 residues (Fig. 1A, overlined sequence). The resulting serum was tested on proteins extracted from *Xenopus* oocytes which had been injected with synthetic RNA transcribed from either *Xbra* or *Xbra3* and was shown to specifically recognise Xbra3 both in Western blots and by immunoprecipitation (data not shown). This antibody was used in whole-mount immunostaining and was found to stain strongly the nuclei of cells expressing *Xbra3* mRNA, in the notochord at stages 25 (not shown) and 35 (Fig. 3E). These observations support the view that *Xbra3* is expressed in embryos, in a pattern distinct from that of *Xbra,* to produce a nuclear protein with a potential role in early mesoderm development.

Regulation of Xbra and Xbra3 by Mesoderm-Inducing Factors

The early expression pattern of *Xbra3* suggested that it may be involved in mesoderm formation and could, like *Xbra,* be a target for induction by mesoderm-inducing factors. To investigate this, animal caps were isolated from embryos at stage 8 and cultured overnight either alone or in the presence of either bFGF or activin. Caps were allowed to develop until control embryos had reached stage 12 or 25, when RNA was extracted and analysed for both *Xbra* and *Xbra3* mRNA by RT-PCR. As has been shown previously (Smith *et al.,* 1991), induction of *Xbra* by both activin and FGF is clearly detectable by stage 12 (Fig. 4). Activin also elicits a moderate response in inducing *Xbra3* when assayed at stage 12, though induction by FGF is weaker at this stage. By stage 25, the differences in the levels of transcripts of the

two genes produced as a result of growth factor stimulation are more marked. At this stage *Xbra* expression is barely detectable in response to either FGF or activin treatment while *Xbra3* expression in response to both inducers is clearly maintained. These results show that the two *Xenopus Brachyury* homologues initially respond similarly to mesoderm-inducing factors with *Xbra3* mRNA showing significantly longer persistence in treated caps, an observation which fits well with the later expression of *Xbra3* in whole embryos.

Induction of Mesodermal Tissue Markers by Xbra and Xbra3

The observed differences in expression patterns observed for *Xbra* and *Xbra3*, together with the significant differences in the C-terminal region of the two predicted polypeptides, give rise to the possibility that the products of the two genes fulfil differing functional roles during embryonic development.

We assessed the mesoderm-inducing activity of Xbra3 by examining the expression of *Xbra* in caps from embryos injected at the two-cell stage with *Xbra3* RNA. As is seen in Fig. 5A, Xbra3 can induce *Xbra* expression in caps, demonstrating a mesoderm-inducing activity of Xbra3 protein. Xbra has been shown to require an intact FGF signalling pathway for its activity in mesoderm formation (Amaya *et al.*, 1993; LaBonne and Whitman, 1994; Schulte-Merker and Smith, 1995), and coexpression of a dominant negative FGF receptor, XFD (Amaya *et al.*, 1991), also abolishes the ability of Xbra3 to induce *Xbra* (Fig. 5A). To compare the activities of Xbra3 and Xbra in inducing the formation of mesoderm, we assayed the expression of a panel of markers in caps taken from embryos injected with either message. Whilst both gene products clearly induce the expression of a range of mesoderm markers, differences between the levels of induction of the various markers by the two Brachyury homologues suggest differences in the nature of the mesoderm induced by each (Fig. 5B). Both proteins are active in the induction of the panmesodermal marker *eFGF* (Isaacs *et al.*, 1992), but when expression of markers of mesoderm of a more dorsal character is analysed at stage 25 Xbra3 is clearly more active than is Xbra. Xbra3 is able to reliably induce expression of *cardiac actin*, a marker of dorsal mesoderm, whereas its induction by Xbra is observed only occasionally and at a low level, in agreement with previous reports (O'Reilly *et al.*, 1995; Taira *et al.*, 1997) (Fig. 5B). Furthermore expression of *Xnot*, a homeobox gene, the expression of which is restricted to notochord and floorplate after gastrulation, remains detectable at stage 25 after injection of *Xbra3* but not after injection of *Xbra*. *Xnot* is widely expressed at early stages in *Xenopus* and was detectable in control caps at stage 12, in agreement with previous reports (Von Dassow *et al.*, 1993).

Expression of *Xnot* is not restricted to the dorsal mesoderm, but is also detected in the floorplate of the neural tube (Von Dassow *et al.*, 1993). To test whether neural

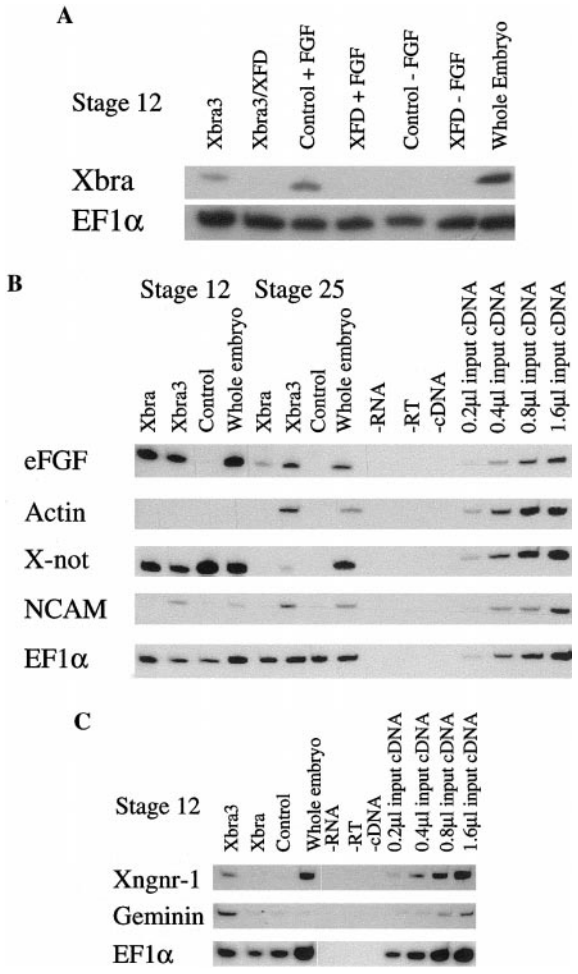

FIG. 5. Mesoderm-inducing activity of Xbra3. Embryos were injected with *Xbra3* message at the two-cell stage and RNA was extracted from caps isolated at the stages indicated and analysed by RT-PCR. (A) Xbra3 induces mesoderm in an FGF-dependent manner. *Xbra* is induced after injection of *Xbra3* mRNA or treatment with FGF in the absence but not in the presence of XFD. (B) Xbra3 induces markers of dorsal mesoderm and neural tissue. While both Xbra and Xbra3 induce eFGF at stages 12 and 25, only Xbra3 induces *cardiac actin* and *Xnot* at stage 25. Induction of *NCAM* is seen at stages 12 and 25 only in response to Xbra3. (C) Xbra3 induces the early neurogenic genes *geminin* and *Xngnr-1* in animal caps isolated from *Xbra3*-injected embryos at stage 12, whereas Xbra does not. The cDNA preparations analysed in C are the same as those analysed in Fig. 7.

tissue was present, we measured the expression of the neural-specific marker, *NCAM*, in caps from *Xbra3*-injected embryos. In agreement with previous reports (Cunliffe and Smith, 1992) we were unable to detect *NCAM* induction after *Xbra* injections, but this marker was clearly expressed at low levels in caps at both stage 12 and stage 25 in response to overexpression of Xbra3. These observations demonstrate a major difference in the inducing activities of

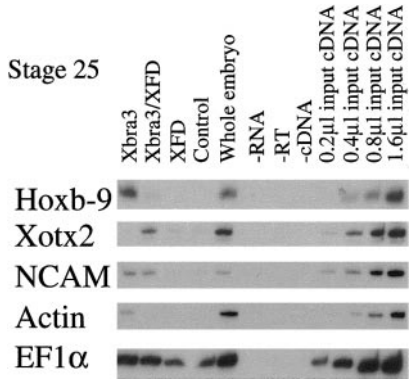

FIG. 6. Xbra3 induces posterior neural markers in an FGF-dependent manner. Embryos were injected at the two-cell stage with the mRNAs indicated and analysed for markers of neural tissue at stage 25 by RT-PCR. *NCAM* is induced by Xbra3 in the presence or absence of XFD. The posterior marker *Hoxb-9* is induced after injection of *Xbra3* alone whereas co-injection of *XFD* abolishes expression of *Hoxb-9* and allows expression of the anterior marker *Xotx2*.

the two proteins (Fig. 5B; see also Fig. 6) and suggest a role for Xbra3 in the induction of neural tissues.

Xbra3 Induces Nervous Tissue

Initial induction of neural differentiation initiated by the inhibition of BMP signalling (see Introduction) is followed by the expression of molecules such as Zic-r1 (Mizuseki *et al.*, 1998) and geminin (Kroll *et al.*, 1998) in a wide dorsal domain. These molecules induce the expression of proneural genes in cells which will contribute to the nervous system. The product of one such gene is Xngnr-1, a bHLH protein expressed at a very early stage of neural differentiation (Ma *et al.*, 1996). We assessed the ability of both Xbra and Xbra3 to induce *geminin* and *Xngnr-1* expression in caps assayed at stage 12, by which time neural induction should have occurred.

As seen in Fig. 5C, both *geminin* and *Xngnr-1* are clearly induced by stage 12 in caps from embryos injected with *Xbra3* message, but not in those injected with *Xbra*. This suggests that Xbra3 induces the expression of genes which act at a very early stage in neural determination, possibly the stage at which the initial distinction is made between cells fated to become epidermis and those which can contribute to the nervous system.

To further characterise the type of neural tissue induced by Xbra3, which expresses the panneural marker *NCAM*, we assayed for the expression of the posterior neural marker, *Hoxb-9* (*XlHbox6*) (Wright *et al.*, 1990), and the anterior neural marker, *Xotx2* (Blitz and Cho, 1995). In addition, as we have shown that induction by Xbra3 of mesoderm which expresses *Xbra* is dependent on FGF signalling, co-injection of *Xbra3* message with *XFD* was performed to investigate whether neural tissue formation in the isolated caps required either the formation of tissue expressing markers of dorsal mesoderm or signalling by FGF. Interference with the formation of mesoderm in caps injected with *XFD* is confirmed by the lack of cardiac actin expression at stage 25 (Fig. 6). These caps also failed to express Xbra at stage 12 (see Fig. 5A). Interestingly, *NCAM* is induced by Xbra3 both in the presence and in the absence of XFD, suggesting that at least part of the neural induction occurring in the caps is via an FGF-independent pathway. In the presence of an intact FGF signalling pathway the neural tissue formed is entirely posterior in character as shown by the induction of the posterior marker *Hoxb-9* in the absence of *Xotx2* induction. Furthermore, this induction of posterior nervous system is totally dependent on FGF signalling, suggesting that neural tissue of a posterior character is induced in an FGF-dependent manner. In contrast, the anterior neural marker *Xotx2* is induced by Xbra3 only when FGF signalling is ablated by co-injection of *XFD*. As is the case for all markers tested, *Xotx2* is not induced after injection of *XFD* alone. This suggests that under conditions under which FGF signalling is inhibited, Xbra3 induces anterior neural tissue. These observations lead to the possibility that the induction of *NCAM* in the presence and absence of FGF signalling reflects the formation of neural tissue of different characters.

To investigate further the mechanism by which Xbra3 induces neural tissue, we assayed the expression of known neural inducers in caps from embryos injected with *Xbra3* mRNA (Fig. 7). *Xngnr-1* and *geminin* are normally expressed at gastrula stages (Chintnis *et al.*, 1995; Ma *et al.*, 1996) and are induced by Xbra3. Thus, if Xbra3 acts through up-regulation of known neural inducers, then differential expression of these molecules should become detectable by these stages in caps from *Xbra3*-injected embryos and not in those injected with *Xbra*. Neither *chordin* nor *Xnr3* was induced by stage 12 in response to either Xbra3 or Xbra. However, *follistatin* and *noggin* mRNAs were observed to an approximately equal extent after injection of either *Xbra* or *Xbra3* mRNAs. As neural induction is not observed following injections with *Xbra*, we conclude that the levels of *follistatin* and *noggin* induced are insufficient to lead to neural tissue formation. An alternative route through which Xbra3 might cause neural induction in caps is through suppression of *BMP4* expression. However, caps from embryos injected with *Xbra3* mRNA exhibit no reduction in expression of *BMP4* mRNA (Fig. 7). Thus, it appears that induction of tissue of a neural character by Xbra3 occurs via a mechanism not involving regulation of expression of these known neural inducers.

Induction of Neural Tissue by Xbra3 Can Operate in a Non-Cell-Autonomous Manner

Xbra3 is expressed in dorsal mesoderm and not in cells fated to contribute to the nervous system. Thus, if the induction of neural markers in caps expressing Xbra3 mim-

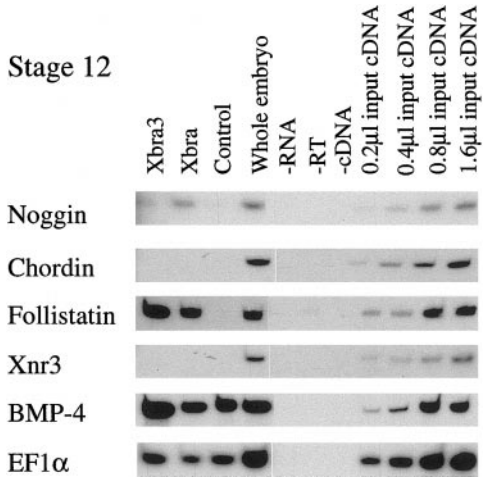

FIG. 7. Xbra3 induces early neural genes by a mechanism independent of known neural inducers. Embryos were injected with either *Xbra* or *Xbra3* message at the two-cell stage and caps analysed for known neural-inducing molecules at stage 12 by RT-PCR. *Chordin* and *Xnr3* are not induced by either Xbra or Xbra3. *Follistatin* and *noggin* mRNAs are induced by both Xbra and Xbra3 to similar levels at the stages tested. *BMP4* expression is not inhibited by the expression of either Xbra or Xbra3. The cDNA preparations analysed are the same as those used in Fig. 5C.

ics a function which operates during normal development, Xbra3 should be able to induce expression of neural markers in cells which themselves are not expressing Xbra3. To test this prediction, we examined whether animal caps from embryos injected with *Xbra3* message were able to induce expression of *Hoxb-9* in caps from uninjected embryos when the two caps were cultured in contact with each other. These experiments were performed using combinations of caps from *X. laevis* and *X. borealis* embryos. In order to detect specifically *Hoxb-9* transcripts expressed in different caps, we cloned portions of the *X. borealis Hoxb-9* cDNA and designed primers specific for the *X. laevis* or *X. borealis Hoxb-9* message. These primers enabled specific detection of *Hoxb-9* expression in either *X. laevis* or *X. borealis* embryos (Fig. 8A). In these experiments, cDNA synthesised for RT-PCR was equalised on the basis of incorporation of ^{32}P-labelled GTP, rather than by amplification of a ubiquitous transcript such as EF1-α (see Materials and Methods). As is the case of *X. laevis*, overexpression of Xbra3 but not Xbra in caps from *X. borealis* embryos results in expression of *Hoxb-9*. To examine the ability of Xbra3-expressing cap cells to induce *Hoxb-9* in a non-cell-autonomous manner, caps from *X. laevis* embryos injected with *Xbra3* or *Xbra* message were cocultured together with caps from uninjected *X. borealis* embryos (see Fig. 8B). Analysis of *X. borealis Hoxb-9* transcripts produced in these sandwiches shows that the *X. borealis Hoxb-9* gene is activated in *X. borealis* caps cocultured with Xbra3-expressing *X. laevis* caps but not when cocultured with Xbra-expressing caps (Fig. 8C). These results indicate that Xbra3-expressing animal cap cells can induce neural tissue in a non-cell-autonomous manner.

DISCUSSION

Xbra has long been established as a homologue of the mouse *Brachyury* gene, acting early in the series of events

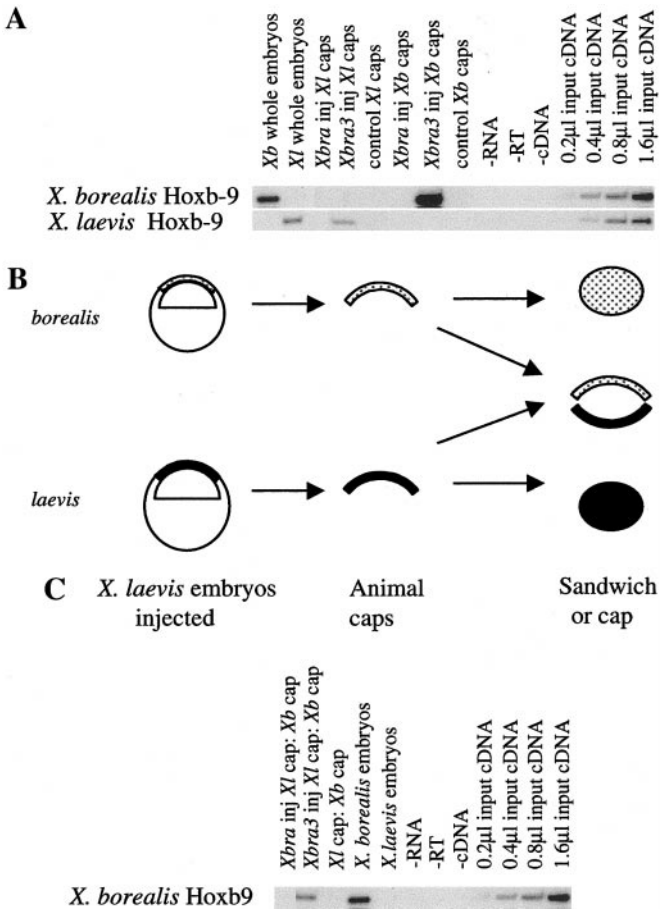

FIG. 8. Xbra3 can induce *Hoxb-9* expression in a non-cell-autonomous manner. A partial cDNA representing a *Hoxb-9* mRNA was cloned from *X. borealis* and primers which amplified specifically *borealis Hoxb-9* were designed. Caps were isolated from embryos injected with *Xbra3* mRNA and cultured to stage 25 by which time *Hoxb-9* expression was detected in both species (A). Caps from *X. laevis* embryos injected with *Xbra* or *Xbra3* RNA were cultured in apposition with caps from uninjected *borealis* embryos (see B), and RNA from the total sandwiches was extracted and amplified using primers specific for *X. borealis Hoxb-9*. *X. borealis Hoxb-9* was found to be expressed in sandwiches containing *laevis* caps expressing Xbra3, but not in sandwiches with Xbra-expressing caps (C). In (A) and (C), cDNA input to the RT-PCR was equalised with respect to [^{32}P]GTP incorporation during cDNA synthesis (see Materials and Methods).

leading to mesoderm formation. In this work, we have demonstrated the existence of a second, distinct *Brachyury* homologue in *Xenopus,* which we have named *Xbra3,* and have shown that it is expressed in the mesoderm at later stages than *Xbra. Xbra3* can function like *Xbra* in responding to mesoderm-inducing signals and inducing the expression of markers of mesoderm formation and differentiation. Unlike *Xbra,* however, *Xbra3* induces the expression in caps of markers of neural tissue and is able to cause expression of genes acting at the earliest known stages of neural determination.

Xbra3 Represents a Second Xenopus Brachyury Homologue

Brachyury homologues have been cloned from several vertebrates including mice, frogs, zebrafish, chicken, and humans (Herrmann *et al.,* 1990; Smith *et al.,* 1991; Schulte-Merker *et al.,* 1994; Kispert *et al.,* 1995b; Edwards *et al.,* 1996). In addition, many additional T-box-containing genes have been identified, possessing regions of homology to the DNA-binding region of Brachyury (Agulnik *et al.,* 1995; Bollag *et al.,* 1994; Knezevic *et al.,* 1997). The gene we describe here represents a second homologue of *Brachyury* in the frog, *Xenopus,* and appears likely to play a functional role during development distinct from that of *Xbra.* We believe that this gene is nonallelic with *Xbra.* This conclusion is supported by the observed lack of sequence similarity in noncoding regions of the two loci, the distinct expression patterns of the two genes, and the differences in consequences of overexpression of their products in developing embryos. The tetraploid nature of the *X. laevis* genome leads to the possibility that *Xbra* and *Xbra3* are pseudoallelic, having been separated by the genome duplication event which is estimated to have occurred about 30 million years ago (Bisbee *et al.,* 1977). The high degree of divergence between Xbra and Xbra3 in coding regions outside the T box, together with a lack of detectable homology between noncoding and intron regions of the two loci, suggests this not to be the case. We propose that *Xbra* and *Xbra3* represent independent *Xenopus* homologues of the *Brachyury* gene which fulfil different functions during development of the embryo.

Xenopus is not the only chordate in which two homologues of *Brachyury* have been found. However, whereas the products of the two amphioxus genes, *AmBra* 1 and *AmBra* 2 (Holland *et al.,* 1995), show 94% amino acid identity, implying their origin results from a relatively recent duplication event rather than representing the ancestral condition for all chordates, the two predicted *Xenopus* proteins show only 73% overall identity while both clearly representing Brachyury homologues. In the chicken, two genes closely related to mouse *Brachyury* have been described (Knezevic *et al.,* 1997), the predicted proteins showing overall identity of 62%. One of these proteins, Ch-T, is clearly the orthologue of mouse Brachyury, showing 97% identity with the mouse protein within the T domain and 82% identity overall. The other chicken protein, Ch TbxT, shows 85% identity with the mouse Brachyury T domain and 60% identity with mouse Brachyury overall. This contrasts with the situation in *Xenopus,* in which Xbra and Xbra3 show similar levels of identity with mouse Brachyury. The identities between the Xbra and Xbra3 T domains and the mouse protein are 89 and 85%, respectively, while the overall identities between the *Xenopus* and the mouse proteins are 76 (Xbra) and 71% (Xbra3). Thus, it may not be meaningful to describe either of these genes as the *Xenopus* orthologue of *Brachyury*. It may be the case that subsequent to duplication, the two genes have taken on different roles in development which encompass the role played by *Brachyury* in the mouse. Whether the two *Xenopus* genes have functions in addition to those played by the mouse gene is not clear.

The later onset and longer duration of expression of *Xbra3* clearly distinguishes this gene from that of *Xbra* and suggests a role for *Xbra3* in postgastrulation events. In the mouse, *Brachyury* is expressed from just prior to the morphological appearance of the primitive streak, when the message is detectable in cells of the embryonic ectoderm (Thomas and Beddington, 1996; D.S., personal observations), and subsequently in the primitive streak-derived mesoderm and the notochord where expression persists at least until day 17.5 (Wilkinson *et al.,* 1990). This expression pattern, particularly the late maintenance of expression in the notochord, is also observed in other vertebrates, including zebrafish and chick (Schulte-Merker *et al.,* 1994; Kispert *et al.,* 1995b). We confirm that in *Xenopus, Xbra* expression in the notochord is down-regulated from a much earlier stage of development, being virtually undetectable in the notochord either by *in situ* hybridisation to tailbud stages or by RT-PCR analysis of notochord RNA. In contrast, *Xbra3* expression is maintained in the notochord beyond stage 35 (Hayata *et al.,* 1999; this work). This difference in the expression patterns of the two *Xenopus Brachyury* homologues suggests that they may play distinct roles in embryogenesis and that *Xbra3* may be involved in the later phases of dorsal mesoderm development and axial patterning.

Like the products of other *Brachyury* homologues (Kispert and Herrmann, 1994; Schulte-Merker *et al.,* 1994), Xbra3 is a nuclear protein and the high similarity of the N-terminal half of the predicted polypeptide to the corresponding regions of Xbra and Brachyury suggests that the DNA binding function demonstrated for this region of Brachyury (Kispert and Herrmann, 1993), Xbra (Conlon *et al.,* 1996; Muller and Herrmann, 1997), and human T (Papapetrou *et al.,* 1997) is conserved. Furthermore, all of the residues shown to be involved in direct interaction with the DNA in an Xbra T domain–DNA complex (Muller and Herrmann, 1997) are identical in Xbra3. Xbra has been shown to contain a transcriptional activation domain between residues 304 and 387, capable of activating transcription via synthetic binding sites for the Brachyury protein when expressed in mouse 3T3 cells (Conlon *et al.,* 1996).

This sequence is only 50.6% identical to the corresponding region of the Xbra3 polypeptide, suggesting either a difference in the nature of the interaction of the two proteins with the transcriptional apparatus or that Xbra3 may not function in this manner.

Xbra3 Induces Mesoderm and Neural Tissue

In previous studies, expression of Xbra alone has not been shown to induce neural markers, but rather to induce mesoderm (Cunliffe and Smith, 1992; O'Reilly *et al.*, 1995; Schulte-Merker and Smith, 1995; Taira *et al.*, 1997), a result consistent with our observations reported here. Xbra3 will also induce mesoderm, although it is unclear whether this relies on its ability to induce *Xbra*. The ability of *Xbra3* to induce *Xbra* expression relies on the presence of an intact FGF signalling pathway. In the presence of XFD, Xbra3 is unable to induce *Xbra* expression, and *cardiac actin* fails to be expressed in caps cultured to later stages. One explanation for these observations is that Xbra3 acts to induce *Xbra*, which, via the previously documented autoregulatory loop (Isaacs *et al.*, 1994), induces expression of *eFGF* and itself, leading to mesoderm formation. An alternative would be that Xbra3 can act directly on *eFGF*, thus inducing both *Xbra* and other mesodermal genes, including *cardiac actin*, indirectly.

Xbra3 Acts Early in Neural Induction

The activity of Xbra3 in inducing expression of neural markers was unexpected and clearly demonstrates a distinct activity of Xbra3 in comparison with Xbra. Current models suggest that the specification of vertebrate neurectoderm proceeds by a mechanism involving the inhibition of BMP class molecules, allowing expression of neurogenic genes in cells destined to become neural (Hemmati-Brivanlou and Melton, 1997). This model does not, however, exclude other routes leading to neural specification, as exemplified by the action of Smad 10, which can directly induce both anterior and posterior nervous tissue by a mechanism which does not appear to involve the inhibition of BMP signalling (Le Seuer and Graff, 1999). The onset of neural development is characterised by the expression of genes such as *geminin* (Kroll *et al.*, 1998) and *Zic-r1* (Mizuseki *et al.*, 1998), in a broad domain which includes cells which will not themselves proceed to a neural fate. Local inhibition of BMP4 signalling, both through inhibition of *BMP4* transcription by proneural gene products and through the action of secreted BMP4 inhibitors, results in expression of neurogenic genes such as *Xngnr-1* (Ma *et al.*, 1996) in cells destined to become neural. Later events, probably including activation and lateral inhibition of neurogenic gene expression through the Notch and Delta pathway, act to select cells which will activate further downstream events in neuronal differentiation, including expression of *XneuroD* and markers of terminal differentiation such as *N-tubulin* (see Ma *et al.*, 1996). Our data show that overexpression of Xbra3 leads to activation of both *geminin* and *Xngnr-1* in caps which later express the neural markers *NCAM* and *Hoxb-9*. Thus, Xbra3 appears to induce neural tissue through a mechanism which involves activating the earliest known steps of *Xenopus* neurogenesis, though the precise mechanism by which this occurs is currently unclear.

Xbra3 Induces Posterior Nervous Tissue by an FGF-Dependent Mechanism

In contrast to the currently known inducers in *Xenopus* which act by inhibition of BMP family molecules to induce anterior neural markers (Hemmati-Brivanlou *et al.*, 1994; Lamb *et al.*, 1993; Sasai *et al.*, 1995), Xbra3 is unusual as the neural tissue formed as a result of Xbra3 overexpression is entirely of posterior character. Xbra3 expression in the presence of a functional FGF signalling pathway leads to the expression of *NCAM* and the posterior marker *Hoxb-9*. Expression of Xbra3 plus XFD abolishes *Hoxb-9* expression and results in the expression of the anterior neural marker *Xotx2*. This complete switch is dramatic and contrasts with other observations in neural patterning in which a gradation in anterior–posterior register is normally seen, as the anterior neural tissue formed by inducers such as noggin, chordin, and follistatin (activation) is transformed to more posterior phenotypes following the effects of FGF and other treatments (transformation) (Barnett *et al.*, 1998; Cox and Hemmati-Brivanlou, 1995; Kengaku and Okamoto, 1995; Lamb and Harland, 1995; Storey *et al.*, 1998; Taira *et al.*, 1997). One possibility is that neural induction by Xbra3 is achieved under conditions of high eFGF, which results in all induced cells adopting a posterior neural phenotype. Direct induction of markers of posterior neural tissue, in the absence of anterior neural markers, has previously been reported in experiments treating chick extraembryonic tissues with FGF (Storey *et al.*, 1998). In this case, *Brachyury* was also induced, in addition to and in advance of posterior neural markers, giving rise to the suggestion that chick *Brachyury* may also be involved in induction of posterior neural tissue (see discussion in Storey *et al*, 1998).

Xbra3 Can Act in a Non-Cell-Autonomous Manner

During normal embryogenesis, Xbra3 is not expressed in cells which contribute to the nervous system. Thus, in order to play a role in neural induction, Xbra3 expression must either directly or indirectly lead to the production of a secreted factor which acts on ectoderm cells. The induction of *Hoxb-9* in a non-cell-autonomous manner by caps from embryos overexpressing Xbra3 suggests that the action of Xbra3 in induction of markers of neural tissue can occur in a non-cell-autonomous manner. This indicates that Xbra3 expression causes the production of a secreted factor which can act to cause adjacent cells to adopt neural characteristics. The nature of this factor remains unclear, but we were unable to detect a difference in the ability of

Xbra and Xbra3 to induce expression of *BMP4* or the neural inducers *noggin, follistatin, chordin,* and *Xnr3.*

At present, the mechanism by which Xbra3 leads to the expression of neural markers in adjacent tissue remains unclear. Xbra3 overexpression might lead to the production of neural markers via a mechanism involving the induction of dorsal mesoderm. However, our observation that coexpression of XFD with Xbra3 inhibits induction of *actin* and *Xbra* whilst maintaining induction of *NCAM* and allowing induction of *Xotx2,* suggests that the neuralising activity of Xbra3 may not rely on the previous induction of mesoderm expressing *Xbra* or *actin.* It is possible that mesoderm of a character not expressing either of these two markers is induced (Graff *et al.,* 1996); at present this cannot be excluded. The alternative is that Xbra3 activates the expression of a secreted neural inducer independent of its role in the production of dorsal mesoderm.

Overall, we show that expression of Xbra3 induces neural development, in addition to inducing *Xbra* and *eFGF,* via the induction of *geminin* and *Xngnr-1.* In the presence of an intact FGF signalling pathway, *Xbra3* expression leads to the production of posterior neural tissue. When the activity of FGF is inhibited by coexpression of XFD, neuralisation still occurs, but tissue of an anterior character is produced. This suggests that Xbra3 has activities distinct from that of Xbra and this, together with the observed presence of the *Xbra3* gene product during neurulation and in the notochord at later stages, suggests that *Xbra3* may play a distinctive role in the induction and patterning of the nervous system. In addition, these observations provide further evidence that FGF can act as one of the posteriorising signals in the anteroposterior patterning of the nervous system (Barnett *et al.,* 1998; Cox and Hemmati-Brivanlou, 1995; Kengaku and Okamoto, 1995; Lamb and Harland, 1995; Storey *et al.,* 1998; Taira *et al.,* 1997; Holowacz and Sokol, 1999).

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