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Bix4 is activated directly by VegT and mediates endoderm formation in *Xenopus* development

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SUMMARY

The maternal T-box gene *VegT*, whose transcripts are restricted to the vegetal hemisphere of the *Xenopus* embryo, plays an essential role in early development. Depletion of maternal *VegT* transcripts causes embryos to develop with no endoderm, while vegetal blastomeres lose the ability to induce mesoderm (Zhang, J., Houston, D. W., King, M. L., Payne, C., Wylie, C. and Heasman, J. (1998) *Cell* 94, 515-524). The targets of VegT, a transcription activator, must therefore include genes involved both in the specification of endoderm and in the production of mesoderm-inducing signals. We recently reported that the upstream regulatory region of the homeobox-containing gene *Bix4* contains T-box binding sites. Here we show that expression of *Bix4* requires maternal VegT and that two T-box binding sites are necessary and sufficient for mesodermal and endodermal expression of reporter genes driven by the *Bix4* promoter in transgenic *Xenopus* embryos. Remarkably, a single T-box binding site is able to act as a mesoderm-specific enhancer when placed upstream of a minimal promoter. Finally, we show that *Bix4* rescues the formation of endodermal markers in embryos in which *VegT* transcripts have been ablated but does not restore the ability of vegetal pole blastomeres to induce mesoderm. These results demonstrate that *Bix4* acts directly downstream of VegT to specify endodermal differentiation in *Xenopus* embryos.

Key words: T-box, VegT, Endoderm, *Xenopus laevis*, Transgenesis, Antisense depletion analysis

INTRODUCTION

The three germ layers of the amphibian embryo are established by mechanisms involving both cytoplasmic localisation and cell-cell interactions (Harland and Gerhart, 1997). The localisation of cytoplasmic determinants occurs during oogenesis, when various mRNAs and proteins become differentially distributed within the oocyte. Prominent among these are the RNAs encoding Vg1, a member of the TGF-β family (Rebagliati et al., 1985; Weeks and Melton, 1987), and VegT (Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996) a member of the T-box family of transcription factors (Papaioannou, 1997; Smith, 1999). Both of these RNAs are restricted to the vegetal hemisphere of the embryo. Cell-cell interactions play a role during blastula stages, when vegetal blastomeres induce overlying equatorial cells to form mesoderm rather than ectoderm (Harland and Gerhart, 1997). This signal may derive from both maternal components, such as Vg1 (Dale et al., 1993; Thomsen and Melton, 1993) and from zygotically activated gene products, such as the nodal-related proteins (Jones et al., 1995; Joseph and Melton, 1997; Smith et al., 1995) and derrière (Sun et al., 1999).

According to this scheme, therefore, the ectoderm of the embryo represents a 'default' pathway – the fate that is adopted by cells that receive no positional cue, whether derived from cytoplasmic determinant or inductive signal. Endoderm, by contrast, forms either as a direct consequence of inheriting particular vegetally localised determinants, or as a result of receiving a particularly high dose of a 'mesoderm-inducing' signal such as Vg1 or activin. Mesoderm, as described above, forms in the equatorial region of the embryo in response to a signal derived from the vegetal hemisphere.

Transcripts encoding the vegetally localised T-box protein VegT (also known as Antipodean, Xombi and Brat) (Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996) play an essential role in early *Xenopus* development. Depletion of maternal *VegT* RNA by injection of antisense oligonucleotides into the oocyte causes embryos to develop with no endoderm and greatly reduced amounts of mesoderm, and vegetal pole blastomeres lose the ability to induce mesoderm from animal pole tissue (Zhang et al., 1998). VegT is a transcription activator (Horb and Thomsen, 1997), and these results suggest that its targets must include genes involved both in the specification of endoderm and in the

production of mesoderm-inducing signals. Identification of such targets would represent a significant advance in coming to understand the molecular basis of endoderm and mesoderm formation.

Members of the *Bix* family of paired-type homeobox-containing genes are strong candidates for direct VegT targets (Tada et al., 1998). *Bix1-Bix4* were originally isolated as targets of the T-box gene *Xbra*, which is expressed at the onset of gastrulation in the prospective mesoderm of the *Xenopus* embryo (Smith et al., 1991). However, like the related genes *Mix.1* (Rosa, 1989), *Mix.2* (Vize, 1996) and *Milk* (Ecochard et al., 1998), *Bix1-Bix4* are expressed in the endoderm as well as the mesoderm of the embryo, and their activation precedes that of *Xbra* (Tada et al., 1998). These results indicate that other genes are also involved in the regulation of *Bix* expression and an obvious candidate is the maternally and vegetally expressed *VegT*. Indeed, misexpression of *VegT* in *Xenopus* animal caps induces expression of *Bix1*, and the upstream regulatory region of *Bix4* contains sites that bind VegT as well as Xbra (Tada et al., 1998).

In this paper, we first show that expression of *Bix4* requires maternal *VegT* and that *VegT* is a potent activator both of endogenous *Bix4* and of reporter constructs driven by the *Bix4* upstream regulatory region. Two T-box binding sites contained within the *Bix4* promoter prove to be both necessary and sufficient for mesodermal and endodermal expression of reporter genes in transgenic *Xenopus* embryos and, remarkably, a single T-box binding site is able to act as a mesoderm-specific enhancer when placed upstream of a minimal promoter.

These results show that *Bix4* is a direct target of VegT and they raise the question of whether *Bix4* mediates the effects of VegT. Our results show that *Bix4* rescues the formation of endodermal markers in embryos in which *VegT* transcripts have been ablated but does not restore the ability of vegetal pole blastomeres to induce mesoderm. These results demonstrate that *Bix4* acts directly downstream of VegT to specify endodermal differentiation in *Xenopus* embryos but that rescue of mesoderm induction requires additional VegT targets.

MATERIALS AND METHODS

Xenopus embryos and microinjection

Xenopus embryos were obtained and fertilised as described (Smith, 1993) and staged according to Nieuwkoop and Faber (1975).

Antisense ablation and RNAase protection analysis

Antisense ablation of maternal *VegT* RNA was performed as described (Zhang et al., 1998). RNAase protection was carried out as described (Tada et al., 1998). A *Bix4* probe was made by linearising pBix4 with *Eco*RV and transcribing with T7 RNA polymerase. An *Xwnt11* probe was made from the plasmid pPCR121, isolated from the subtracted cDNA library previously described (Tada et al., 1998). The plasmid was linearised with *Hin*fI and transcribed with T3 RNA polymerase. Other probes, including *Xwnt8* (Christian et al., 1991), *Xbra* (Smith et al., 1991), *ornithine decarboxylase* (Isaacs et al., 1992), *Xvent1* (Gawantka et al., 1995), *Xsox17α* (Hudson et al., 1997), muscle-specific *actin* (Mohun et al., 1984), *αT4-globin* (Walmsley et al., 1994), *IFABP* (Shi and Hayes, 1994) and endodermin (Sasai et al., 1996) were as described (Tada et al., 1998).

Reporter constructs and luciferase assays

A Green Fluorescent Protein cDNA (Zernicka-Goetz et al., 1997) was cloned into the *Hin*dIII/*Xba*I sites of pGL3-basic (Promega). The *Bix4* promoter (Tada et al., 1998) was then cloned into the *Nhe*I and *Hind*III sites of this construct and of pGL3-basic to generate GFP and luciferase reporter gene constructs. Point mutations were generated with the Stratagene QuickChange site-directed mutagenesis kit. The *eFGF* and *Bix4* T-box binding sites (see Fig. 3A) were cloned as annealed oligonucleotides into the *Xba*I or *Sal*I sites of pA48.pBLCAT3T. All constructs were sequenced. Luciferase assays used the Promega Dual Luciferase kit. Five animal caps were lysed in 50 μl of passive lysis buffer (Promega) and 5 or 10 μl were assayed for luminescence.

Transgenesis and in situ hybridisation

Transgenic *Xenopus* embryos were generated as described (Kroll and Amaya, 1996). Typically, 5-35 copies of the reporter gene are integrated into the host genome, with 2-6 copies at a single site (Kroll and Amaya, 1996). In situ hybridisations were as described (Tada et al., 1998). To make in situ hybridisation probes, GFP and luciferase plasmids were linearised with *Nco*I and CAT plasmids were linearised with *Sal*I or *Pst*I.

RESULTS

Bix4 expression is abolished in embryos lacking maternal *VegT* RNA and is activated in animal caps by *Xbra* and *VegT*

Expression of *Bix4* was analysed in embryos in which maternal *VegT* transcripts were depleted by injection of antisense oligonucleotides into the oocyte. Previous work indicates that *endodermin*, a pan-endodermal marker (Sasai et al., 1996), *Xsox17α*, an endoderm-specific transcription factor (Hudson et al., 1997), and *IFABP*, a marker of small intestine (Shi and Hayes, 1994) are not expressed in such embryos, and that expression of mesodermal markers is delayed (Zhang et al., 1998). Fig. 1 shows that *Bix4* (and *Bix1*, not shown) is also undetectable in *VegT*-depleted embryos, indicating that maternal *VegT* is essential for initiation of transcription of this gene. Consistent with this observation, *VegT* and (to some extent) the related T-box gene *Xbra*, as well as a combination of the two, induce expression of *Bix4* in animal caps (Fig. 2A). They also activate expression in animal caps of a luciferase reporter gene driven by 1.6 kb of sequence upstream of the *Bix4* transcription start site (Fig. 2B and Tada et al., 1998).

Although *Xbra* and *VegT* are both eventually expressed in maternal *VegT*-depleted embryos (Fig. 1 and Zhang et al., 1998), their activation at stage 14 is apparently too late to induce *Bix4*; previous work has demonstrated that blastomeres lose the ability to respond to *Xbra* by stage 13 (Tada et al., 1997).

T-box sites in the *Bix4* promoter are required for expression in mesoderm and endoderm

The *Bix4* regulatory region contains two 10 bp T-box binding sites within 70 bp of the transcription start site, and a related sequence is positioned a further 15 bp upstream (Fig. 3A and Tada et al., 1998). We refer to these sites as 'distal', 'middle' and 'proximal' (T_d, T_m and T_p). In vitro binding assays indicate that both Xbra and VegT bind to the middle site, while only Xbra binds strongly to the proximal site (Tada et al., 1998). Interaction between VegT and the proximal site can be

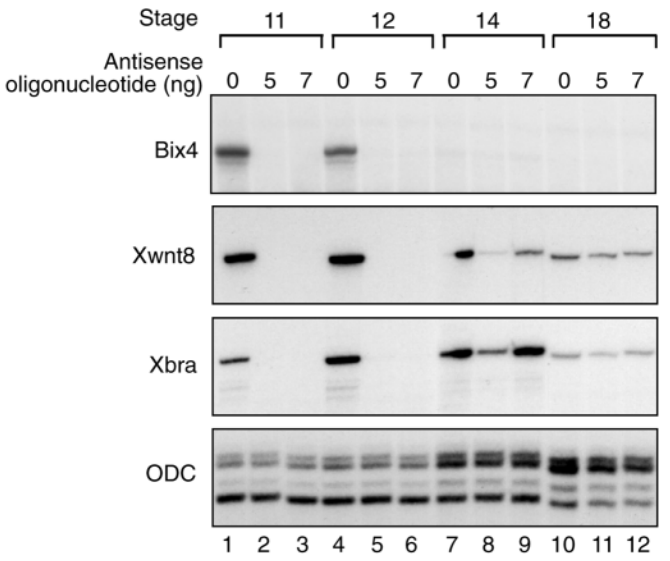


Fig. 1. *Bix4* expression is abolished in embryos depleted of maternal *VegT* RNA. Embryos derived from uninjected oocytes or from oocytes injected with 5 or 7 ng *VegT* antisense oligonucleotides were analysed by RNAase protection at stages 11, 12, 14 or 18 for expression of *Bix4*, *Xwnt8*, *Xbra* and *ODC*. Depletion of *VegT* RNA prevents expression of *Bix4* and delays expression of *Xwnt8* and *Xbra*.

detected, however, with increased levels of $MgCl_2$ and VegT protein in the binding reaction (not shown).

To ask whether VegT activates *Bix4* expression through direct interaction with these sites, we made transgenic *Xenopus* embryos in which the 1.6 kb *Bix4* upstream regulatory sequence was used to drive expression of reporter genes. Analysis was carried out at the early gastrula stage, and three reporter genes were used: Firefly Luciferase (LUC), Green Fluorescent Protein (GFP) and chloramphenicol acetyltransferase (CAT). GFP fluorescence is difficult to visualise in gastrula-stage embryos due to yolk autofluorescence, so the spatial expression of this and other reporters was analysed by in situ hybridisation.

Transgenic *Xenopus* embryos carrying the *Bix4*-GFP or *Bix4*-LUC construct expressed reporter genes in both mesoderm and endoderm, in a pattern resembling that of endogenous *Bix4* (Fig. 3B; Table 1). Interestingly, vegetal expression of the *Bix4*-GFP construct was higher than that of *Bix4*-LUC. This may reflect a difference in RNA stability or in the ability of the in situ probe to penetrate endoderm. Since here we are particularly interested in identifying elements required for endodermal expression of *Bix4*, we have concentrated on use of the *Bix4*-GFP construct.

To investigate directly the roles of the T-box binding sites in the regulation of *Bix4*, they were mutated individually and these constructs were tested for the ability to drive expression of GFP in transgenic embryos (Fig. 3B; Table 1). Mutation of the middle T-box site (T_m) resulted in the most striking change in expression pattern; transgenic embryos containing this reporter construct lacked expression in the endoderm even after prolonged staining. In contrast, mutation of the distal or proximal sites (T_d or T_p) did not affect the strong endodermal expression. In addition, we note that all three mutations caused a decrease in intensity but an increase in extent of reporter gene expression in the marginal zone, that mutations in the T_p site frequently caused higher levels of reporter gene expression on the dorsal side of the embryo, and that mutations in the T_d site caused elevated levels of reporter gene expression in the vegetal hemisphere.

These experiments demonstrated that the T_m site is necessary for expression of *Bix4* reporter genes in the endoderm, but the functions of the T_p and T_d sites were harder to define. In an effort to elucidate their roles, double mutations with the T_m site were made (Fig. 3B). Embryos with the T_d/T_m double mutation construct exhibited low, diffuse reporter gene expression; there is little or no expression in the endoderm and weak expression throughout the ectoderm and marginal zone. Embryos with the T_m/T_p double mutation exhibit complete loss of reporter gene expression.

Together, our results indicate that the middle (T_m) site, to which both VegT and Xbra bind, is required for endodermal expression and for strong expression in the mesoderm. The proximal (T_p) site, which Xbra binds strongly and VegT only weakly, is required together with the middle site for expression in the mesoderm. Finally, since mutation of the T_d

Table 1. Expression patterns of *Bix* reporter gene constructs

Construct	Number of experiments	Embryos examined	Expression				
			Mesoderm and endoderm	Mesoderm alone	Endoderm alone[#]	Other*	No expression
Bix4.GFP	6	132	51	0	41	21	19
ΔT_d *Bix4*.GFP	5	143	52	11	37	24	19
ΔT_m *Bix4*.GFP	3	77	10	52	0	11	4
ΔT_p *Bix4*.GFP	3	78	52	3	8	9	6
$\Delta T_d\,\Delta T_m$ *Bix4*.GFP	5	102	21	29	11	8	33
$\Delta T_m\,\Delta T_p$ *Bix4*.GFP	2	71	0	0	0	8	63
T_mT_p-CSKA.CAT	7	131	41	24	0	25	41
ΔT_mT_p-CSKA.CAT	5	75	2	37[‡]	0	16	20
$T_m\Delta T_p$-CSKA.CAT	6	191	6[‡]	96[‡]	0	34	55
$\Delta T_m\Delta T_p$-CSKA.CAT	5	130	0	7[‡]	0	49	74
eFGF-CSKA.CAT	6	105	8	42	0	15	40

*Other includes one or two isolated 'spots' of expression which may derive from unintegrated plasmid.
Endodermal expression consisted only of a few weak spots of staining, while mesodermal expression was very strong.
[‡]Mesodermal expression in these cases was very weak.
[#]In cases where staining appeared to be confined to endoderm, it is likely that more protracted incubation would also have revealed mesodermal expression.

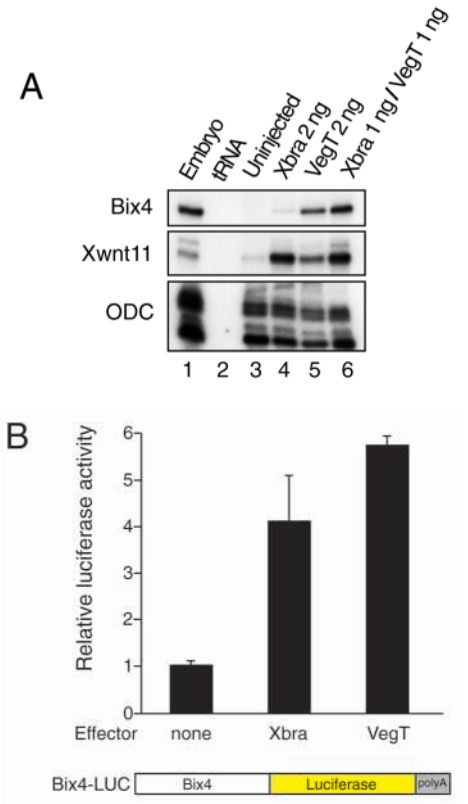


Fig. 2. *VegT* and *Xbra* induce expression of endogenous *Bix4* and of *Bix4* reporter constructs. (A) Expression of *Bix4* is induced in animal caps in response to *Xbra* (weakly), *VegT*, and a combination of both T-box genes. Embryos at the 2-cell stage were injected with the indicated RNAs. Animal pole regions were dissected at stage 8, cultured to stage 10.5, and then assayed by RNAase protection for expression of *Bix4*, *Xwnt11* and *ODC*. (B) Both *VegT* and *Xbra* activate expression of a *Bix4* reporter construct. Embryos at the 2-cell stage were injected with 20 pg *Bix4*-LUC, 10 pg pRLTK and, where indicated, 250 pg *Xbra* or *VegT* RNA. Animal caps were dissected at stage 8 and groups of five were assayed in triplicate for Firefly and Renilla luciferase activities 3.5 hours later. Firefly luciferase activities were then normalised to Renilla activities. Error bars indicate standard deviations.

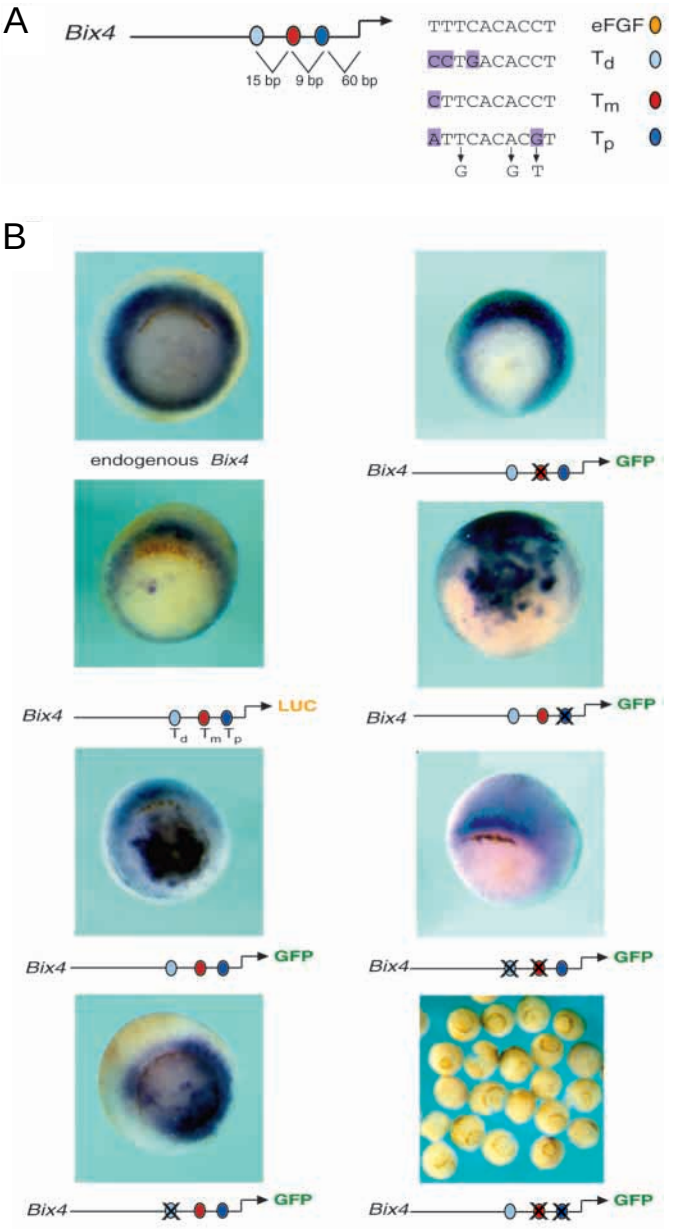


Fig. 3. Three T-box-related binding sites are required for expression of *Bix4* reporter constructs in the mesoderm and endoderm of *Xenopus* early gastrulae. (A) Locations (left) and sequences (right) of three 10 bp sites in the *Bix4* promoter that show homology to the T binding site (Tada et al., 1998): T_d, the site most distal to the transcription start site (light blue oval); T_m, the middle site (red oval); T_p, the site most proximal to the transcription start site (dark blue oval). The sequence of the Xbra binding site in the *eFGF* promoter (Casey et al., 1998) (orange oval) is shown for comparison. Purple boxes indicate base pairs that differ between the four sequences and arrows indicate the mutations made in each site. (B) In situ hybridisation analysis of transgenic *Xenopus* embryos carrying the indicated reporter gene constructs. X indicates that the site has been mutated as shown in A.

site leads to higher and more diffuse expression of reporter genes, this distal site appears to be required to restrict expression to the correct cells and to repress levels of expression. All three T-box binding sites are necessary for correct expression of *Bix4* in the mesoderm and endoderm of the early gastrula.

T-box sites in the *Bix4* promoter are required for activation by VegT

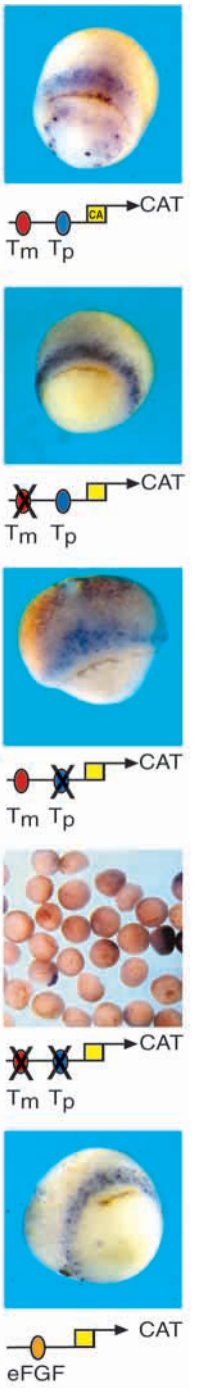
To address the effects of mutating the T-box sites in a more quantitative fashion, we studied the ability of VegT to activate wild-type and mutated *Bix4*-LUC constructs in animal caps (Fig. 4). Consistent with our observations in transgenic embryos, mutation of the middle site (T_m) essentially abolishes luciferase induction by VegT, while mutation of the proximal site (T_p) has little effect. Most significantly, mutation of the distal site (T_d) causes a four-fold increase in basal reporter gene expression while retaining significant VegT inducibility. These results suggest that the levels of staining in transgenic embryos are related to the levels of expression of *Bix4* reporter constructs. They also confirm that the distal T-box binding site (T_d) plays a role in repressing expression of *Bix4*.

A single T-box site is sufficient for expression in mesoderm while both sites in the *Bix4* promoter are required for expression in endoderm

The importance of the T-box binding sites in the expression of *Bix4* during early *Xenopus* development was emphasised in experiments in which a short sequence from the *Bix4* promoter containing the T_m and T_p sites was placed upstream of the minimal cytoskeletal actin (CSKA) promoter driving expression of CAT. This element proved to be sufficient to drive expression of CAT in both mesoderm and endoderm (Fig. 5). Mutation of either site caused loss of expression in the endoderm and, in the case of mutations in the proximal site (T_p), a decrease in mesodermal expression. Mutation of both sites resulted in loss of specific expression in both germ layers. These results suggest, remarkably, that a single T-box site is sufficient to drive mesoderm-specific expression of reporter constructs. To verify this idea, the 10 bp T-box site identified in the *eFGF* promoter (Casey et al., 1998) was placed upstream of the CSKA or thymidine kinase (TK) minimal promoters and these constructs were used to make transgenic *Xenopus* embryos. In both cases, expression of the CAT reporter gene during gastrula stages was confined to the mesoderm (Fig. 5; data not shown for the TK minimal promoter). Together, these experiments demonstrate that T-box sites are sufficient, as well as necessary, for reporter gene expression in mesoderm and endoderm. Constructs such as those described above might be used to drive specific expression of dominant-negative or other genes in the mesoderm and endoderm, and we are presently testing T-box sites to see if they function as mesodermal or endodermal enhancers in other organisms.

Bix4 restores expression of endodermal markers to embryos depleted of maternal VegT but does not rescue the ability to induce mesoderm

The results described above indicate that *Bix4* is a direct target of *VegT* and they raise the question of whether *Bix4* mediates the effects of *VegT*. Preliminary experiments demonstrated that *Bix4*, like *Bix1* (Tada et al., 1998) and members of the related *Mix* family (Ecochard et al., 1998; Henry and Melton, 1998; Latinkic and Smith, 1999; Rosa, 1989; Vize, 1996), can activate endodermal markers such as *endodermin*, *Xsox17α* and *IFABP* in animal caps, as well as the late ventral mesodermal marker *αT4-globin* (Fig. 6A). *Bix4* also, in a dose-dependent manner, restored expression of *Xsox17α*, *endodermin* and *Xbra* to whole embryos depleted of maternal *VegT* RNA (Fig. 6B and data not shown), with lower doses tending to rescue *Xbra* and higher doses rescuing *Xsox17α*. We note, however, that this rescue is both delayed and incomplete; elevated expression of both *Xbra* and *Sox17α* in response to *Bix4* is only detectable by stage 12.5. This indicates that *Bix4* cannot be the sole mediator of the effects of *VegT*. Consistent with this conclusion, *Bix4* cannot effect morphological rescue of *VegT*-depleted embryos (data not shown), and, unlike *VegT* itself, it does not restore to *VegT*-depleted vegetal pole regions the ability to induce mesoderm. Thus, wild-type animal caps do not elongate when juxtaposed with VegT-depleted vegetal bases expressing *Bix4* and muscle actin is not expressed in these conjugates (Fig. 7A,B). This indicates that rescue of *Xbra* expression by *Bix4* (Fig. 6B) is a cell-autonomous effect of *Bix4* rather than an indirect effect mediated by vegetal pole blastomeres.



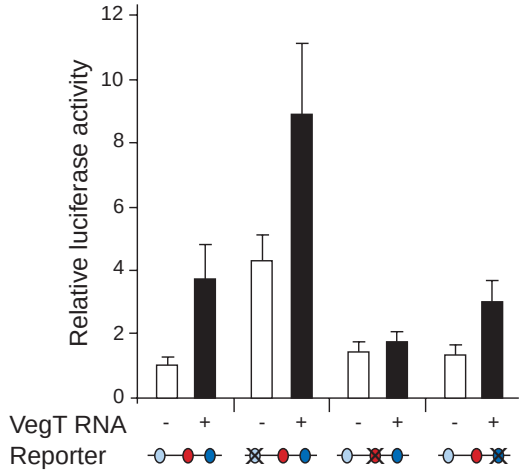


Fig. 4. Mutation of any of the three T-box sites alters the activity of *Bix4*-LUC in response to *VegT* in animal caps. Embryos at the 2-cell stage were injected with 20 pg *Bix4*-LUC, 10 pg pRLTK and, where indicated, 250 pg *VegT* RNA. Animal caps were dissected at stage 8 and groups of five were assayed in triplicate for Firefly and Renilla luciferase activities 3.5 hours later. Firefly luciferase activities were then normalised to Renilla activities. Each measurement represents the average of three separate experiments; error bars indicate standard errors.

DISCUSSION

The results described in this paper demonstrate that the paired-like homeobox gene *Bix4* is a direct target of the T-box transcription factor *VegT*. Depletion of maternal *VegT* transcripts prevents *Bix4* expression and *VegT* proves to be a potent activator both of endogenous *Bix4* and of reporter

Fig. 5. A 42 bp *Bix4* promoter element containing two T-box binding sites (T_m and T_p) drives reporter gene expression in the mesoderm and endoderm of the *Xenopus* early gastrula. Mutation of either site (represented by Xs) causes loss of expression in the endoderm, with mutation of the T_p site also causing a reduction in mesodermal expression. Mutation of both sites abolishes germlayer-specific expression. A single 10 bp T-box binding site derived from the *eFGF* promoter is sufficient to drive expression throughout the presumptive mesoderm. T_m, T_p and eFGF T-box binding sequences are shown in Fig. 3A. Yellow box indicates the CSKA minimal promoter.

constructs driven by the *Bix4* promoter. Analysis of transgenic *Xenopus* embryos expressing *Bix4* reporter constructs shows that the T_m site (Fig. 3A), to which VegT binds strongly, is necessary for expression in the endoderm, while two other sites in the promoter (T_d and T_p) are also required for correct spatial expression at the early gastrula stage.

Additional experiments show that a short sequence from the *Bix4* promoter containing the T_m and T_p sites, when placed upstream of a minimal promoter, is sufficient to drive expression of reporter constructs in both mesoderm and endoderm. Furthermore, a single T-box binding site derived from the *eFGF* promoter, comprising just 10 nucleotides, is sufficient to drive expression of reporter constructs exclusively in the mesoderm.

Finally, we observe that *Bix4* can restore, to some extent, the expression of endodermal and mesodermal markers in *VegT*-depleted embryos but cannot rescue the ability of the vegetal pole to induce mesoderm. These and other issues are discussed below.

The *Bix* genes as targets of Xbra and VegT

The four *Bix* genes were isolated in a screen for targets of Xbra, although it is clear from our previous work (Tada et al., 1998) and from work described here that they are also activated by other T-box gene products, including VegT. In this connection, we note that expression of *Bix1* is induced equally strongly by Xbra and VegT (Tada et al., 1998), while *Bix4* is much more efficiently activated by VegT (Fig. 2A). This indicates that *Xbra* plays a more significant role in the regulation of *Bix1* than in *Bix4* and, consistent with this suggestion, we observe that mesodermal expression of *Bix1* is higher than that of *Bix4* (M. T., unpublished observations). A comparison of the regulatory regions of *Bix1* and *Bix4* may therefore shed light on T-box gene specificity.

Analysis of the *Bix4* promoter has identified three 10 base-pair motifs that resemble the T site in the upstream regulatory region of the Xbra target *eFGF* (Casey et al., 1998). These motifs, T_m, T_p and T_d, differ from the *eFGF* motif with respect to 1, 2 and 3 base pairs, respectively (Fig. 3A). VegT appears to have different affinities for each site (ESC, unpublished) and each site has a distinct role in the regulation of *Bix4* expression. The site with the highest affinity for VegT, T_m, is required for expression of reporter genes in the endoderm (Fig. 3B) and for activation of reporter genes in response to VegT (Fig. 4), while the lower affinity T_p site is necessary for normal expression in the mesoderm (Fig. 3B). Mutation of the latter site does not affect the response of *Bix4* to VegT (Fig. 4).

The T_d site, which differs from the consensus T site with respect to three nucleotides, does not appear to bind VegT or Xbra (E. S. C., unpublished observations) and mutation of this site suggests that it is required to restrict or repress expression of *Bix4*. One possibility, therefore, is that the T_d site binds another member of the T-box family which acts as a transcriptional repressor and, indeed, Tbx2 has recently been shown to repress the melanocyte-specific TRP-1 promoter (Carreira et al., 1998). We note, however, that the CACCT sequence contained within all the T sites is also the binding site for a family of two-handed zinc finger/homeodomain proteins which includes δEF1 (Higashi et al., 1997; Takagi et al., 1998), Zfh-1 (Lai et al., 1993) and, most recently, SIP1 (Verschueren et al., 1999). Members of this family act as transcriptional

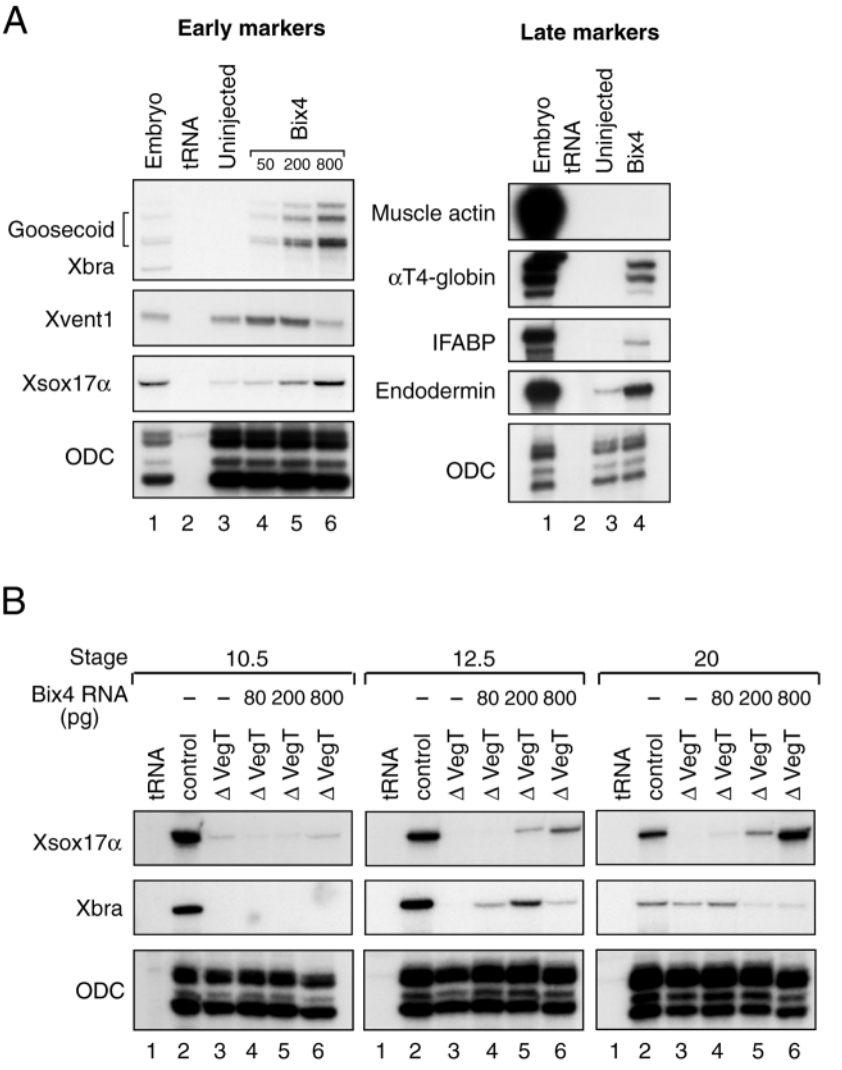


Fig. 6. *Bix4* induces endodermal and some mesodermal markers in animal caps and rescues (albeit belatedly) expression of *Xsox17α* and *Xbra* in *VegT*-depleted embryos. (A) Misexpression of low concentrations of *Bix4* elevates expression of *Xvent1* in animal caps assayed at stage 10.5, while high concentrations induce *goosecoid* and *Xsox17α*. At stage 34, 200 pg *Bix4* induces expression of *αT4-globin*, *IFABP* and *endodermin*. *Bix4* RNA was injected into *Xenopus* embryos at the 2-cell stage, and animal caps were dissected at stage 8 and cultured to stages10.5 (early) or 38 (late) when they were analysed by RNAase protection. (B) *Bix4* causes belated rescue of mesodermal and endodermal markers in embryos depleted of maternal *VegT* RNA. Embryos lacking maternal *VegT* (ΔVegT) were injected with the indicated amounts of *Bix4* RNA at the 2-cell stage into the vegetal hemisphere. They were allowed to develop to stage 10.5, 12.5 or 20 and were then analysed by RNAase protection for expression of *Xsox17α*, *Xbra* and *ODC*.

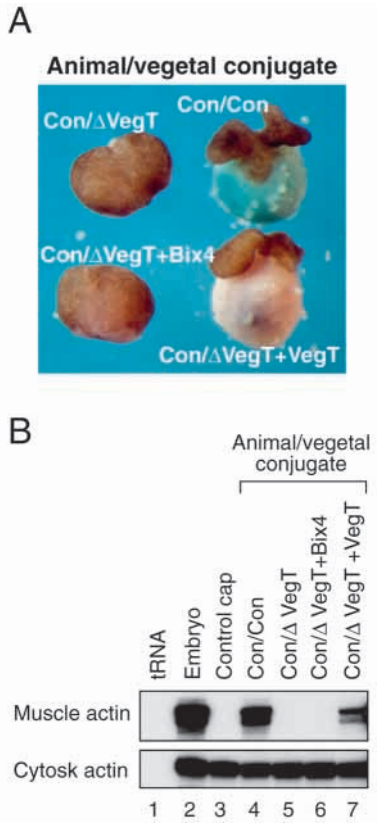


Fig. 7. *Bix4* does not restore the ability of *VegT*-depleted vegetal pole regions to induce mesoderm. (A) Unlike *VegT*, *Bix4* cannot restore to VegT-depleted vegetal pole regions the ability to induce animal caps to undergo mesoderm-specific morphological movements (Symes and Smith, 1987). VegT-depleted embryos were left uninjected (ΔVegT) or received injections of 200 pg *Bix4* or 200 pg *VegT* RNA. Vegetal pole regions derived from such embryos or from control embryos (Con) were dissected at stage 8 and juxtaposed with animal pole regions from control embryos. They were cultured to stage 16 and photographed. (B) Unlike *VegT*, *Bix4* cannot restore to *VegT*-depleted vegetal pole regions the ability to induce animal caps to express muscle-specific actin. Animal cap-vegetal pole conjugates were prepared as in (A), but animal pole regions were separated from the vegetal tissue after 2 hours (Zhang et al., 1998) and cultured alone to stage 25 when they were analysed by RNAase protection.

repressors, and it will be of interest to discover whether such proteins are expressed during early *Xenopus* development.

T-box sites function as mesodermal and endodermal enhancers

To simplify analysis of the roles of the T-box binding sites in the regulation of *Bix4* expression, we have placed them upstream of a minimal promoter. Our results demonstrate that one T-box site linked to a minimal promoter, whether it be T_p, T_m, or the site derived from the *eFGF* promoter, is sufficient for activation of a reporter gene in the mesoderm, while the combined action of the T_m and T_p sites results in expression in the endoderm (Figs 3B, 5).

These observations provide no simple rationalisation of a model that proposes that endoderm and mesoderm are specified by a gradient of VegT, with high concentrations of VegT specifying endoderm and low concentrations mesoderm (Kimelman and Griffin, 1998; Stennard, 1998; Zhang et al., 1998). Indeed, from the model one might predict the opposite of what we observe – that is, a reporter driven by a single T-box site should be expressed more strongly in the endoderm, where levels of VegT are highest, than in the mesoderm.

One interpretation of the inability of a single T-box binding site to drive expression of reporter genes in the endoderm is that the vegetal hemisphere contains repressor molecules that compete with VegT for the T-box site. As discussed above, these might include T-box family members such as Tbx2 (Carreira et al., 1998) or members of the δEF1 family (Higashi et al., 1997; Lai et al., 1993; Takagi et al., 1998; Verschueren et al., 1999). According to this idea, the ability of two T-box binding sites to drive expression in the endoderm might be due to more efficient binding of VegT to two sites than to one. An alternative view is that the ability of a single T-box binding site to drive expression of reporter genes in the mesoderm and not the endoderm is that equatorially expressed T-box genes, such as *Xbra* and *eomesodermin* (Ryan et al., 1996), also play a role in the activation of *Bix4* through this site and that their gene products are more efficient activators than VegT. This is under investigation.

Role of *Bix4* in the formation of endoderm and mesoderm

Maternal *VegT* is required for proper formation of both the endoderm and the mesoderm of the *Xenopus* embryo (Zhang et al., 1998). Our data demonstrate that, in rescuing endoderm formation in *VegT*-depleted embryos, *Bix4* can mediate at least some of the effects of *VegT*. The fact that *Bix4* is unable to restore mesoderm-inducing activity to vegetal pole regions derived from *VegT*-depleted embryos, however, suggests that there are additional VegT targets, which may include other members of the *Bix* and *Mix* families and even the inducing factors themselves. In the future, we plan to isolate such targets, investigating first those genes that restore mesoderm-inducing activity to *VegT*-depleted vegetal pole regions. Candidates under investigation include the *nodal*-related genes (Jones et al., 1995; Joseph and Melton, 1997; Smith et al., 1995) and *derrière* (Sun et al., 1999). We also plan to investigate the extent to which inducing factors such as these also contribute to the regulation of *Bix* gene expression (Tada et al., 1998).

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