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Inhibition of Xbra transcription activation causes defects in mesodermal patterning and reveals autoregulation of Xbra in dorsal mesoderm

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SUMMARY

The *Brachyury* (*T*) gene is required for formation of posterior mesoderm and for axial development in both mouse and zebrafish embryos. In this paper, we first show that the *Xenopus* homologue of *Brachyury, Xbra,* and the zebrafish homologue, *no tail* (*ntl*), both function as transcription activators. The activation domains of both proteins map to their carboxy terminal regions, and we note that the activation domain is absent in two zebrafish *Brachyury* mutations, suggesting that it is required for gene function. A dominant-interfering *Xbra* construct was generated by replacing the activation domain of Xbra with the repressor domain of the *Drosophila* engrailed protein. Microinjection of RNA encoding this fusion protein allowed us to generate *Xenopus* and zebrafish embryos which show striking similarities to genetic mutants in mouse and fish. These results indicate that the function of *Brachyury* during vertebrate gastrulation is to activate transcription of mesoderm-specific genes. Additional experiments show that Xbra transcription activation is required for regulation of Xbra itself in dorsal, but not ventral, mesoderm. The approach described in this paper, in which the DNA-binding domain of a transcription activator is fused to the engrailed repressor domain, should assist in the analysis of other *Xenopus* and zebrafish transcription factors.

Key words: *Xenopus*, mesoderm, *Brachyury*, transcription activation, *engrailed* repressor, zebrafish, *no tail*

INTRODUCTION

The *Brachyury* (*T*) gene is required for formation of posterior mesoderm and for axial development in both mouse and zebrafish embryos (Herrmann et al., 1990; Halpern et al., 1993; Schulte-Merker et al., 1994; Conlon et al., 1995, 1996). In these species, and in *Xenopus*, the gene is expressed transiently throughout the presumptive mesoderm and later in the notochord and in posterior tissues (Wilkinson et al., 1990; Herrmann, 1991; Smith et al., 1991; Schulte-Merker et al., 1992). In *Xenopus*, over-expression of *Brachyury* causes ectopic posterior mesoderm formation (Cunliffe and Smith, 1992, 1994; Rao, 1994), with different concentrations of Brachyury inducing different types of mesoderm (Cunliffe and Smith, 1992; O'Reilly et al., 1995). Since *Brachyury* acts cell-autonomously (Rashbass et al., 1991) and encodes a nuclear sequence-specific DNA-binding protein (Schulte-Merker et al., 1992; Kispert and Herrmann, 1993; Cunliffe and Smith, 1994), it is possible that it functions by regulating the transcription of downstream mesoderm-specific genes.

In this paper, we show that the *Xenopus* homologue of *Brachyury*, *Xbra*, and the zebrafish homologue, *no tail* (*ntl*), both function as transcription activators. The activation domains of the proteins map to their carboxy terminal regions and they function efficiently in yeast and in mouse fibroblasts, suggesting that activation does not require tissue-specific auxiliary transcription factors. The Brachyury activation domain is absent in two zebrafish *Brachyury* mutants, suggesting that it is required for Brachyury function. We have investigated this idea in *Xenopus* by creating an interfering *Xbra* construct in which the activation domain of Xbra is replaced by the repressor domain of the *Drosophila* engrailed protein (Jaynes and O'Farrell, 1991; Han and Manley, 1993). Over-expression of this fusion protein causes the formation of *Xenopus* and zebrafish embryos that show striking similarity to genetic mutants in mouse and fish, showing that the function of *Brachyury* during vertebrate gastrulation is to activate transcription of mesoderm-specific genes.

Additional experiments studied the expression of endogenous *Xbra* and of *Pintallavis* and *sonic hedgehog* (*shh*) in embryos in which *Xbra* function is inhibited. Expression of *Xbra* in dorsal mesoderm requires transcription activation by Xbra, while ventral and posterior expression is independent of autoregulation. By contrast, *Pintallavis* and *shh*, which are expressed exclusively in dorsal tissues (Ruiz i Altaba and Jessell, 1992; Ruiz i Altaba et al., 1995), are expressed normally in such embryos.

Finally, the approach described in this paper, in which the DNA-binding domain of a transcription activator is fused to the engrailed repressor domain, may assist in the analysis of other *Xenopus* and zebrafish transcription factors.

MATERIALS AND METHODS

Yeast transcription assays and transposition mutagenesis

GAL4^{DBD}-Xbra and GAL4^{DBD}-Ntl were generated by mutating the initiating methionine of Xbra (Smith et al., 1991) and Ntl (Schulte-Merker et al., 1992, 1994) to an *Eco*RI site by PCR mutagenesis. The corresponding fragments were cloned in-frame to the *Eco*RI site of the GAL4 DNA-binding domain (GAL4^{DBD}) contained in the yeast vector pGBT9. Fusions were confirmed by sequencing. Transposon TnXR, a 4888 bp derivative of Tn1000 (Morgan et al., 1996) was introduced into the GAL4^{DBD}-Xbra and GAL4^{DBD}-Ntl plasmids (Sedgwick and Morgan, 1994). Targeted constructs were identified by restriction mapping and PCR analysis using primers specific for GAL4 and TnXR. Exact positions of the insertions were determined by transposon-primed nucleotide sequencing into flanking DNA. 5′ and internal deletions were created by conventional techniques. Transcription activation was assessed by the expression of *GAL4^{UAS}-lacZ* and *GAL4^{UAS}-Ura* reporters in *S. cerevisiae* strains YN190 and Y166. At least three transformations were performed for each deletion, with a minimum of 250 colonies scored per transformation.

Transient transfection analyses

Expression plasmids encoding full-length Xbra and Ntl were derived from the corresponding pGBT9 constructs (see above). Xbra and Ntl deletion constructs were generated by PCR or conventional techniques. The Xbra-En^{R} construct was generated by fusing the Xbra DNA-binding domain (amino acids 1–232) to a fragment encoding amino acids 2–298 of the *Drosophila* engrailed protein. This region of engrailed was derived from plasmid MEnT (Badiani et al., 1994) and the Myc tag (Evan et al., 1985) of MEnT was included in Xbra-En^{R}. All constructs were cloned in-frame into the *Eco*RI site in the plasmid MLVplink (Dalton and Treisman, 1992). Fusions were confirmed by sequencing and in vitro translation of all constructs gave proteins of the correct size (not shown). The CAT reporter plasmid pBLCAT2 (Luckow and Schutz, 1987) was modified such that two copies of an oligonucleotide containing the Brachyury-binding site TTTCACACCTAGGTGTGAAA (Kispert and Herrmann, 1993) were inserted into the *Sal*I site upstream of the promoter region. Lipofections were carried out according to Marais et al. (1995). Cells were cultured for 48 hours and extracts were then assayed for CAT activity. MLV*lacZ* was cotransfected as a control for transfection efficiency (Hill et al., 1993). All constructs were translated in a reticulocyte lysate system and shown by band-shift assays to bind to a Brachyury-binding site with equivalent affinity. Truncation of Xbra and Ntl did not affect the stability or nuclear localization of either protein, as judged by in vivo competition experiments in which the truncated proteins were able to displace wild-type Xbra and Ntl from their cognate binding sites.

Xenopus embryos and microinjection

Xenopus embryos were obtained by in vitro fertilisation (Smith and Slack, 1983). They were maintained in 10% Normal Amphibian Medium (NAM; Slack, 1984) and staged according to Nieuwkoop and Faber (1975). Embryos for microinjection were transferred to 75% NAM containing 4% Ficoll Type 400 (Sigma). Fragments encoding Xbra-En^{R} and En^{R} were subcloned into the vector pSP64T (Krieg and Melton, 1984) and RNA was synthesised according to Smith (1993). In vitro translation of this material gave products of the correct size (data not shown). RNA (a total of 0.5 ng in approximately 10 nl water) was injected at the 1-cell stage or into both cells of the 2-cell stage embryo using an air-driven system (Smith, 1993). After injection, embryos were transferred gradually to 10% NAM and allowed to develop to the desired stage.

Histology, immunocytochemistry and in situ hybridization

For histological analysis, specimens were fixed and sectioned as described by Smith (1993). Sections (7 μm) were stained by the Feulgen /Light Green/Orange G technique (see Smith, 1993). Whole-mount immunocytochemistry with antibodies MZ15 (Smith and Watt, 1985), Tor70 (Bolce et al., 1992) and 12/101 (Kintner and Brockes, 1984) was carried out essentially as described by Smith (1993). Whole-mount in situ hybridisation was carried out essentially as described by Harland (1991). A digoxigenin-labelled riboprobe specific for the 3′ region of *Xbra* was prepared by digesting pXT1 (Smith et al., 1991) with *Cla*I and transcribing with T7 RNA polymerase. This probe does not recognise the Xbra-En^{R} construct described above. A probe specific for *Pintallavis* was prepared according to O'Reilly et al. (1995). A *Xenopus sonic hedgehog* probe was kindly provided by J-P. Concordet and P. Ingham (ICRF, London).

Zebrafish embryos

Each cell of zebrafish embryos at the 2- to 8-cell stage was injected with approximately 2 pg RNA encoding Xbra-En^{R} or En^{R} using an gas-driven system. Embryos were allowed to develop for 20 hours before being fixed in 4% paraformaldehyde and photographed.

RESULTS

Xbra and no tail are transcription activators whose activation domains map to their C termini

The abilities of Xbra and Ntl to activate transcription were first assayed in the budding yeast *S. cerevisiae*. Full-length Xbra and Ntl proteins were expressed as N-terminal fusions with the DNA-binding domain of *S. cerevisiae* GAL4 protein. Both the GAL4-Xbra and the GAL4-Ntl constructs caused yeast to grow in the absence of uracil and stimulated expression of a

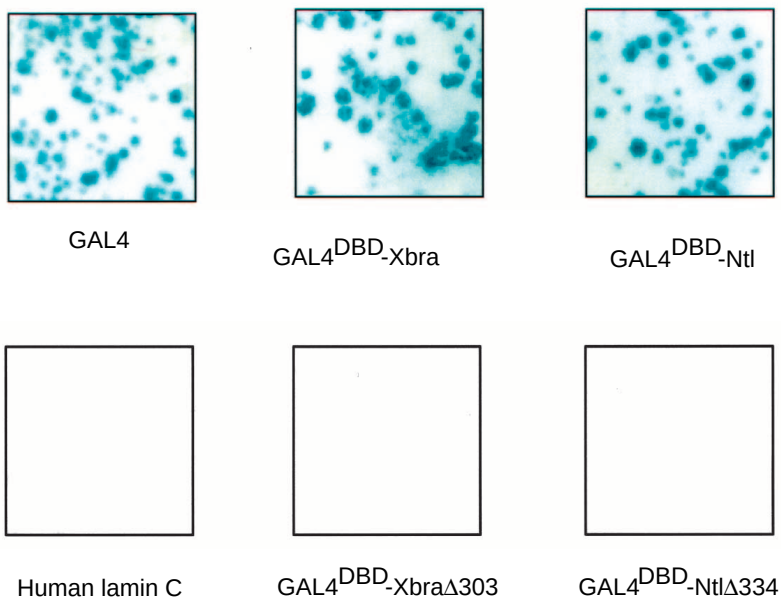

Fig. 1. Transcription activation by Xbra and Ntl. Transcription activation by Xbra and Ntl in yeast was demonstrated by induction of the expression of a *GAL4^{UAS}-lacZ* reporter gene in the presence of a plasmid encoding the *S. cerevisiae* GAL4 DNA-binding domain fused to either Xbra or Ntl coding sequences. Top row: transcription activation by full-length GAL4 (positive control) and by fusions of GAL4^{DBD} to Xbra and Ntl, respectively. Bottom row: lack of transcription activation by fusions of GAL4^{DBD} to human lamin C (negative control), to Xbra truncated at amino acid 303, and to Ntl truncated at amino acid 334.

GAL4^{UAS}*-lacZ* reporter sequence (Fig. 1), indicating that Xbra and Ntl proteins are transcription activators in this system.

The transcription activation domains of Xbra and Ntl were mapped using an extensive truncation series of the Xbra (n=25) and Ntl (n=55) coding sequences generated by transposition mutagenesis in *E. coli* (Sedgwick and Morgan, 1994; Morgan et al., 1996) (Figs 1, 2). These experiments identified a domain in the carboxyl-terminal portion of Xbra (amino acids 303 to 387) and of Ntl (amino acids 312 to 407) which is necessary and sufficient for transcription activation (Fig. 2).

To confirm these results, and to test whether Xbra and Ntl function as transcription activators when contacting DNA directly, plasmids encoding full-length and truncated versions of Xbra and Ntl were transfected into NIH3T3 cells along with a chloramphenicol acetyl transferase (CAT) reporter plasmid carrying two copies of a Brachyury-binding site (Kispert and Herrmann, 1993) upstream of a minimal promoter. Like mouse Brachyury (Kispert et al., 1995), both Xbra and Ntl caused strong activation of transcription (Fig. 3, lanes 2 and 4), while truncated versions of the proteins that lack the putative activation domains, including mutations in Ntl corresponding to the *ntl*^{b160} and *ntl*^{b195} alleles, had little or no activity (Fig. 3, lanes

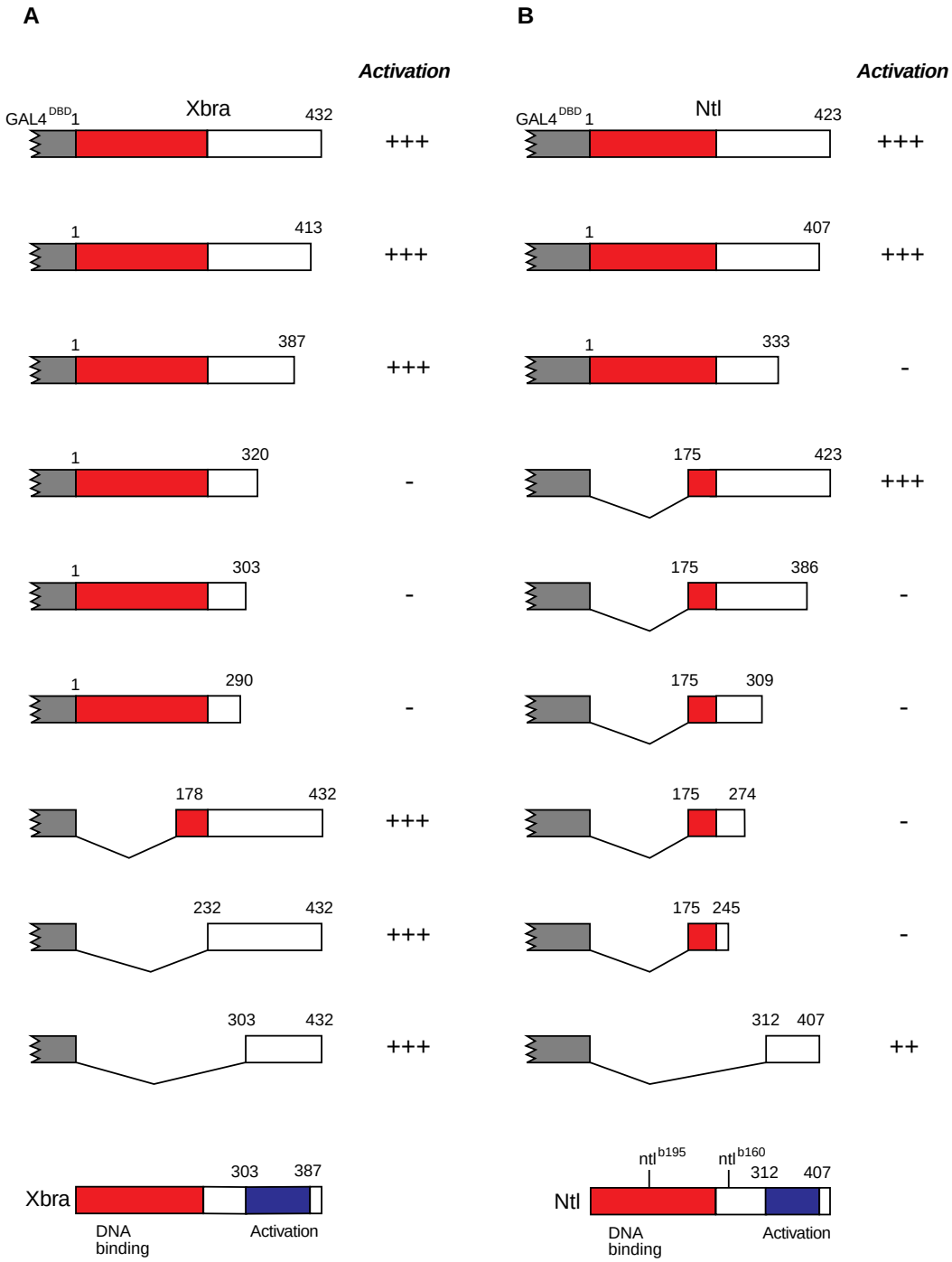

Fig. 2. Mapping of the Xbra and Ntl transcription activation domains using a modification of the assay described in Fig. 1. The GAL4 DNA-binding domain (DBD) was fused to a 3′ deletion series of Xbra or Ntl generated by transposition mutagenesis or to 5′ truncations and internal deletions generated by conventional techniques. Strong activation: '+++'; weaker activation: '++'; no activation: '-'.
(A) Representative examples of results obtained with Xbra deletions. The region mapped as the Xbra activation domain is shown at the bottom. This region gives slightly lower activation than that obtained by the full-length protein, and it is likely that a small number of flanking amino acids are required for full induction of transcription.
(B) Representative examples of results obtained with Ntl deletions. The region mapped as the Ntl activation domain is shown at the bottom. For both Xbra and Ntl, additional deletions and truncations gave results consistent with those described here. Interruptions of coding sequence due to the *ntl*^{b160} and *ntl*^{b195} mutations are indicated. In both A and B, the DNA-binding domain, shown in red, is based on Kispert and Herrmann (1993) together with band-shift analyses using Xbra and Ntl deletions (not shown).

3 and 5, and data not shown). Recent experiments have demonstrated that Xbra also activates transcription of a reporter gene in *Xenopus* oocytes (T. Mohun and M.-A. O'Reilly, personal communication).

Xbra-En^R inhibits transcription activation by Xbra

Genetic analysis in zebrafish suggests that the activation domain of Brachyury is necessary for biological function: the truncated protein encoded by *ntl^{b160}* cannot activate transcription and the phenotype of this mutant allele is similar to that of *ntl^{b195}*, whose more severely truncated protein product would not be predicted even to bind DNA (Kispert and Herrmann, 1993; Schulte-Merker et al., 1994). It is therefore likely that the function of Brachyury in vivo is to activate transcription of downstream mesoderm-specific genes. To investigate this question directly, we created an Xbra fusion protein (Xbra-En^R) designed to interfere with the transcription activation function of wild-type Xbra (Fig. 4). In this construct, the DNA-binding domain of Xbra is fused to the repressor domain of the *Drosophila* engrailed protein. This domain of engrailed is an active repressor of transcription that impairs the ability of a wide variety of transcription activators to interact with the basal transcription machinery (Han and Manley, 1993). The domain is functional when fused to heterologous DNA-binding domains, and chimeric proteins of this sort can act in *cis* to repress the activity of adjacent enhancer elements (Jaynes and O'Farrell, 1991; Badiani et al., 1994). Accordingly, Xbra-En^R is unable to activate transcription in transient transfection assays (Fig. 3, lanes 6 and 8) and it interferes with transcription activation by wild-type Xbra in a dose-dependent fashion, with complete inhibition obtained with a two-fold excess of Xbra-En^R over Xbra (Fig. 3, lanes 7 and 9). Transfection of a plasmid encoding only the DNA-binding domain of Xbra also inhibited transcription due to wild-type Xbra, but this required a ten-fold excess to achieve even slight inhibition (data not shown).

Inhibition of Xbra function in *Xenopus* embryos

In an effort to interfere with transcription activation by *Xbra*, *Xenopus* embryos were injected at the 2-cell stage with RNA encoding Xbra-En^R (n›800) or with RNA encoding En^R alone (n›200). All embryos developed perfectly normally until the early gastrula stage, but those injected with RNA encoding Xbra-En^R failed to complete gastrulation (Fig. 5), and by tadpole stages posterior structures were absent (Fig. 6B,C). Embryos injected with RNA encoding the engrailed repressor domain alone (En^R) were indistinguishable from uninjected controls (Figs 5, 6A,D), as are embryos injected with RNA encoding just the DNA-binding domain of Xbra (Cunliffe and Smith, 1992). Histological analysis confirmed the absence of posterior mesoderm at tadpole stages and indicated that muscle and, in some specimens, notochord, were present in anterior regions (Fig. 6D,E). Whole-mount immunocytochemistry using the notochord-specific antibody MZ15 (Smith and Watt, 1985) showed that approximately 60% of embryos injected with Xbra-En^R RNA contained patches of notochord in anterior regions (Fig. 6G); no MZ15-positive cells were detected in the remaining 40% (Fig. 6H). Additional experiments using the notochord-specific antibody Tor70 (Bolce et al., 1992) confirmed that notochord differentiation was inhibited in embryos injected with RNA encoding Xbra-En^R (Fig. 7). Finally, whole-mount immunocytochemistry using the muscle-specific antibody 12/101 (Kintner and Brockes, 1984) revealed that all embryos injected with Xbra-En^R RNA formed muscle, but that there was a graded loss of somitic tissue along the antero-posterior axis (Fig. 6I,J).

The phenotype of *Xenopus* embryos injected with Xbra-En^R RNA resembles that of genetic mutations in mouse and zebrafish embryos that lack functional *Brachyury* gene product; such embryos also show posterior truncations, a loss of mature notochord and defects in somite patterning (Herrmann et al., 1990; Halpern et al., 1993; Herrmann and

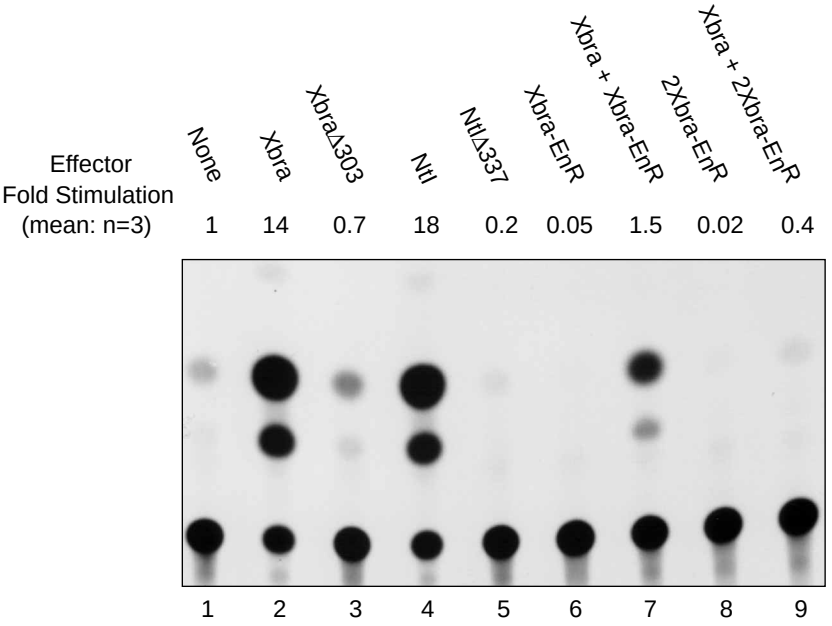

Fig. 3. Transcription activation by Xbra and Ntl and repression of activation by a dominant interfering Xbra-En^R construct. NIH3T3 cells were lipofected with the indicated effector plasmids together with a chloramphenicol acetyl transferase (CAT) reporter plasmid carrying two copies of the Brachyury-binding site (Kispert and Herrmann, 1993) upstream of a minimal promoter. Plasmids encoding wild-type Xbra (lane 2) or wild-type Ntl (lane 4) cause activation of transcription while the Xbra and Ntl alleles lacking the putative activator domain (see Fig. 2) have little or no effect (lanes 3 and 5). A plasmid encoding an Xbra allele in which the activation domain is replaced by the *Drosophila engrailed* repressor domain (Xbra-En^R) causes no activation (lanes 6 and 8) and it inhibits activation due to Xbra in a dose-dependent manner, with complete inhibition obtained with a two-fold excess of Xbra-En^R over Xbra (lanes 7 and 9). This experiment was carried out three times, with similar results obtained each time. Loading was normalised by reference to levels of β-galactosidase activity derived from the co-transfected MLV*lacZ* plasmid. Mean levels of stimulation are indicated. Xbra did not cause transcription activation in the absence of a Brachyury-binding site (not shown).

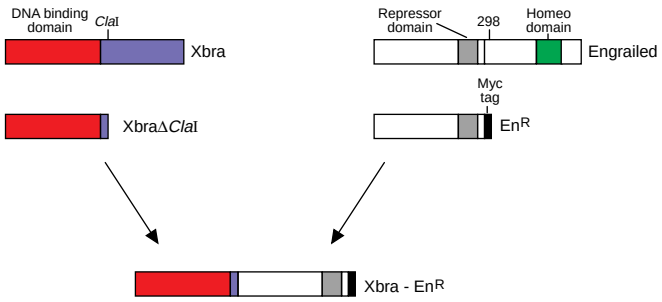

Fig. 4. Construction of Xbra-En^R. The entire carboxy terminal of Xbra, including the transcription activation domain, was removed by digestion with *Cla*I and the DNA-binding domain was then fused to amino acids 2-298 of the *Drosophila* engrailed protein.

Kispert, 1994; Schulte-Merker et al., 1994; Conlon et al., 1995). To test the specificity of this Xbra-En^R phenotype, RNA encoding Xbra-En^R was coinjected with RNA encoding wild-type Xbra (Fig. 8). Increasing levels of the wild-type RNA rescued the posterior defects caused by Xbra-En^R in a dose-dependent manner, although an Xbra-induced posteriorization of anterior structures occurred before the posterior defects were completely rescued (Fig. 8C).

Further evidence for the specificity of the Xbra-En^R phenotype was obtained by injecting Xbra-En^R RNA into each cell of zebrafish embryos at the 2- to 8-cell stage. Injected embryos displayed many abnormalities associated with genetic *ntl* mutants, including a lack of posterior mesoderm, inhibition of notochord differentiation and disruption of somite formation (Fig. 9). These observations, together with those described above, suggest that injection of Xbra-En^R RNA blocks Brachyury function in a specific manner, and indicate that the role of *Brachyury* in the vertebrate embryo is to activate transcription of genes required for normal mesodermal patterning and differentiation.

Autoregulation of Xbra in dorsal, but not in ventral, mesoderm

Previous studies in mouse and zebrafish embryos have suggested that maintenance of expression of *Brachyury* requires functional Brachyury protein (Herrmann, 1991; Schulte-Merker et al., 1994). We have examined this question in *Xenopus* by studying expression of endogenous *Xbra* in embryos injected with RNA encoding Xbra-En^R. At the early gastrula stage, comparison with controls revealed that expression of *Xbra* in such embryos was strongly down-regulated in dorsal, but not in ventral, mesoderm (compare Fig. 10A and B) and dorsal expression remained down-regulated in these tissues throughout gastrulation and neurulation (Fig. 10D; n›100). By contrast, *Xbra* remained strongly expressed in the notochord of control embryos (Fig. 10C).

The dorsal down-regulation of *Xbra* in embryos injected with Xbra-En^R might be due to complete loss of dorsal *Xbra*-expressing cells, or it may reflect a requirement for Xbra in maintaining its own expression in this region of the embryo. To distinguish between these possibilities, we studied expression of *Pintallavis*, which is normally expressed in dorsal mesoderm and in the developing notochord of *Xenopus* (Ruiz i Altaba and Jessell, 1992; O'Reilly et al., 1995). Injection of RNA encoding Xbra-En^R had no effect on expression of *Pintallavis* between stages 10 and 17 (Fig. 10E,F; n›50), indicating that the absence of *Xbra* in the dorsal region of such embryos is due not to cell loss, but to autoregulation of *Xbra* expression. Histological sections confirmed that expression of *Pintallavis* in Fig. 10H occurred in the notochord (data not shown). Similar results were obtained using a probe specific for *Xenopus sonic hedgehog* (*shh*) which, like *Pintallavis*, is expressed in dorsal mesoderm and the floorplate of *Xenopus* (Ruiz i Altaba et al., 1995; R. Ladher, J-P. Concordet, F. L. C., J. C. S. and P. Ingham, unpublished observations). As discussed below, these results are similar to those obtained in mouse and zebrafish embryos that lack functional *Brachyury* gene product.

DISCUSSION

Xbra and Ntl are transcription activators

The results described in this paper show that Xbra and Ntl are transcription activators, and that the activation domains of both proteins map to their carboxy-terminal regions (Fig. 2). There is little sequence conservation between the activation domains of the two proteins, although we note that acidic residues occur with high frequency in both.

Recently, Kispert and Hermann (1995) found that mouse Brachyury (T) also functions as a transcription activator, and further demonstrated that the C-terminal half of the protein may be divided into four domains, consisting of two transcription activators and two repressors. Our yeast assay (Fig. 2 and data not shown) does not reveal a second activation domain in Xbra or in Ntl, but more detailed analysis is required to determine whether this represents a real difference between Brachyury and Xbra and Ntl.

Interference with Xbra function

In zebrafish, a genetic mutation (*ntl*^{b160}) that lacks the activation domain described above displays a phenotype indistinguishable from a putative loss-of-function mutation (*ntl*^{b195}), suggesting a fundamental role for this region of the protein in mesodermal patterning (see Fig. 2B). Further evidence implicating the activation domain of Brachyury in mesoderm formation comes from work of Rao (1994), who finds that truncation of Xbra at amino acid 304 (XbraΔ304), which abolishes transcription activation (Fig. 2 and data not shown), also

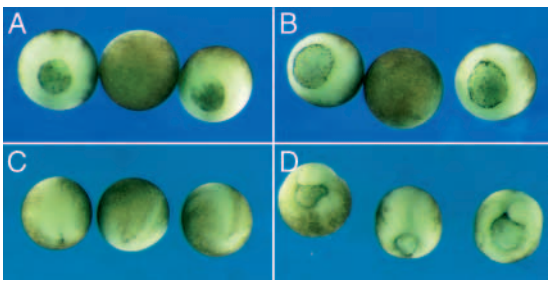

Fig. 5. Xbra-En^R interferes with gastrulation movements in *Xenopus*. (A,C) Control embryos. (B,D) Embryos injected with RNA encoding Xbra-En^R. Embryos are at stages 10.5 (A,B) and stage 14 (C,D). Note that the blastopore is slow to close in embryos injected with Xbra-En^R.

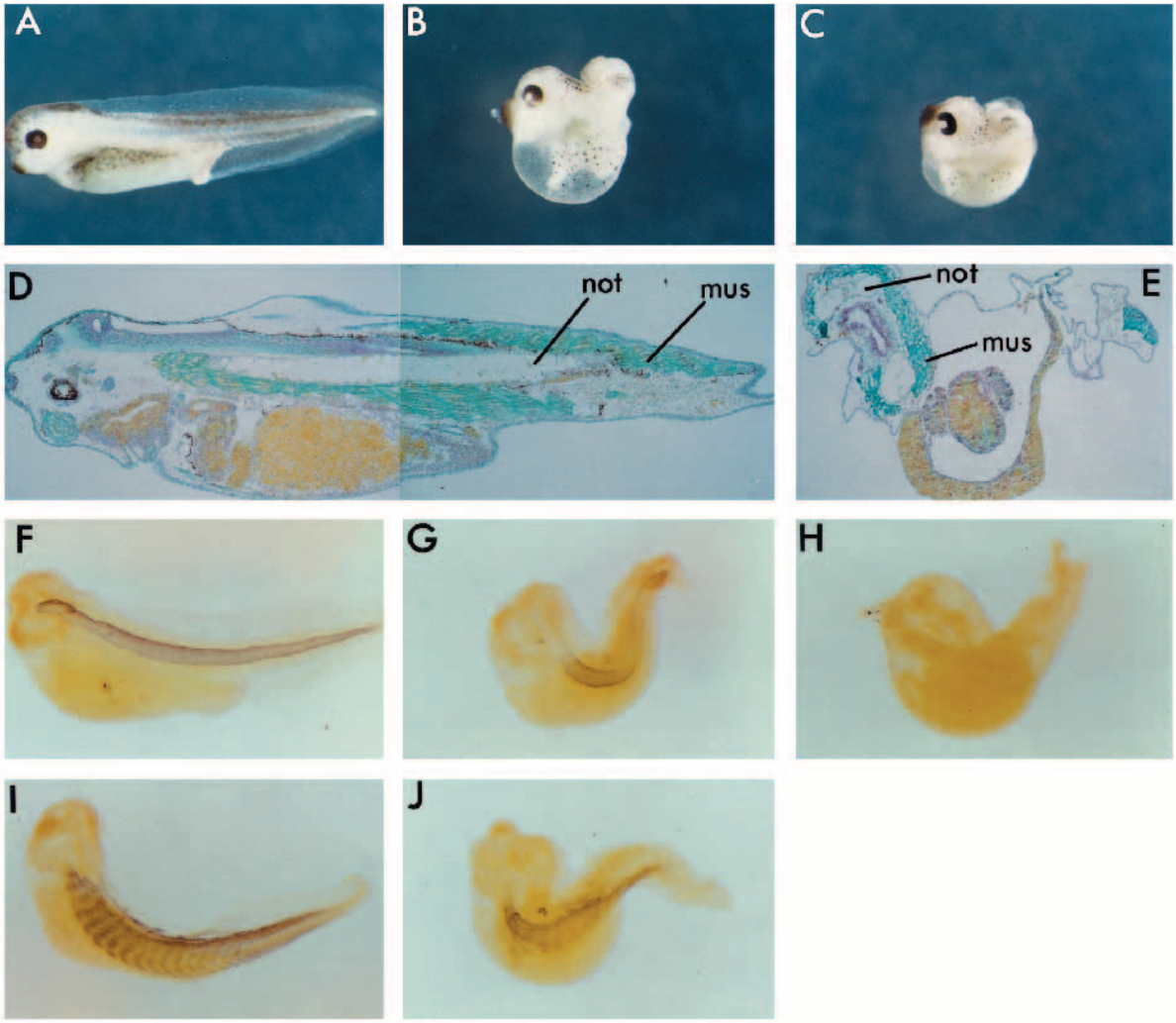

Fig. 6. Phenotype of *Xenopus* embryos injected with RNA encoding Xbra-En^R. (A) Control, uninjected embryo. Embryos injected with RNA encoding En^R alone are indistinguishable from such controls. (B,C) Embryos injected with 0.5 ng RNA encoding Xbra-En^R lack posterior structures. (D) Histological section of an embryo injected with 0.5 ng RNA encoding En^R alone. This embryo is indistinguishable from sections of control embryos. Note notochord (not) and muscle (mus). (E) Histological section of an embryo injected with RNA encoding Xbra-En^R. This embryo lacks posterior structures but has formed notochord (not) and somitic muscle (mus) anteriorly. (F–H) Embryos stained with the notochord-specific antibody MZ15. (F) Control embryo. (G) Embryo injected with RNA encoding Xbra-En^R; notochord is present anteriorly. (H) Embryo injected with RNA encoding Xbra-En^R; notochord is absent. (I,J) Embryos stained with the muscle-specific antibody 12/101. (I) Control embryo. (J) Embryo injected with RNA encoding Xbra-En^R; muscle is present anteriorly. Results similar to these were obtained following injection of 0.2 and 1.0 ng Xbra-En^R RNA (not shown).

prevents induction of mesoderm in animal caps. Surprisingly, injection of high concentrations of XbraΔ304 RNA induces the caps to form neural tissue (Rao, 1994); at present we can offer no explanation for this observation.

The significance of the Xbra activation domain was investigated in *Xenopus* embryos by designing a dominant-interfering *Xbra* construct (Xbra-En^R) in which the activation domain of Xbra is replaced by the repressor domain of the *Drosophila* engrailed protein. Injection of RNA encoding Xbra-En^R causes *Xenopus* embryos to develop abnormally: the blastopore fails to close, posterior structures are greatly reduced and notochord differentiation is impaired. These embryos resemble zebrafish

Fig. 7. Inhibition of notochord differentiation by Xbra-En^R revealed using Tor70 staining. (A) Control, uninjected embryo. (B,C) Embryos injected with 0.5 ng RNA encoding Xbra-En^R. Note normal notochord in A, anterior notochord in B and lack of notochord in C.

and mouse embryos that lack functional *Brachyury* gene product (Herrmann et al., 1990; Halpern et al., 1993; Herrmann and Kispert, 1994; Schulte-Merker et al., 1994; Conlon et al., 1995; Wilson et al., 1995), and further similarities come from comparison of the expression patterns of *Brachyury* itself, of *Pintallavis* (HNF3β) and of *shh* in these embryos.

Fig. 8. Co-injection of RNA encoding wild-type Xbra brings about partial rescue of the effects of Xbra-En^R. (A) Control embryo. (B) Embryo injected with 1 ng RNA encoding Xbra-En^R. (C) Embryo injected with 1 ng RNA encoding Xbra-En^R together with 2 ng RNA encoding wild-type Xbra. Note that posterior structures are more complete in C than in B, although anterior structures in C are reduced.

Xenopus embryos injected with RNA encoding Xbra-En^R closely resemble zebrafish *ntl* embryos in that expression of *Brachyury* is down-regulated in the notochord while transcripts persist in posterior tissues (Schulte-Merker et al., 1994). In addition, *sonic hedgehog* is expressed in axial structures at early stages in both types of embryo, as are the HNF3β homologues *Pintallavis* (Fig. 10) and *axial* (Strähle et al., 1993, 1996). T^{Wis}/T^{Wis} mouse embryos, which lack functional *Brachyury* gene product, differ slightly from zebrafish and *Xenopus* in that expression of *Brachyury* itself is down regulated throughout the embryo, but it may be significant that expression persists for longest in posterior structures (Herrmann, 1991). Finally, as in zebrafish and *Xenopus* embryos that lack *Xbra* function, T^{Wis}/T^{Wis} mouse embryos express *shh* (Conlon et al., 1995) and HNF3β (F. L. C., R. Arkell and R. Beddington, unpublished observations).

Together, these results suggest that the role of *Brachyury* in the vertebrate embryo is to activate transcription of genes required for normal gastrulation, for formation of posterior mesoderm and for terminal differentiation of the notochord. Initial formation of the notochord appears to occur normally, at least as judged by the markers *shh* and HNF3β. The ability of *Xbra* to induce different mesodermal cell types at different concentrations (Cunliffe and Smith, 1992; O'Reilly et al., 1995) suggests that some targets of Brachyury have high-affinity binding sites while others have low-affinity sites (Jiang and Levine, 1993). In future work we intend to identify such targets of Brachyury.

Although notochord differentiation is reduced by the Xbra-En^R construct, patches of notochord remain in many embryos, particularly in anterior regions (Fig. 7). This may be due to slight variations in the amounts of injected Xbra-En^R RNA, to different amounts of RNA being inherited by different blastomeres, or to decay of Xbra-En^R as development proceeds. All these suggestions are consistent with the idea that the function of *Xbra* in notochord differentiation is a relatively late function. We also note that although notochord is absent in the zebrafish ntl^{b160} and ntl^{b195} alleles (Halpern et al., 1993), a weak *ntl* allele discovered in the large-scale screen of Nüsslein-Volhard and colleagues (Mullins et al., 1994) does have small notochord cells (J. Odenthal and N. Nüsslein-Volhard, personal communication). Furthermore, there may be notochord present in the caudal regions of mouse *T*/*T* mutant embryos (see Beddington et al., 1992).

The effects of Xbra-EnR resemble those of a truncated FGF receptor: autoregulation of *Xbra* expression

We have shown previously that the ability of *Xbra* to induce mesoderm in isolated animal pole tissue depends on an intact FGF signalling pathway (Schulte-Merker and Smith, 1995), and we suggested that *Xbra* and eFGF are components of an indirect autoregulatory loop in which *Xbra* induces expression of eFGF, and eFGF is required for maintenance of expression of *Xbra*. Consistent with this idea, the expression patterns of

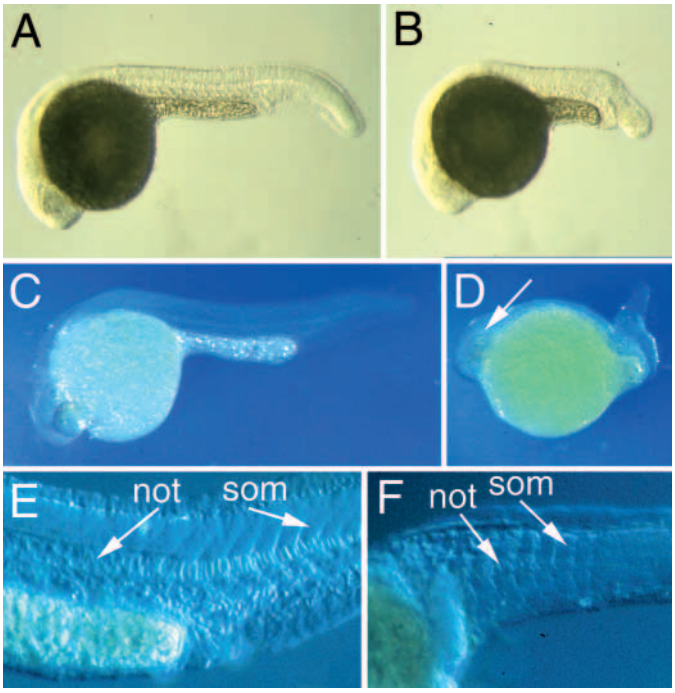

Fig. 9. Phenotype of zebrafish embryos injected with RNA encoding Xbra-En^R. (A) Wild-type zebrafish embryo approximately 24 hours after fertilization. (B) Sibling *ntl* mutant embryo at the same stage as that in A. (C) Control uninjected zebrafish embryo approximately 24 hours after fertilisation. (D) Sibling zebrafish embryo injected with RNA encoding Xbra-En^R at the same stage as that in C. Posterior tissue is greatly reduced but anterior structures such as the eye (arrow) are present. (E) High-power view of the somites and notochord of a control zebrafish embryo in the trunk region. Note the highly vacuolated cells of the notochord (not) and the chevron-shaped somites (som). (F) High-power view of the trunk region of a zebrafish embryo injected with RNA encoding Xbra-En^R. The notochord (not) is poorly differentiated, the somites (som) are only slightly chevron-shaped and they are fused at the ventral midline. In all these respects embryos injected with RNA encoding Xbra-En^R resemble the *ntl* mutation (Halpern et al., 1993).

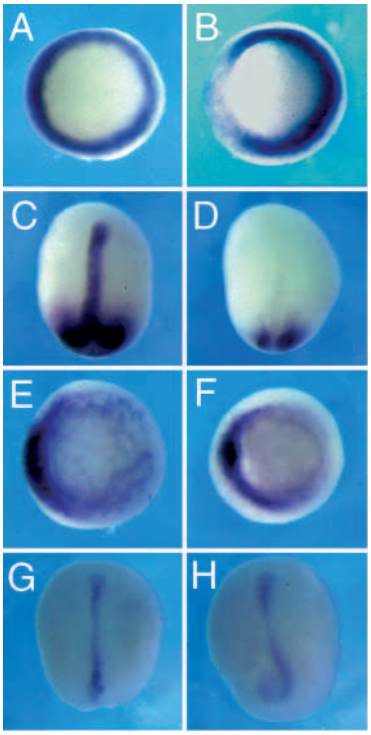

Fig. 10. In situ hybridization analysis of embryos injected with RNA encoding Xbra-En^R. (A–D) Embryos hybridised with an *Xbra* probe at stage 10 (A,B) and stage 14 (C,D). Control embryos at the early gastrula stage express *Xbra* throughout the marginal zone (A). Control embryos at the neurula stage express *Xbra* in the notochord and in posterior cells (C). Embryos injected with RNA encoding Xbra-En^R do not express *Xbra* in the organizer at the early gastrula stage (B; see left-hand side of embryo) or in the notochord at the neurula stage (D). The greater intensity of staining in the ventral region of the embryo shown in B is not a consistent observation and decreases in endogenous levels of *Xbra* have been confirmed by RNAase protection (not shown). (E–H) Embryos hybridised with a *Pintallavis* probe at stage 10 (E,F) and stage 14 (G,H). Expression of *Pintallavis* in embryos injected with RNA encoding Xbra-En^R (F,H) is similar to that in controls (E,G).

Xbra and of eFGF are remarkably similar (Isaacs et al., 1996) and the phenotype of *Xenopus* embryos injected with RNA encoding Xbra-En^R resembles that of embryos injected with RNA encoding a truncated FGF receptor (Amaya et al., 1991, 1993; Isaacs et al., 1994) and is also similar to that of embryos in which signalling through the MAP kinase pathway is inhibited (MacNicol et al., 1993; Gotoh et al., 1995; LaBonne et al., 1995; Umbhauer et al., 1995).

A prediction of the indirect autoregulatory loop model is that expression of *Xbra* should be down-regulated in embryos injected with Xbra-En^R. In situ hybridisation analysis (Fig. 10) reveals that such down-regulation does occur, but only in dorsal mesoderm. In this respect, *Xenopus* embryos expressing Xbra-En^R resemble mutations in zebrafish embryos that lack the Brachyury transcription activation domain (Schulte-Merker et al., 1994). These results indicate that ventral expression of *Xbra* is independent of autoregulation and is, therefore, controlled differently from dorsal expression. This difference in the control of expression of *Xbra* in dorsal and ventral tissue is under investigation.

Interference with transcription factor action

The strategy that we describe in this paper, in which the DNA-binding domain of a transcription activator is fused to the active repressor domain of the engrailed protein, should be readily applicable to other *Xenopus* and zebrafish transcription factors. A particular advantage of this approach is that interfering alleles of this sort are likely to be at least ten times more efficient than simple competitive inhibitors (see legend to Fig. 3). Indeed, we have shown previously that injection of 5 ng RNA encoding just the DNA-binding domain of Xbra has no effect on *Xenopus* development (Cunliffe and Smith, 1992), whereas 0.2 ng of RNA encoding Xbra-En^R is sufficient to generate the phenotype shown in Figs 5–10. This effect requires the DNA-binding domain of Xbra, because injection of RNA encoding En^R alone has no effect (Fig. 6). We next intend to study *Pintallavis*, a homologue of HNF3β (Ruiz i Altaba and Jessell, 1992) whose expression does not depend on *Xbra* (Fig. 9E-H). We have recently demonstrated that *Pintallavis* is a transcription activator (Morgan et al., 1996) that acts synergistically with *Xbra* to pattern the mesoderm in *Xenopus* (O'Reilly et al., 1995).

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