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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), and that activin is capable of acting as a long-range signal in the embryo (Gurdon et al., 1996; Jones et al., 1996). Together, these results suggest that activin may function as a long-range morphogen, with high activin concentrations in the vegetal hemisphere inducing endoderm and lower concentrations in the equatorial region inducing mesoderm.

Activin treatment of prospective ectodermal tissue induces the expression of several target genes in an immediate-early fashion. Of these, *Xenopus Brachyury* (*Xbra*), which is expressed in a widespread fashion throughout the mesoderm (Smith et al., 1991), is particularly interesting. *Brachyury* function is required for posterior mesoderm and notochord differentiation in mouse, zebrafish and *Xenopus* embryos (Conlon et al., 1996; Herrmann et al., 1990; Schulte-Merker et al., 1994), and mis-expression of *Xbra* in prospective ectodermal tissue of *Xenopus* is sufficient to cause ectopic mesoderm formation in a dose-dependent fashion; low concentrations induce formation of ventral mesoderm, while high concentrations induce dorsal tissues including muscle (Cunliffe and Smith, 1992). Co-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 cycloheximide was applied continuously at a concentration of 10 μg/ml.

Construction of a subtracted library and screening of libraries

Xenopus embryos were injected at the one-cell stage with 50 pg RNA encoding Xbra-GR (Tada et al., 1997) and animal caps were dissected from these embryos at late blastula stage 9. They were cultured for 2.5 hours in the presence or absence of dexamethasone (Tada et al., 1997) and poly(A)$^+$ RNA was then prepared from 1500 untreated or 1500 dexamethasone-treated caps. cDNA was cloned in a directional fashion into the *Xho*I and *Not*I sites of the modified pSP64T vector pGEM64TXBT3 to prepare ‘induced’ and ‘uninduced’ cDNA libraries. A subtracted library, enriched for *Xbra*-inducible cDNAs, was prepared using the PCR-Select cDNA subtraction kit (CLONTECH), which makes use of the technique of suppression PCR (Hubank and Schatz, 1994). Subtracted fragments were cloned into pBluescriptIISK(−). Full-length cDNAs were obtained by screening a *Xenopus* gastrula cDNA library (Cho et al., 1991). Four independent positive clones were excised in vivo and designated pBix1, pBix2, pBix3 and pBix4.

Plasmid constructs and in vitro transcription

To construct pSP64T-Bix1, a full-length Bix1 cDNA was inserted into the *Spe*I-*Xho*I sites of pSP64TBX, a modified version of pSP64T. *Xbra-GR* RNA was prepared from pSP64T-Xbra-GR as described (Tada et al., 1997), *Xbra-En^R* RNA was prepared from pSP64T-Xbra-En^R as described (Conlon et al., 1996), *Eomesodermin* RNA was prepared from pEOMES/RN3 as described (Ryan et al., 1996), *VegT* RNA was prepared from pCS2+VegT as described (Zhang and King, 1996) and *BMP-4* RNA was prepared from pSP64T-hBMP4 as described (Jones et al., 1992).

RNA preparation, northern blotting and RNAse protection

RNA preparation and RNAse protection analyses were performed as described (Tada et al., 1997) except that lithium precipitation was omitted. Samples were analysed with probes specific for *Xbra* (Smith et al., 1991), *goosecoid* (Blumberg et al., 1991), *Xwnt8* (Christian et al., 1991), *Xvent1* (Gawantka et al., 1995), *chordin* (Sasai et al., 1994), muscle actin (Mohun 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* (Walmsley et al., 1994). To make a *Bix1* probe, pBix1 was linearized with *Ssp*I 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 *Bam*HI 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 *Xba*I, self-ligated with T4 DNA ligase and then digested with *Bam*HI (the *Bix1* cDNA contains a *Bam*HI site). PCR was carried out using one tenth of this material as a template with the *Bix*-specific primers: 5′-AGTCCAGGGCAGAACTGAAGC-3′ (directed 5′) and 5′-CATCATTCAGGGAATGTGTCT-3′ (directed 3′). The PCR product obtained was digested with *Xba*I, cloned into pBluescriptIISK(−) 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′-CGCGAAGCTTCTTCCAAATCTGCCTCTTCCC-3′, which contains a *Hin*dIII site for cloning. The amplified fragment was cloned into the *Nhe*I and *Hin*dIII sites of pGL3-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:

B: 5′-TCGACTTCATCTTCACACCTTCTGGAGATATTCACACG-TTTCCCCT-3′;
M1: 5′-TCGACTTCATCT**G**CAC**G**C**T**TTCTGGAGATATTCACAC-GTTTCCCCT-3′;
M2: 5′-TCGACTTCATCTTCACACCTTCTGGAGATAT**G**CAC**G**C-**T**TTTCCCCT-3′;
M1/M2: 5′-TCGACTTCATCT**G**CAC**G**C**T**TTCTGGAGATAT**G**CA-C**G**C**T**TTTCCCCT-3′.

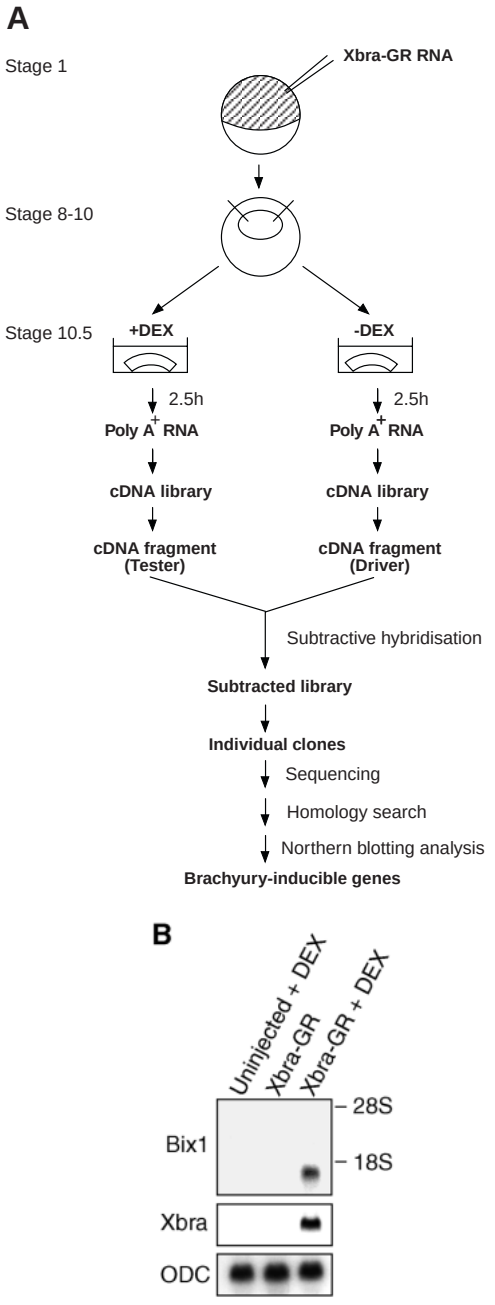

Fig. 1. Design of screen for Xbra targets. (A) Schematic illustration of a strategy to isolate Xbra target genes. Details are described in the text and Materials and methods. (B) Induction of *Bix1* in a screen by northern blotting analysis. Animal caps derived from embryos injected with 50 pg *Xbra-GR* RNA or uninjected 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.

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 *Eco*RV/*Xba*I fragment derived from pXT1 (Smith et al., 1991) and VegT was cloned into the same vector as a *Hin*dIII/*Not*I 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.

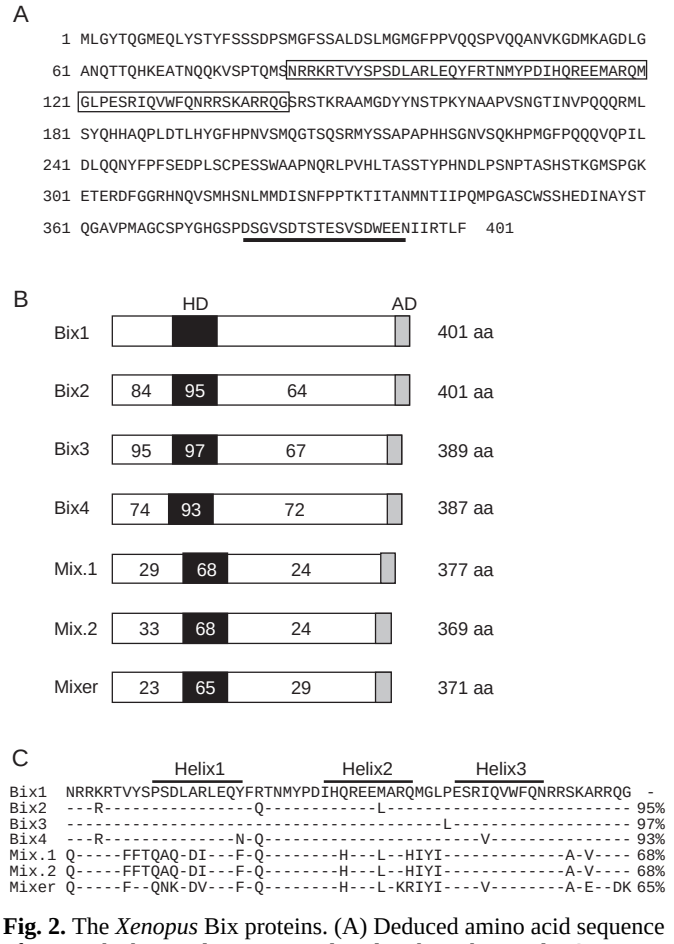

Fig. 2. The *Xenopus* Bix proteins. (A) Deduced amino acid sequence of Bix1. The homeodomain is enclosed within a box and a C-terminal acidic region is underlined. (B) Comparison of the four Bix proteins and *Xenopus* Mix.1 (Rosa, 1989), Mix.2 (Vize, 1996) and Mixer (Henry and Melton, 1998). Figures indicate percentage amino acid identity. HD, homeodomain; AD, acidic domain.
(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.

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 *Bix1-4* 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, to some extent, Mixer.

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

Expression of *Bix1* is first detectable by RNAse 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.

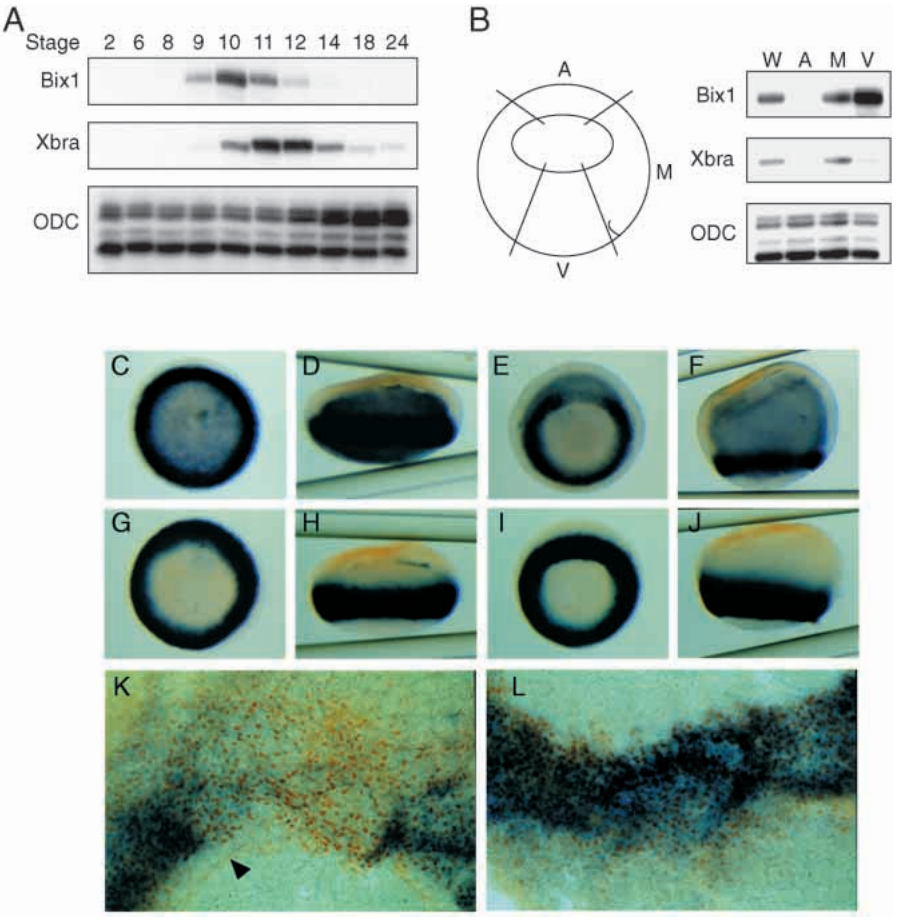

Fig. 3. Expression of *Bix1*. (A) RNAse protection analysis reveals that *Bix1* expression begins between stages 8 and 9 (late blastula) and precedes that of *Xbra*. (B) Dissection of *Xenopus* early gastrulae, followed by RNAse protection analysis, reveals that high levels of *Bix1* expression occur in the vegetal pole region of the embryo, with lower levels present in the marginal zone. A, animal pole; M, marginal zone; V, vegetal pole. (C-J) Comparison of expression patterns of *Bix1* (C-F) and *Xbra* (G-J) at early gastrula stage 10 (C,D,G,H) or stage 10.5 (E,F,I,J). Note expression of *Bix1* in the vegetal hemisphere of the embryo at the early gastrula stage (C,D), and the decline of *Bix1* expression in the vegetal region and the dorsal marginal zone by the mid-gastrula stage (E,F). The decline in *Bix1* expression in the dorsal marginal zone is also illustrated (K,L), and show an embryo that is also stained using an anti-Xbra antibody (brown). (K) Dorsal marginal zone. Note Xbra-positive and *Bix1*-negative cells. The dorsal blastopore lip is marked by an arrowhead. (L) Ventral marginal zone. Cells express both *Xbra* and *Bix1*.

3C,E,K,L). Expression of *Bix1* also declines in the vegetal pole region (Fig. 3C-F). The expression pattern of *Bix1* at the early gastrula stage was confirmed by dissection of embryos followed by RNAse protection analysis. *Bix1* transcripts were absent from the animal cap and present in the marginal zone but the strongest expression of *Bix1* occurred in the vegetal pole region of the embryo (Fig. 3B).

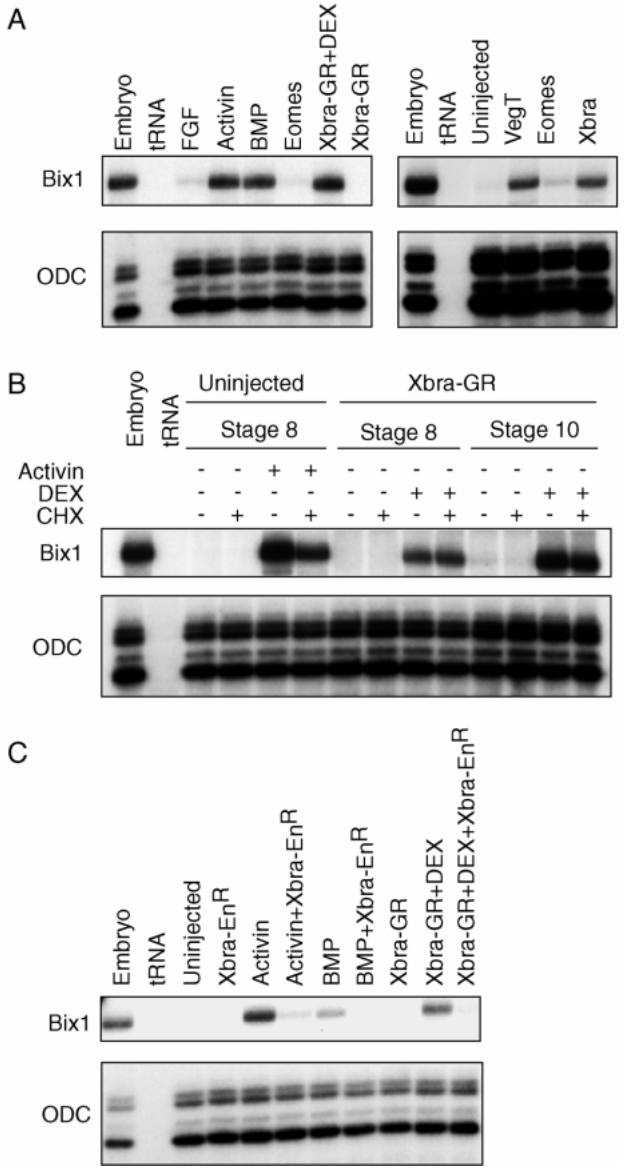

Fig. 4. Induction of *Bix1* expression by inducing factors and T box-containing genes. (A) Expression of *Bix1* is induced in animal caps by 100 ng/ml FGF (weakly), 8 units/ml activin (see Cooke et al. (1987) for the definition of a unit of activin activity), BMP-4 (1 ng RNA), eomesodermin (2 ng RNA), VegT (2 ng RNA), Xbra (2 ng RNA) and Xbra-GR (50 pg RNA). (B) Induction of *Bix1* expression by activin and by Xbra-GR does not require protein synthesis. Cycloheximide (CHX) treatment was sufficient to reduce incorporation of [^{35}S]methionine into acid-precipitable material by 94% during the period of induction. (C) Expression of *Bix1* in response to activin and BMP-4 is reduced by 500 pg RNA encoding Xbra-En^{R}, a dominant-negative *Xbra* construct, and *Bix1* expression in response to Xbra-GR is completely inhibited by *Xbra-En^{R}*.

Regulation 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-En^{R}* (Conlon et al., 1996) (Fig. 4C). In the experiment shown, *Xbra-En^{R}* 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 CTTCACACCT 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 will bind 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 (*). Mutation of either element (creating probes M1 and M2) caused loss of the lower-mobility band, suggesting that the upper complex contains two molecules of XDBD while the lower complex contains just one. Binding to the M1 probe, in which the distal site is mutated, is weaker than binding to M2, indicating that XDBD interacts more strongly with the distal site.

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 $MgCl_2$ concentration of the binding buffer from 1 mM to 5 mM appears to facilitate binding to the proximal site (data not shown).

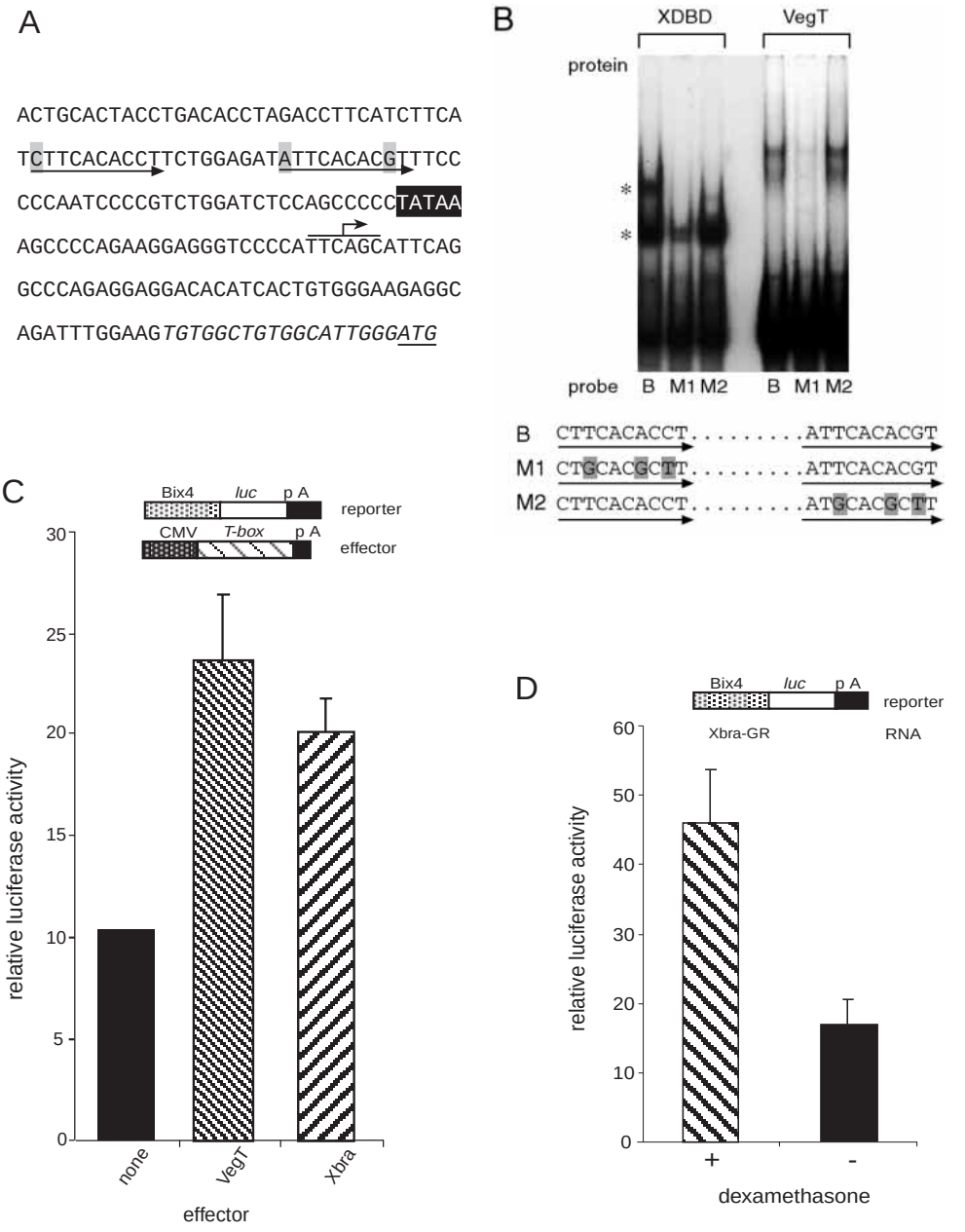

Fig. 5. Xbra and VegT bind to Brachyury half sites in the 5′ regulatory region of *Bix4* and activate transcription of a minimal promoter construct. (A) Upstream regulatory region of *Bix4* (GenBank accession number AF079563). The two 10 bp T-box response elements are underlined with arrows. Nucleotides 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.

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 derived from embryos injected with the *Bix4*-luciferase construct together with RNA encoding Xbra-GR; addition of dexamethasone caused a 3-fold induction of luciferase activity (Fig. 5D).

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 *Xsox17α* 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 later 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

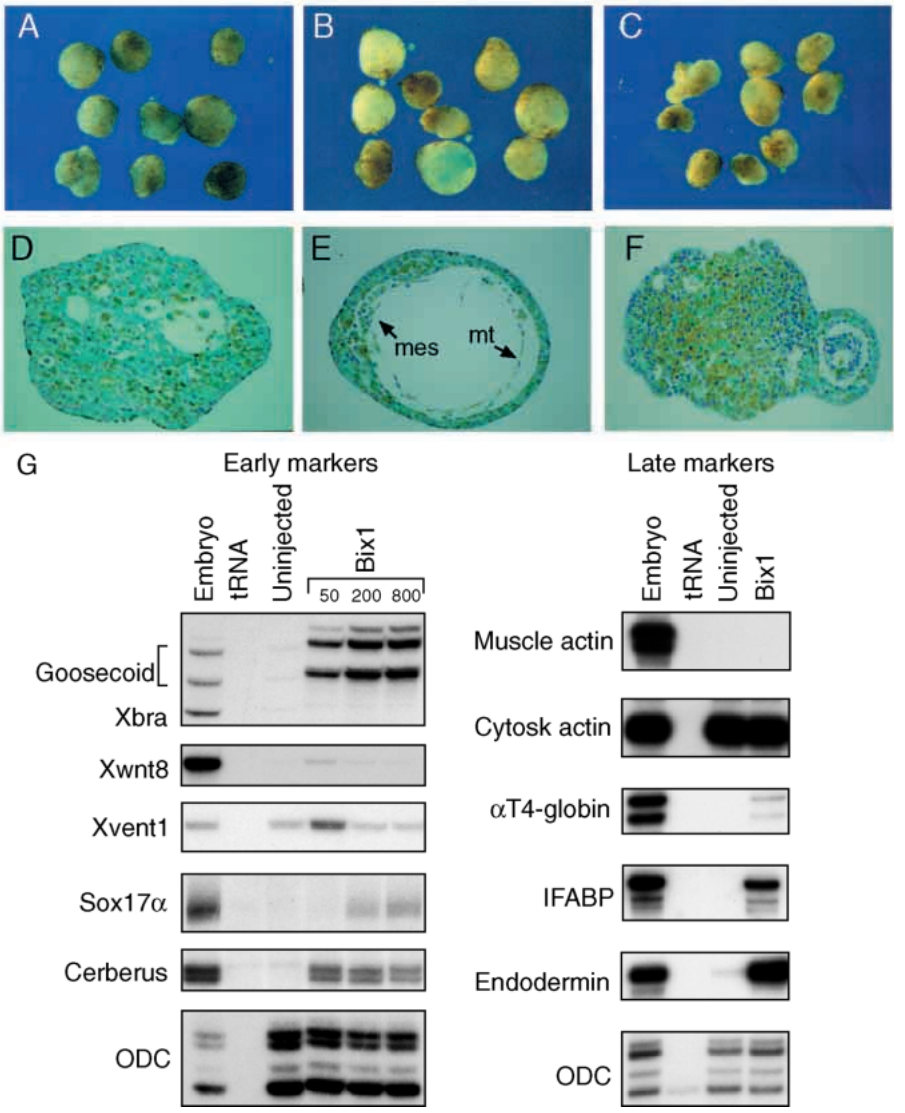

Fig. 6. Low levels of *Bix1* cause animal caps to form ventral mesoderm, and high levels induce formation of endoderm. (A-F) Histological sections of animal caps expressing low (100 pg) and high (1 ng) levels of *Bix1*. Control caps (A,D) form atypical epidermis. Caps expressing low levels of *Bix1* (B,E) form ventral mesoderm including mesenchyme (mes) and mesothelium (mt). Caps expressing high levels of *Bix1* form yolky masses resembling endoderm (C,F). (G) RNAse protection analysis of animal caps *Bix1* RNA. 'Early markers' were analysed at stage 10.5 and received the indicated amounts of RNA; 'Late markers' were analysed at stage 34 and received 200 pg RNA.

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*,

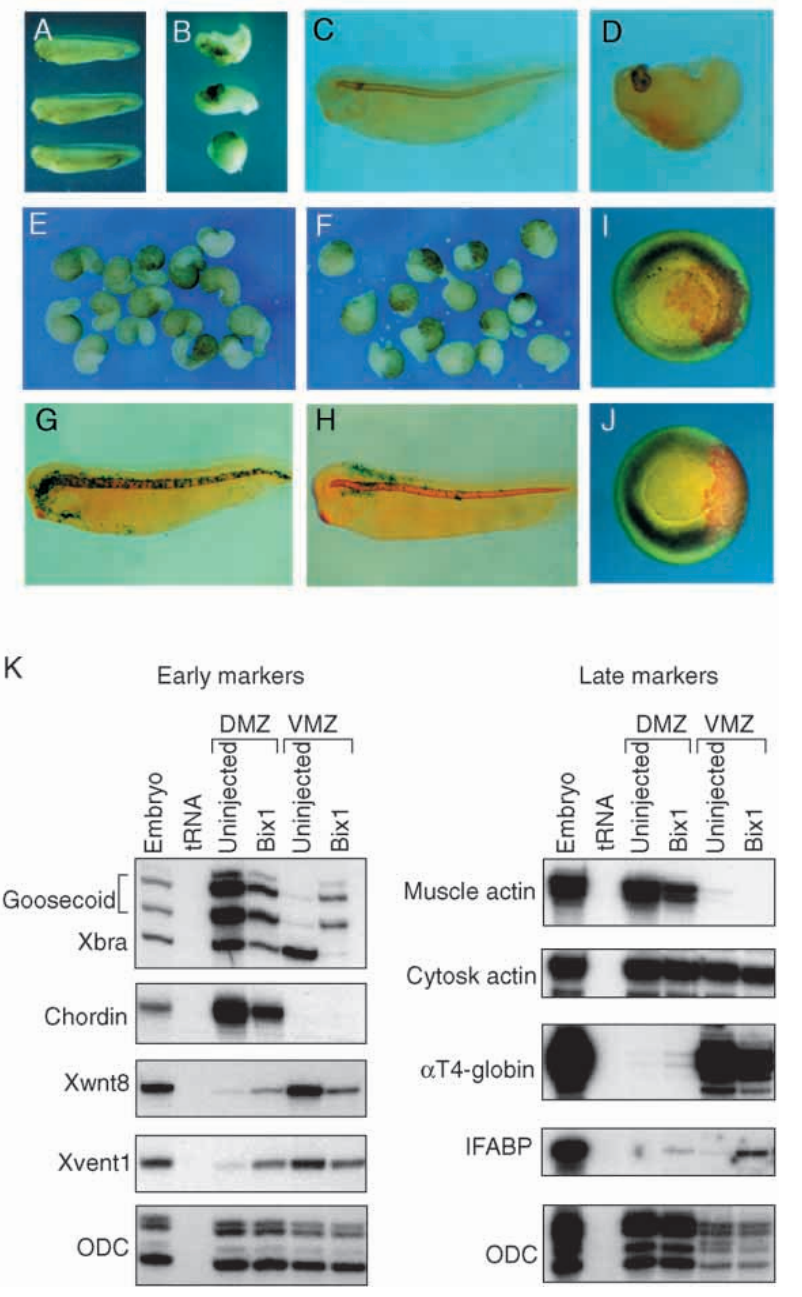

Fig. 7. Effects of *Bix1* over-expression on whole embryos and marginal zone regions. (A) Control embryos at stage 33. (B) Embryos injected with 400 pg *Bix1* RNA at the same stage as those in (A). Note lack of head and axial structures. (C) Control embryo at stage 33 stained with the monoclonal antibody 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 FLDx lineage label alone (red staining) does not affect *Xbra* expression. (J) Injection of FLDx 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. 7J) which, in a negative autoregulatory loop, may then cause a decrease in expression of *Bix1* itself (see Fig. 4C). By contrast, *Bix1* is not normally expressed in the dorsal marginal zone, and when ectopically expressed in this region the amount of *Bix* gene product is not sufficient to induce endoderm, but does cause ventralization of dorsal mesoderm. This ventralizing effect of the *Bix* genes is also revealed by injection of *Bix1* RNA into dorsal blastomeres of the 32-cell stage *Xenopus* embryo; the presence of the *Bix* gene product prevents these cells from contributing to notochord (Fig. 7H). In the future we plan to investigate the mechanisms by which the *Bix* genes exert these effects, an investigation which, like our analysis of the T-box genes, will include a search for their transcriptional targets.

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