**Bix1**, a direct target of *Xenopus* T-box genes, causes formation of ventral mesoderm and endoderm

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**SUMMARY**

Brachyury, a member of the T-box gene family, is required for posterior mesoderm and notochord differentiation in vertebrate development, and mis-expression of *Xenopus* Brachyury causes ectopic mesoderm formation. Brachyury is a transcription activator, and its ability to activate transcription is essential for its biological function, but Brachyury target genes have proved difficult to identify. Here we employ a hormone-inducible Brachyury construct and subtractive hybridization to search for such targets. Using this approach we have isolated *Bix1*, a homeobox gene expressed both in the marginal zone of *Xenopus* and in the vegetal hemisphere. Expression of *Bix1* is induced in an immediate-early fashion by mesoderm-inducing factors such as activin as well as by the products of the T-box genes Xbra and VegT (also known as Antipodean, Brat and Xombi). Activation of *Bix1* in response to Xbra is direct in the sense that it does not require protein synthesis, and both Xbra and VegT activate expression of a reporter gene driven by the *Bix* 5′ regulatory region, which contains an Xbra/VegT binding site. Mis-expression of low levels of Bix1 causes formation of ventral mesoderm, while high levels induce endodermal differentiation. These results suggest that *Bix1* acts downstream of both VegT and Xbra to induce formation of mesoderm and endoderm.

Key words: *Xenopus*, Mesoderm, Endoderm, Brachyury, VegT, T-box gene, Activin, Target gene

**INTRODUCTION**

The mesoderm of the amphibian embryo arises through an inductive interaction in which blastomeres of the vegetal hemisphere of the embryo act on overlying equatorial cells (Nieuwkoop, 1969). Candidates for endogenous mesoderm-inducing signals include members of the transforming growth factor-β family such as activin and Vg1 (Slack, 1994). Treatment of prospective ectoderm with activin, for example, causes the formation of ventral and dorsal mesoderm in a concentration-dependent manner (Green and Smith, 1990), and inhibition of activin signalling in the intact embryo, by over-expression of truncated activin receptors, interferes with mesoderm formation (Dyson and Gurdon, 1996; Hemmati-Brivanlou and Melton, 1992). Recent work indicates that high concentrations of activin also induce ectodermal tissue to form endoderm (Henry et al., 1996; Stennard et al., 1996; Zhang and King, 1996), and mis-expression of Xbra with Pintallavis, the *Xenopus* homologue of HNF-3β, induces formation of notochord (O’Reilly et al., 1995). Together, these results indicate that Brachyury plays a key role in vertebrate mesoderm formation.

Brachyury encodes a sequence-specific DNA-binding protein (Kispert and Herrmann, 1993) and it functions as a transcription activator (Conlon et al., 1996; Kispert et al., 1995). Its DNA-binding domain defines the so-called T-box gene family, members of which have been isolated from *Caenorhabditis elegans* and *Drosophila*, as well as from the vertebrates (Papaioannou, 1997; Smith, 1997). In *Xenopus* the T-box family includes VegT (also known as Antipodean, Brat and Xombi), which is first expressed maternally in the vegetal hemisphere of the embryo (Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996), and eomesodermin, which is expressed in the prospective mesoderm of the late blastula (Ryan et al., 1996).
Although the ability of Brachyury to activate transcription is essential for its biological function (Conlon et al., 1996), little is known about Brachyury target genes. To investigate this issue, we have made an inducible version of Xbra called Xbra-GR, in which the Xbra open-reading frame is fused to the ligand-binding domain of the human glucocorticoid receptor. When expressed in Xenopus embryos, this construct is completely inactive unless dexamethasone (DEX) is added to the culture medium (Tada et al., 1997). In this paper, we use this hormone-inducible construct together with subtractive hybridization to search for Brachyury target genes. This approach has allowed us to isolate Bix1-4, novel homeobox genes which, like Xbra, are expressed throughout the marginal zone but are also expressed in the vegetal hemisphere.

The latter observation, together with the fact that expression of the Bix genes precedes that of Xbra, suggests that Bix expression might be regulated by VegT as well as by Xbra. Consistent with this idea, both Xbra and VegT induce expression of Bix1 in prospective ectodermal tissue, both proteins interact with an element present in the Bix4 5′ regulatory region, and both activate expression of a reporter gene driven by this region.

Mis-expression of low concentrations of Bix1 in prospective ectodermal tissue causes formation of ventral mesoderm while high concentrations induce endodermal differentiation. These results suggest that in the marginal zone Bix1-4 genes function downstream of Xbra and are involved in mesoderm formation, and that in the vegetal hemisphere they act downstream of VegT and are involved in regulation of endodermal differentiation.

Finally, we note that the use of hormone-inducible constructs like Xbra-GR may provide a powerful approach for isolating transcription factor targets in Xenopus development.

MATERIALS AND METHODS

Embryonic manipulations

Fertilisation, culture and microinjection of Xenopus embryos were as described (Tada et al., 1997). Embryos were staged according to Nieuwkoop and Faber (1975). Protein synthesis inhibition experiments were performed as described (Smith et al., 1991), except that lithium precipitation was omitted. Samples were analysed with probes specific for Xbra (Smith et al., 1991), goosecoid (Blumberg et al., 1991), Xvent1 (Christian et al., 1991), Xvent1 (Gawantka et al., 1995), chordin (Sasai et al., 1994), muscle actin (Mohn et al., 1984), Xsox17α (Hudson et al., 1997), cerberus (Bouwmeester et al., 1996), ODC (Isaacs et al., 1992), IFABP (Shi and Hayes, 1994), endodermin (Sasai et al., 1996) and αT4-globin (Walmshley et al., 1994). To make a Bix1 probe, pBix1 was linearized with SspI and transcribed with T7 RNA polymerase. Northern blotting was performed as described (Sambrook et al., 1989), using randomly primed probes.

In situ hybridization, immunohistochemistry and β-gal staining

Whole-mount in situ hybridization was performed as described (Harland, 1991), except that BM purple was used as substrate and RNAse treatment was omitted. A Bix1 probe was prepared by linearizing pBix1 with BamHI and transcribing with T7 RNA polymerase, thus excluding the N-terminal portion and homeodomain of the protein. The Xbra probe was as described (Tada et al., 1997). For histological analysis, specimens were fixed, sectioned and stained as described (Tada et al., 1997). Whole-mount immunohistochemistry was carried out using the monoclonal antibody MZ15, specific for notochord, and a rabbit polyclonal antibody prepared by Brenda Price and Steve Smerdon (NIMR) specific for Xbra protein. β-gal staining was performed as described (Tada et al., 1997). Immunolocalization of the lineage tracer FLDx was carried out as described (Jones and Smith, 1998).

Isolation of the Bix4 regulatory region by inverse PCR

To isolate the 5′ regulatory regions of the Bix genes, inverse PCR was performed according to a protocol kindly provided by Paul Krieg (University of Texas, Austin). Xenopus genomic DNA (1 μg) was digested with XhoI, self-ligated with T4 DNA ligase and then digested with BamHI (the Bix1 cDNA contains a BamHI site). PCR was carried out using one tenth of this material as a template with the Bix-specific primers: 5′-AGTCCAGGGCAAGTGAAGC-3′ (directed 5′) and 5′-CATCTACGGAATGTTGCT-3′ (directed 3′). The PCR product obtained was digested with XhoI, cloned into pBlueScriptIIISK(−) and sequenced. It comprised 1.8 kb and contained sequences identical to the 5′ portion of the Bix4 cDNA. The transcription start site of the Bix promoter was determined by RNase protection and is located approximately 72 bp upstream of the initiation codon and 24 bp downstream of the TATA box.

To make a luciferase reporter plasmid, the promoter region (~1.6 kb to +54) was isolated by PCR using a T3 primer and the oligonucleotide 5′-CGCGAAGCTTCTCTCAAAATCTGCCTCTTCCC-3′, which contains a HindIII site for cloning. The amplified fragment was cloned into the NheI and HindIII sites of pG3-L3-Basic (Promega).

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays were carried out as described previously (Casey et al., 1998), using purified Xbra DNA-binding domain (XDBD) (Casey et al., 1998) or VegT prepared by in vitro translation in a reticulocyte lysate (Promega). Radiolabelled and competitor probes were as follows:
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Mesoderm and endoderm induction by the T-box target gene Bix1

B: 5′-TCGACTTCTCATCAGTACCGCTTCTCTGGAGATATCCAGCTGTTCCCT-3′;
M1: 5′-TCGACTTCTCATCGACGCTTCTCTGGAGATATCCACGCTTTCCCT-3′;
M2: 5′-TCGACTTCTCATCAGTACCGCTTCTCTGGAGATATCCAGCTGTTCCCT-3′;
M1/M2: 5′-TCGACTTCTCATCGACGCTTCTCTGGAGATATCCAGCTGTTCCCT-3′.

COS cell transfections

COS cells were transfected by lipofection with 400 ng effector, 100 ng Bix-luciferase, 100 ng pRL-TK (Promega) and 400 ng pcDNA3 (Invitrogen). Cells were lysed and Renilla and firefly luciferase activities were determined according to the manufacturer's instructions (Dual-Luciferase Kit, Promega). Xbra was cloned into pcDNA3 as an EcoRV/XbaI fragment derived from pXT1 (Smith et al., 1991) and VegT was cloned into the same vector as a HindIII/NotI fragment derived from pCS2+VegT (Zhang and King, 1996).

Reporter gene studies in animal cap assays

Both cells of a Xenopus embryo at the two-cell stage were injected with 2 pg SV40-RL (Promega), 2 pg Bix-luciferase and 100 pg Xbra-GR RNA. Animal caps were dissected at stage 8 and left untreated or exposed to dexamethasone for 4 hours. 10 μl of 1X passive lysis buffer (Promega) was then added per cap and the luminescence of 10 μl was measured. Each sample consisted of 5 caps.

Fig. 1. Design of screen for Xbra targets. (A) Schematic illustration of a strategy to isolate Xbra target genes. Details are described in the text and Materials and methods. (B) Induction of Bix1 in a screen by northern blotting analysis. Animal caps derived from embryos injected with 50 pg Xbra-GR RNA or un.injected were dissected at blastula stages, then treated with 10⁻⁶ M dexamethasone (DEX) for 3 hours or left untreated. ODC and Xbra served as loading or positive controls, respectively. Note DEX alone does not induce Bix1 expression.

Fig. 2. The Xenopus Bix proteins. (A) Deduced amino acid sequence of Bix1. The homeodomain is enclosed within a box and a C-terminal acidic region is underlined. HD, homeodomain; AD, acidic domain. (B) Comparison of the four Bix proteins and Xenopus Mix.1 (Rosa, 1989), Mix.2 (Vize, 1996) and Mixer (Henry and Melton, 1998). Figures indicate percentage amino acid identity. (C) Comparison of the homeodomains of Xenopus Bix1-4, Mix.1, Mix.2 and Mixer. Amino acid identities (%) are indicated. Bars indicate identical amino acids to those of Bix1. Note that the gene called milk (Ecochard et al., 1998) is identical to Bix2. The accession numbers of Bix1-4 are, respectively, AF079559, AF079560, AF079561 and AF079562.

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RESULTS

The Bix genes are paired-like homeobox genes inducible by Xenopus Brachyury

We have made use of a hormone-inducible Xbra construct, Xbra-GR (Tada et al., 1997), to isolate genes that are induced within 2.5 hours of Xbra activation and are therefore likely to be direct targets of this transcription factor. Xenopus embryos at the one-cell stage were injected with RNA encoding hormone-inducible Xbra. Animal caps were dissected at the late blastula stage, and half were treated with dexamethasone (DEX) for 2.5 hours. RNA was extracted from each population of cells and suppression PCR (Hubank and Schatz, 1994) was used to construct a cDNA library enriched for Brachyury-inducible genes (Fig. 1A). In a preliminary screen, 53 randomly picked clones were sequenced and subjected to a BLAST search. The abilities of 37 of these genes to be induced by Xbra were then tested by northern blotting analysis, using RNA derived from DEX-treated or untreated animal caps (Fig. 1B). Four independent clones were identified as Brachyury-inducible genes (BIG). Of these, BIG4 (comprising 166 nucleotides) proved to be particularly strongly induced by Xbra and to have no homology with known sequences in GenBank. Screening of a gastrula cDNA library using this clone as a probe yielded four novel homeobox-containing genes, which we designate Bix1, Bix2, Bix3 and Bix4 (Brachyury-inducible homeobox-containing gene) (Fig. 2). The predicted amino acid sequences of the proteins encoded by Bix genes reveal greater than 95% identity in the homeodomain and about 90% identity in the N-terminal region, but only 60-70% identity in the C-terminal domain (Fig. 2B,C). Bix1 and Bix3 are most closely related to the original cDNA, with each showing just 1 nucleotide change out of 166. The spatial and temporal expression patterns of Bix1-Bix4 are identical, and expression of each is induced in animal caps by Xbra-GR (data not shown). The homeodomain of Bix1 is, respectively, 68%, 68% and 65% identical to those of Xenopus Mix.1, Mix.2 and Mixer, encoded by the genes most closely related to Bix1 in the GenBank database (Fig. 2C). A glutamine is present at position 9 of the third helix, and there is an acidic domain at the C terminus, a domain which is conserved amongst all the Bix proteins and is also present in Mix.1, Mix.2 and Mixer, encoded by the genes most closely related to Bix1 in the GenBank database.

Bix1 is a downstream target gene of Brachyury and of mesoderm-inducing factors

Expression of Bix1 is first detectable by RNAsa protection analysis at mid-blastula stage 8.5 (Fig. 3A and data not shown). It reaches a peak at the early gastrula stage and transcripts are not detectable by the early neurula stage. Northern blotting analysis reveals a single Bix1 transcript of approximately 1.8 kb (Fig. 1B). Although Bix1 was identified as a target of Xbra, its expression precedes that of Xenopus Brachyury (Fig. 3A), indicating that its initial expression does not require Xbra. It is possible that activation of Bix1 occurs in response to the maternal and vegetally located T-box gene VegT (also known as Antipodean, Brat and Xombi), or in response to inducing factors such as activin (see below).

The spatial expression of Bix1 was studied by in situ hybridization (Fig. 3C-J), which revealed that Bix1 is first expressed throughout the equatorial region of the embryo and in the vegetal pole region, and that transcripts disappear from the dorsal marginal zone by the mid-gastrula stage (Fig. 3K-L).
incorporation of \(^{35}\)S methionine into acid-precipitable material by Cycloheximide (CHX) treatment was sufficient to reduce by activin and by Xbra-GR does not require protein synthesis. RNA) and Xbra-GR (50 pg RNA). (B) Induction of RNA), eomesodermin (2 ng RNA), VegT (2 ng RNA), Xbra (2 ng RNA), Bix1 expression by inducing factors and T box- genes are activated directly by Xbra and VegT Activation of Bix1 expression by Xbra-GR occurs in the absence of protein synthesis, suggesting that the Bix genes are direct targets of Xbra and perhaps of other T-box gene products such as VegT. To investigate this question, Bix 5' regulatory sequences were isolated by inverse PCR (see Materials and methods). Sequencing of a 1.8 kb clone, which proved to derive from Bix4, revealed a 10-bp element CTTCACACGT, centred 66 nucleotides 5' upstream of the transcription start site, and a related sequence, CTTCACACCT centred approximately 85 nucleotides from the transcription start site, and a related sequence, ATTCACACGT, centred 66 nucleotides 5' of the transcription start site (Fig. 5A). The former sequence represents only half of the previously identified palindromic Brachyury binding site (Kispert and Herrmann, 1993), but we have recently demonstrated that a similar motif is present in the regulatory region of Xenopus eFGF, that the Xbra DNA-binding domain binds to this half-site, and that it confers Xbra responsiveness to a linked reporter gene (Casey et al., 1998).

The abilities of Xbra and VegT to interact with these elements were investigated in electrophoretic mobility shift assays using a 46 bp probe that contains both sites (Fig. 5B). Incubation of the Xbra DNA-binding domain (XDBD) with the wild-type probe (B) resulted in the formation of two complexes (1). Mutation of either element (creating probes M1 and M2) caused loss of the lower-mobility band, suggesting that the upper complex contains just one. Binding to the M1 probe, in contrast to the lower complex, which the distal site is mutated, is weaker than binding to M2, indicating that XDBD interacts more strongly with the distal site.

The regulatory role of Bix1 expression was studied using the animal cap assay, which showed that Bix1 is induced by activin and BMP-4 and, to some extent, by FGF. Xbra and VegT also activate Bix1 expression, but the T-box gene eomesodermin (Ryan et al., 1996) does so only weakly (Fig. 4A). Induction of Bix1 expression by Xbra-GR and by activin is immediate-early in the sense that it does not require protein synthesis (Fig. 4B) and will occur in dissociated cells (data not shown). Induction of Bix1 expression by Xbra-GR and DEX is inhibited, as would be predicted, by the dominant-negative Xbra construct Xbra-EnR (Conlon et al., 1996) (Fig. 4C). In the experiment shown, Xbra-EnR also reduced expression of Bix1 in response to activin and BMP-4 (Fig. 4C); this may reflect a requirement for Xbra activity in the maintenance of Bix1 expression. Taken together with the temporal and spatial expression patterns of Bix1, these results suggest that Bix1 expression is initiated by VegT or an activin-like molecule in the vegetal hemisphere. In the marginal zone, the similarities in the expression patterns of the two genes (Fig. 3K,L) suggest that Xbra may also play a role in the initiation and maintenance of Bix1 transcription. Additional experiments (not shown) indicate that Bix2-4 are regulated in a similar manner to Bix1.

Bix genes are activated directly by Xbra and VegT

Activation of Bix1 expression by Xbra-GR occurs in the absence of protein synthesis, suggesting that the Bix genes are direct targets of Xbra and perhaps of other T-box gene products such as VegT. To investigate this question, Bix 5’ regulatory sequences were isolated by inverse PCR (see Materials and methods). Sequencing of a 1.8 kb clone, which proved to derive from Bix4, revealed a 10-bp element CTTCACACGT, centred approximately 85 nucleotides upstream of the transcription start site, and a related sequence, ATTCACACGT, centred 66 nucleotides 5’ of the transcription start site (Fig. 5A). The former sequence represents only half of the previously identified palindromic Brachyury binding site (Kispert and Herrmann, 1993), but we have recently demonstrated that a similar motif is present in the regulatory region of Xenopus eFGF, that the Xbra DNA-binding domain binds to this half-site, and that it confers Xbra responsiveness to a linked reporter gene (Casey et al., 1998).

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VegT also proved to interact with the wild-type probe, but mutation of the distal site (M1) essentially abolished binding, while mutation of the proximal site (M2) had little effect. VegT, therefore, binds only the distal site under these conditions. We note, however, that increasing the MgCl2 concentration of the binding buffer from 1 mM to 5 mM appears to facilitate binding to the proximal site (data not shown).
Specificity of the observed complexes was confirmed by competition with a 50-fold excess of unlabelled wild-type probe B, while a similar excess of unlabelled oligonucleotide M1/M2, in which both the proximal and the distal sites are mutated, had no effect (data not shown).

To confirm that T-box proteins can interact with the Bix genes to activate transcription, a luciferase reporter gene was placed under the control of 1.6 kb of the Bix4 5’ regulatory region and it was transfected into COS cells together with an effector construct containing Xbra or VegT driven by the CMV promoter. Both T-box constructs caused a 2.5-fold induction of luciferase activity (Fig. 5C). A similar result was obtained in animal caps injected with Xbra-GR and left untreated or treated with dexamethasone. The graph represents a single experiment carried out in triplicate. Error bars indicate standard deviations.

**Fig. 5.** Xbra and VegT bind to Brachyury half sites in the 5’ regulatory region of Bix4 and activate transcription of a minimal promoter construct.

(A) Upstream regulatory region of Bix4 (GenBank accession number AF079563). The two 10 bp T-box response elements are underlined with arrows. Nucleotides that do not match the consensus half-site (Kispert and Herrmann, 1993) are boxed in gray. The approximate transcription start site is marked by a small arrow and the TATA box is outlined in black. The nucleotides in the 5’ UTR that are omitted from the Bix4-luciferase construct are in italics and the first ATG is underlined.

(B) Electrophoretic mobility shift assays of XDBD and VegT incubated with 46 bp radiolabelled probes including the two half-sites underlined in (A). B represents the wild-type probe, M1 contains a mutation in the distal response element and M2 contains a mutation in the proximal response element (see Materials and methods). Asterisks mark the two complexes formed with XDBD and probe B. (C) Induction of Bix4-luciferase by Xbra and VegT in COS cells. COS cells were transfected with Bix4-luciferase and effector constructs containing VegT and Xbra driven by the CMV promoter. ‘None’ represents expression from cells transfected with the reporter construct only. In all experiments the luciferase activity of cells containing only the reporter construct is set at 10 and other values are shown relative to this. The hatched bars represent an average of five separate experiments in which each transfection was carried out in duplicate. Error bars represent standard deviations.

(D) Expression of Bix4-luciferase in animal caps injected with Xbra-GR and left untreated or treated with dexamethasone. The graph represents a single experiment carried out in triplicate. Error bars indicate standard deviations.

**Mis-expression of Bix1 causes the formation of ventral mesoderm and endoderm**

Different concentrations of Xbra induce different types of mesoderm: low concentrations cause the formation of ventral mesoderm, while higher concentrations induce muscle (Cunliffe and Smith, 1992; O’Reilly et al., 1995). These results inspired us to ask whether mis-expression of the Xbra target Bix1 also leads to mesoderm formation in prospective ectodermal tissue and whether, bearing in mind that it is also expressed in the vegetal hemisphere and responds to VegT, it can induce endodermal differentiation as well. Ectopic expression of low concentrations of Bix1 in animal caps is sufficient to induce ventral mesoderm containing mesenchyme and mesothelium (33%; n=15), resembling that induced by Xbra (Fig. 6B,E), while high concentrations induce yolky tissue that resembles endoderm (Fig. 6C,F).
These histological observations were confirmed by RNAse protection analysis. At low concentrations, Bix1 induces expression of Xwnt8 and elevates expression of Xvent1, and eventually animal caps expressing Bix1 go on to express αT4-globin (Fig. 6G). Higher concentrations of Bix1 induce the endoderm-specific marker Sox17α and eventually such caps express the endodermal markers IFABP and endodermin (Fig. 6G). At early stages Bix1 also efficiently induces expression of the dorsal mesendodermal markers goosecoid and cerberus (Fig. 6G), but does not activate Xbra (Fig. 6G), eFGF, VegT, pintallavis, Xnot, chordin or siamois (data not shown).

We next tested the function of Bix1 in whole embryos. Overexpression of Bix1 in whole embryos causes a disruption of gastrulation that results in the absence of head and axial structures (see Fig. 7A-D). Severe phenotypes such as this are difficult to interpret, but experiments in which Bix1 is mis-expressed in isolated dorsal or ventral marginal zone tissue suggest that it is capable both of ventralizing dorsal mesoderm and of causing ventral mesoderm to form endoderm. The ventralizing effects of Bix1 are revealed by the fact that it inhibits the convergent extension of isolated dorsal marginal zone tissue (Fig. 7E,F) and, when expressed specifically in the dorsal marginal zone of the intact embryo, it prevents cells from differentiating as notochord (Fig. 7G,H). Expression of Bix1 in dorsal marginal zone explants also reduces expression of the dorsal markers goosecoid and chordin and elevates expression of the early ventral markers Xwnt8 and Xvent1 as well as the late ventral marker αT4-globin (Fig. 7K). At this later stage Bix1-expressing dorsal marginal zone tissue also expresses low levels of the endoderm-specific gene IFABP (Fig. 7K).

Interestingly, Bix1 induces much higher levels of IFABP in isolated ventral marginal zone regions than it does in dorsal tissue (Fig. 7K). This may reflect the fact that high levels of Bix1 are required for endodermal differentiation, and that it is easier to achieve these levels by over-expression in ventral marginal zone tissue, where endogenous Bix1 is expressed, than in dorsal tissue, where it is not. Consistent with the idea that high levels of Bix1 causes ventral marginal zone tissue to adopt an endodermal fate, levels of early mesodermal markers, such as Xbra, Xwnt8 and, to a lesser extent, Xvent1, decline, as do levels of the late marker αT4-globin (Fig. 7K). The induction of goosecoid expression by Bix1 in ventral marginal zone tissue is probably due to the effects of Bix1 on residual animal pole tissue remaining in these explants (see Fig. 6G).

To ask whether the conversion of mesoderm to endoderm by Bix1 occurs in a cell-autonomous manner, equatorial blastomeres of Xenopus embryos at the 32-cell stage were injected with Bix1 RNA together with the lineage marker fluorescein-lysine-dextran (FLDx). Xbra expression was extinguished only in cells expressing Bix1 RNA (70%, n=30; see Fig. 7I,J), indicating that Bix1 does indeed act cell-autonomously.

**DISCUSSION**

The results described in this paper introduce a novel approach for the identification of transcription factor targets in the early Xenopus embryo (Fig. 1). Using this approach we have identified the Bix gene family (Fig. 2), homeobox-containing genes Bix1 and Bix2 that induce formation of endoderm. These genes are expressed in the marginal zone and are required for the conversion of mesoderm to endoderm. The induction of endodermal markers by Bix1 is cell-autonomous, indicating that Bix1 acts directly on endodermal progenitor cells. Further studies are needed to determine the mechanisms by which Bix1 regulates endodermal gene expression and how this process is coordinated with other transcription factors during early development.
genes which are expressed both in the mesoderm and in the endoderm of the early frog embryo (Fig. 3). Experiments using cycloheximide (Fig. 4) and dispersed cells (not shown) suggest that the Bix genes are direct targets of Xbra, and indeed Xbra, as well as VegT, drives the expression of reporter genes linked to the 5’ regulatory region of Bix4, which contains an Xbra/VegT binding-site (Fig. 5). Mis-expression of low levels of Bix1 in animal caps causes the formation of ventral mesoderm, while higher levels induce endoderm (Fig. 6). Mis-expression in whole embryos reveals that the Bix gene products also prevent blastomeres from differentiating as notochord (Fig. 7).

**Isolation of Xbra targets**

Many transcription factors are expressed in the early Xenopus embryo, and understanding the functions of these gene products depends on identifying their targets. In general, this has proved no easy task, and it has been necessary to rely on intelligent guesswork based on knowledge of known genes (Casey et al., 1998; Laurent et al., 1997), rather than on a screen that would allow the identification of previously unknown transcripts. In this paper we describe a novel approach to identifying transcription factor targets, which takes advantage of a hormone-inducible Xbra construct (Tada et al., 1997). The technique identifies genes that are activated shortly after induction of Xbra activity, and it is possible to ask whether they are activated by Xbra directly by determining whether induction is sensitive to cycloheximide. This approach has allowed us to identify, among other novel cDNAs (M. T. and L. F., unpublished), the Bix gene family, members of which are induced by Xbra and also by the T-box family member VegT. The 5’ regulatory region of Bix4.

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**Fig. 7.** Effects of Bix1 over-expression on whole embryos and marginal zone regions. (A) Control embryos at stage 33. (B) Embryos injected with 400 pg Bix1 RNA at the same stage as those in (A). Note lack of head and axial structures. (C) Control embryo at stage 33 stained with the monoclonal antibody MZ15, which recognises notochord. (D) Embryo injected with Bix1 RNA stained with MZ15. Notochord differentiation in the trunk is abolished. The small patch of MZ15 staining in the anterior region of the embryo derives predominantly from the otic vesicle. (E) Dorsal marginal zone tissue cultured to the equivalent of stage 18. Note convergent extension. (F) Dorsal marginal zone tissue derived from embryos injected with Bix1 RNA undergoes less convergent extension (88%; n=17). (G) Injection of RNA encoding β-galactosidase into blastomere B1 of the 32-cell stage embryo reveals that this cell forms predominantly notochord (Dale and Slack, 1987), revealed here by staining with MZ15. (H) Co-injection of RNA encoding Bix1 causes cells to be diverted from notochord formation (100%; n=16). (I,J) Down-regulation of Xbra expression in response to Bix1 occurs in a cell-autonomous fashion. (I) Injection of FLEx lineage label alone (red staining) does not affect Xbra expression. (J) Injection of FLEx lineage label together with RNA encoding Bix1 causes cell-autonomous down-regulation of Xbra. (K) RNase protection analyses illustrating the effects of Bix1 on gene expression in dorsal and ventral marginal zone regions of the Xenopus embryo. Early markers are analysed at stage 10.5 and late markers at stage 34.
like that of eFGF (Casey et al., 1998), contains Xbra/VegT binding sites, confirming that our technique identifies bona fide T-box targets that play a role in mesoderm and endoderm formation.

Control of Bix gene expression

Although the Bix genes were identified as targets of Xbra, their initial expression, at least in the endoderm, seems not to require Xbra function, because Bix1 activation precedes that of Xbra and indeed Xbra is not expressed in the vegetal hemisphere of the embryo (Smith et al., 1991). The onset of Bix expression may therefore be regulated by secreted factors such as activin or by the product of the maternal gene VegT. The latter possibility is attractive for several reasons. First, VegT is capable of inducing Bix1 expression in animal caps (Fig. 4A), and, like Xbra, it binds to sequences present in the Bix4 promoter. Second, the expression pattern of VegT resembles that of Bix1, and in particular we note that both are down-regulated in the prospective notochord (Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996). And finally, expression of VegT in animal pole explants, like mis-expression of Bix1, causes formation of both mesoderm and endoderm (Horb and Thomsen, 1997), whereas Xbra has not been reported to induce endoderm.

While these considerations suggest that VegT plays an important role in Bix gene expression, a role for Xbra is not excluded. In particular, Xbra may be required for maintenance of Bix1 expression (Fig. 4C), and it is also possible that the two T-box genes co-operate in the regulation of Bix expression. Both Xbra and VegT interact with sequences that correspond to half of the previously identified palindromic Brachyury binding site (Kispert and Herrmann, 1993), and the close proximity of two Xbra/VegT sites in the Bix promoter (Fig. 5A) may allow the formation of Xbra/VegT heterodimers; this is under investigation.

Finally, we note that Bix1 is induced by activin in an immediate-early fashion (Fig. 4B), suggesting that TGF-β family members may co-operate with T-box genes in the control of Bix gene expression.

Functions of the Bix genes

The Bix gene family is likely to have at least three functions in the early Xenopus embryo. The first two may be inferred from mis-expression of Bix1 RNA in animal pole tissue. Low levels of Bix1 induce formation of ventral mesodermal cell types, while higher levels induce endoderm. These observations are in accord with the levels of expression of Bix1 in the embryo, for at the early gastrula stage Bix1 RNA is more abundant in prospective endoderm than in prospective mesoderm (Fig. 3B). The results suggest that Bix1 and perhaps the other Bix genes induce cells to become mesoderm or endoderm in a dose-dependent fashion. Indeed, Bix2, or Milk, has recently been shown to act as an endoderm inducer (Ecochard et al., 1998), as has the more distantly related gene Mixer (Henry and Melton, 1998).

Mis-expression of Bix1 in the marginal zone produces results that are consistent with this model, and it also reveals the third function of Bix1. When Bix1 is mis-expressed in the ventral marginal zone of the embryo, where the endogenous gene is expressed at moderate levels, the amount of Bix1 gene product becomes high enough to induce endoderm. This induction of endoderm is accompanied by a down-regulation of Xbra (Fig. 7I) which, in a negative autoregulatory loop, may then cause a decrease in expression of Bix1 itself (see Fig. 4C).

REFERENCES


