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Region-specific activation of the *Xenopus Brachyury* promoter involves active repression in ectoderm and endoderm: a study using transgenic frog embryos

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SUMMARY

Tissue specification in the early embryo requires the integration of spatial information at the promoters of developmentally important genes. Although several response elements for signalling pathways have been identified in *Xenopus* promoters, it is not yet understood what defines the sharp borders that restrict expression to a specific tissue. Here we use transgenic frog embryos to study the spatial and temporal regulation of the *Xbra* promoter. Deletion analysis and point mutations in putative transcription factor-binding sites identified two repressor modules, which exert their main effects at different stages during gastrulation. One module is defined by a bipartite binding site for a Smad-interacting protein (SIP1) of the δEF1 repressor family and acts to confine expression to the marginal zone early in gastrulation. The other module is defined by two homeodomain-binding sites and is responsible for repression in dorsal mesoderm and ectoderm at mid-gastrula stages. In addition, an upstream region of the promoter is necessary to repress expression in neural tissues later in development. Together, our results show that repression plays an important role in the restriction of *Xbra* expression to the mesoderm, and we suggest that similar mechanisms may be involved in the spatial regulation of other genes in early embryonic development.

Key words: *Brachyury*, Mesoderm, Repression, SIP1, Transcriptional regulation, Transgenesis, *Xbra*, *Xenopus*

INTRODUCTION

The *Xenopus* blastula is patterned into three domains: the animal pole region, the marginal zone and the vegetal hemisphere (see reviews by Slack, 1994; Harland and Gerhart, 1997). During gastrulation, these regions become rearranged to form, respectively, the ectoderm, mesoderm and endoderm of the embryo. The identity of these germ layers is established by a developmental program laid down in the genome of the organism, which requires the correct spatial distribution of transcription factors (see, for example, Zhang et al., 1998).

Xenopus Brachyury (*Xbra*) is a member of the T-box transcription factor family of genes (Herrmann et al., 1990; Papaioannou and Silver, 1998; Smith, 1999) and is expressed in the marginal zone of the embryo shortly before the beginning of gastrulation (Smith et al., 1991). During gastrulation, transcription of *Xbra* is downregulated in involuting mesoderm, while expression is maintained in the prospective notochord (Smith et al., 1991). By the end of gastrulation, *Xbra* can be detected only in the tailbud and notochord (Gont et al., 1993). This expression pattern of *Brachyury* is conserved throughout the vertebrates (Papaioannou and Silver, 1998; Smith, 1999).

The importance of the correct spatial expression of *Brachyury* is highlighted by the effects of its absence or misexpression. On the one hand, mouse or zebrafish embryos lacking a functional *Brachyury* gene do not form posterior mesoderm or a properly differentiated notochord (Chesley, 1935; Halpern et al., 1993), and inhibition of the transcription activation function of *Xbra* causes a similar phenotype in *Xenopus* (Conlon et al., 1996). On the other hand, mis-expression of *Xbra* in *Xenopus* causes animal pole tissue to form mesoderm, with a concomitant perturbation of gastrulation (Cunliffe and Smith, 1992). These experiments show that *Brachyury* is both necessary and sufficient for the formation at least of posterior mesoderm. Thus, if one were able to understand how expression of *Brachyury* is regulated, and then how this transcription factor exerts its effects at the level of its target genes, this would represent a significant step forward in our understanding of mesoderm induction. We have recently begun to search for *Xbra* target genes in an attempt to ask how *Brachyury* specifies mesoderm (Casey et al., 1998, 1999; Tada et al., 1998). In this paper, we address the regulation of *Xbra* expression.

Xbra expression can be ectopically activated in the animal hemisphere of the *Xenopus* embryo by members of the TGF-

β and FGF families of signalling polypeptides (Smith et al., 1991; Isaacs et al., 1994; Stennard et al., 1996). Induction of *Xbra* by at least some of these factors is direct in the sense that it can occur in the presence of the protein synthesis inhibitor cycloheximide (Smith et al., 1991). Interestingly, addition of FGF also leads to ectopic activation of *Xbra* in the vegetal hemisphere of the embryo (Cornell et al., 1995). Somehow, these signalling pathways must be integrated at the *Xbra* promoter to result in the proper expression of *Xbra* in the marginal zone and later in the posterior mesoderm and notochord of the embryo. This issue is complicated by the fact that many of the signalling pathways that can induce ectopic expression of *Xbra* in animal or vegetal pole tissue are active in these regions. For example, BMP signalling occurs at high levels in the animal hemisphere during gastrulation (Fainsod et al., 1994), while nodal-related genes (Jones et al., 1995) and derrière (Sun et al., 1999) are expressed in the vegetal hemisphere. Similarly, the MAP kinase pathway, which mediates the activation of *Xbra* in response to FGF (Gotoh et al., 1995; LaBonne et al., 1995; Umbhauer et al., 1995), is active in the vegetal hemisphere (LaBonne and Whitman, 1997; Christen and Slack, 1999). How then are the sharp borders of *Xbra* expression created that confine its transcripts to the mesoderm?

Attempts to isolate activin- and FGF-response elements in the *Xbra* promoter have defined a 150 bp region in the 5′ flanking region, which confers responsiveness to both factors in embryos injected with *Xbra*-based reporter constructs (Latinkic et al., 1997). This region also contains homeodomain-binding sites, which confer repression of *Xbra* at high doses of activin (Green et al., 1992; Gurdon et al., 1994, 1995). However, a more precise localisation of response elements in these experiments was hampered by the fact that injected DNA reporter constructs do not integrate into the genome. This causes considerable variation of expression from experiment to experiment, probably because of the mosaic distribution of the injected DNA (Vize et al., 1991).

To address this difficulty and to investigate the spatial regulation of *Xbra*, we have now created transgenic *Xenopus* embryos in which exogenous DNA is integrated into the genome during the first cell cycle. This technique results in the correct spatial and temporal transcription from the cardiac actin and N-tubulin promoters and allows the simian cytomegalovirus and cytoskeletal actin promoters to drive ubiquitous expression of reporter genes (Kroll and Amaya, 1996). Our results show that the proximal *Xbra* 5′ flanking region is sufficient to drive expression of a reporter gene throughout the marginal zone at the early gastrula stage. Interestingly, expression in the presumptive notochord domain is rapidly downregulated thereafter, suggesting, as in the mouse, that expression in the notochord is mediated by an element that is remote from the proximal 5′ promoter (Clements et al., 1996). Deletion analysis and the creation of point mutations in putative transcription factor-binding sites in the proximal *Xbra* 5′ flanking region have identified two distinct repressor elements within this region. Disruption of these elements causes widespread expression of reporter constructs at different stages of gastrulation. Our results suggest that the restriction of *Xbra* to the marginal zone is achieved predominantly by specific repression in areas where it is not required, rather than activation in areas where it is expressed. A similar mechanism may be involved in the regulation of other key genes in early vertebrate development.

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).

Reporter constructs

Xbra promoter fragments used in this study are from the *Xbra2* gene (Latinkic et al., 1997). All promoter constructs except *Xbra*-4.1 were based on a pGL3 vector in which the Luciferase gene was replaced by a GFP reporter (Zernicka-Goetz et al., 1996). *Xbra*-2.1 is as described (Latinkic et al., 1997) and contains 2165 bp of 5′ flanking sequence and 50 bp of 5′ untranslated region. *Xbra*-970 was created by deleting a *Mlu*I-*Pst*I fragment from *Xbra*-2.1. Shorter deletion constructs were created by removing the 2165 bp promoter fragment using *Mlu*I and *Xho*I and replacing it with shorter fragments created by PCR using the sequence CCGCTCGAGCAGGTAGTAAATCC as 3′ primer (*Xho*I site is underlined) and 5′ primers as indicated below, in which an *Mlu*I site is underlined:

Xbra-381:5′ CGAACGCGTCATCTGCCATTATACCA 3′
Xbra-303:5′ GGAACGCGTCAGTTCTTACTGGATG 3′
Xbra-153:5′ GGAACGCGTCATAGAGCTCTCTGG 3′

Xbra-231 was created by excising a 231 bp fragment from *Xbra*-231.Luc (Latinkic et al., 1997) using *Hin*dIII and *Mlu*I and inserting it into the pGL3.GFP vector.

Xbra-4.1 contained a *Bam*HI fragment of *Xbra2* genomic sequence including 2.1 kb of 5′ flanking region, the first exon, the first intron and part of the second exon. In this case, GFP was fused in-frame to the coding region of the second exon.

Constructs containing point mutations were made using the Stratagene® QuikChange™ Site-Directed Mutagenesis Kit, which uses a pair of complementary oligonucleotides containing the desired point mutations. *Xbra*-2.1ABmut-GFP, in which two homeodomain-binding sites (underlined) are mutated at positions −169 (A→G mutation indicated in **bold**) and −154/−155 (AT→CC mutation indicated in **bold**), in the context of the 2.1 kb promoter, was made using the primer:

5′ CCTCTGACTTGCAATTGAATTCCCAGGATCCTCATAGAGCTCTCTG 3′
5′ CAGAGAGCTCTATGAGGATCCTGGGAATTCAATTGCAAGTCAGAGG 3′

Xbra-2.1Amut-GFP, in which an Antennapedia-type homeodomain-binding site at position −169 is mutated in the context of the 2.1 kb promoter, was made using the following primers:

5′ CCTCTGACTTGCAATTGAATTCCCAGGATTATCATAGAGC 3′
5′ GCTCTATGATAATCCTGGGAATTCAATTGCAAGTCAGAGG 3′

Other constructs carrying point mutations were already available in the background of the pGL2.Luc vector and were excised using *Hin*dIII and *Mlu*I and cloned into the pGL3.GFP vector (see above). These constructs were as follows:

Xbra-2.1TATA/SRFmut-GFP from *Xbra*2.1M2-Luc (Branko Latinkic, unpublished)
Xbra-381ABmut-GFP from *Xbra*381AB-Luc (Latinkic et al., 1997)
Xbra-381Bmut-GFP from *Xbra*381B-Luc (B. V. L., unpublished)
Xbra-2.1mut1-GFP from *Xbra*-2.1mut1-Luc (Remacle et al., 1999)
Xbra-2.1mut2-GFP from *Xbra*-2.1mut2-Luc (Remacle et al., 1999)
Xbra-2.1mut3-GFP from *Xbra*-2.1mut3-Luc (Remacle et al., 1999)
Xbra-2.1mut4-GFP from *Xbra*-2.1mut4-Luc (Remacle et al., 1999)

The constructs *Xbra*-381TATA/SRFmut-GFP and *Xbra*-381Amut-GFP were derived from the equivalent 2.1 kb constructs by PCR, making use of the primers used to create the *Xbra*-381 deletion construct above. All constructs were checked by sequencing.

For transgenesis, plasmids were linearised with either *Not*I or *Mlu*I. In some experiments, a fragment containing just the promoter and

reporter was released by additional digestion with *Sac*I. No difference in transgenic frequency was observed with these procedures, although expression of shorter constructs appeared more consistent if the vector sequence was removed.

Transgenesis

Transgenic *Xenopus* embryos were generated essentially as described (Kroll and Amaya, 1996). Sperm nuclei were incubated with linearised promoter constructs for 5 minutes followed by addition of interphase egg extract and *Not*I restriction enzyme (20 U/ml) 10-15 minutes prior to injection. A Drummond Nanoinject was used to inject 5 nl of sperm nucleus suspension per egg, at a concentration of two nuclei per 5 nl. This procedure caused 5-30% of eggs to cleave normally, of which 40-60% completed gastrulation and 5-30% formed normal swimming tadpoles if allowed to continue development. Frequency of transgenesis, as analysed by expression of reporter genes, varied between 50 and 90%.

Whole-mount in situ hybridisation

In situ hybridisation to detect GFP was carried out as described (Latinkic et al., 1997). After colour detection, some embryos were dehydrated and cleared in a 2:1 mixture of benzyl alcohol/ benzyl benzoate to visualise internal staining.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays were carried out as described (Latinkic et al., 1997). The probe containing the SRF/TATA domain of the *Xbra* promoter was made from the following oligonucleotides:

5′ GACTCTTGTCCAGGCCTATTTATGGAAGA 3′

5′ GACTTCTTCCATAAATAGGCCTGGACAAG 3′

The non-specific competitor (NS) was the AB probe previously described (Latinkic et al., 1997). Input proteins were made by in vitro translation.

RESULTS

Spatial and temporal expression of the *Xbra* promoter

The *Xbra* promoter used in this study is derived from *Xbra2*, a gene closely related to the originally identified *Xbra* and which has identical spatial and temporal expression patterns (Latinkic et al., 1997). Preliminary experiments using DNA injection into *Xenopus* embryos have suggested that reporter gene expression driven by 2.1 kb of *Xbra* 5′ flanking region is localised to the marginal zone at the early gastrula stage (Latinkic et al., 1997). To investigate the properties of the 2.1 kb *Xbra* promoter in more detail, we performed a comparison of Green Fluorescent Protein (GFP) reporter gene expression in transgenic embryos with expression of the endogenous *Xbra* gene at different stages of development (Fig. 1A). To obtain maximum sensitivity, expression of GFP was analysed by in situ hybridisation; detection of GFP fluorescence can be difficult at early embryonic stages because yolk autofluorescence can mask GFP expression, including that driven by the *Xbra*-2.1 construct used here.

At pregastrula stages 9-10, promoter activity appeared not to be regulated and expression was often detectable throughout the embryo, or in segments of it, in both animal and vegetal hemispheres (Fig. 1A). In some embryos, animal and vegetal expression persisted until the very early gastrula stage, when GFP transcripts were located either to the right or left side of the embryo and restricted marginal zone expression began in the other half. We noted a similar, albeit weak, widespread activation of endogenous *Xbra* at stage 9 (Fig. 1B; see also Panitz et al., 1998), but this soon resolved into specific expression in the dorsal marginal zone and then the entire marginal zone by the beginning of gastrulation.

By stage 10.25, expression of the *Xbra* reporter construct also became confined to the marginal zone, with slightly stronger expression on the dorsal side of the embryo (Fig. 1A). After stage 10.5, however, expression became downregulated in the dorsal marginal zone and was absent in the notochord thereafter. Nevertheless, the reporter construct remained active in the circumblastoporal region and GFP transcripts were detectable in the tailbud at stage 28. This expression pattern resembles that of *Xbra* itself, except that the endogenous gene is expressed additionally in the notochord (Gont et al., 1993).

In an attempt to find an element regulating notochord-specific expression of *Xbra*, we also analysed embryos transgenic for *Xbra*-4.1, a construct that comprises the 2.1 kb 5′ flanking region, the first intron and part of the second exon of *Xbra* fused in frame to GFP. Expression was markedly enhanced in these embryos such that GFP fluorescence was readily visible under the fluorescence microscope during gastrulation (Fig. 1C). The spatial and temporal expression pattern of GFP, as well as activin inducibility, was, however, identical to that obtained with the 2.1 kb 5′ flanking region alone (not shown). Expression in the notochord was not detected, suggesting that a notochord-specific regulatory element is located elsewhere in the *Xbra* gene.

Deletion study of the *Xbra* promoter

As a first step towards understanding the regulation of *Xbra* in vivo, we created transgenic embryos using constructs carrying deletions of the 5′ regulatory region. A fragment containing 970 bp of the *Xbra* 5′ flanking region showed no alteration of reporter gene expression during gastrulation, but did drive consistent expression of GFP in neural tissues at later stages (Fig. 2B; 100%, n>25). Neural expression of this sort was observed in only 20% (n=29) of embryos carrying *Xbra*-2.1 (Fig. 2A), suggesting that an element that suppresses neural expression is positioned between base pairs –2165 and –970 of the *Xbra* 5′ flanking region.

A construct containing 381 bp of the *Xbra* 5′ flanking region (*Xbra*-381) also drove expression of GFP in the marginal zone (Fig. 3A,B; 72%, n=43), although the increased staining time suggested that expression was slightly weaker than that obtained with *Xbra*-2.1. Some embryos also had very weak reporter gene expression in the inner ectodermal layer, which was only visible in cleared embryos (not shown). In contrast, when a construct containing only 303 bp of the *Xbra* 5′ flanking region (*Xbra*-303) was employed, expression was stronger, but it was no longer restricted to the marginal zone (Fig. 3C,D; 100%, n>40). Rather, expression extended somewhat into the vegetal pole and strong expression was detected in the inner layer of the ectoderm. At late gastrula stages reporter gene expression in the prospective notochord was downregulated while ectodermal expression persisted (Fig. 3D).

Further deletion of the promoter to 231 bp (*Xbra*-231) caused a spatial expression pattern similar to that of *Xbra*-303 (Fig. 3E,F), but expression was much weaker and tended to occur in patches (100%; n=32). In half of the stained embryos

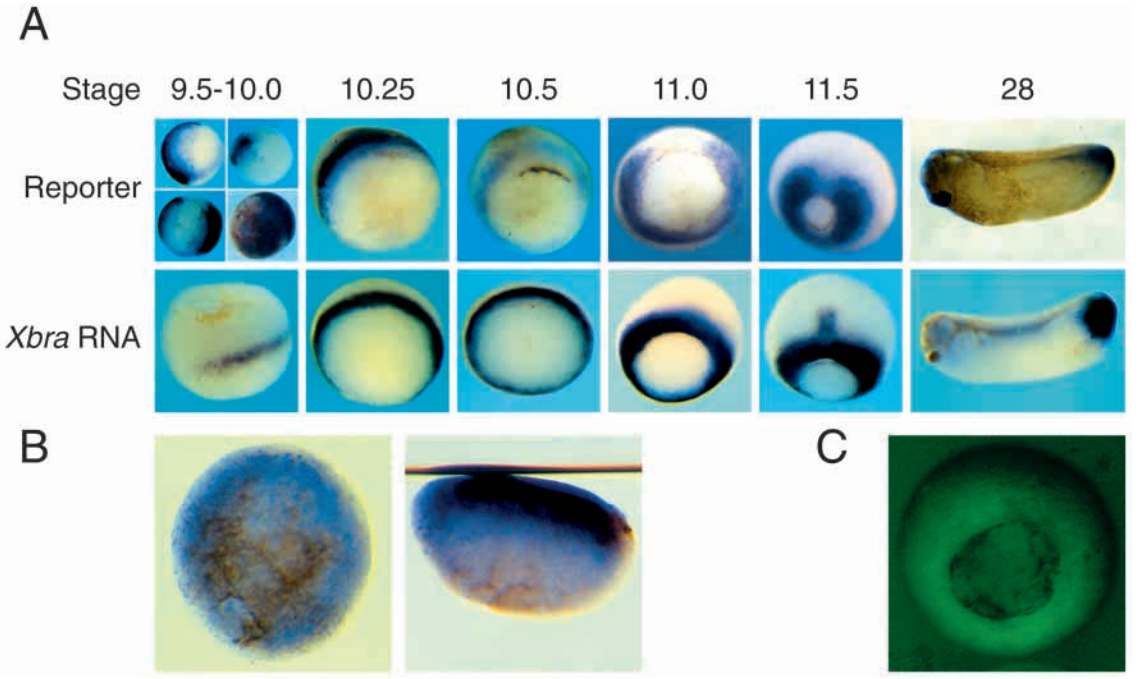

Fig. 1. Comparison of *Xbra* reporter gene expression with that of the endogenous gene. (A) Time course of *Xbra*-2.1 expression compared with that of endogenous *Xbra*. Dorsal is upwards in all panels, except wild-type stage 9.5 (dorsal to the right). The colour reaction to detect reporter gene expression took 24 to 48 hours compared with 5 hours to detect endogenous *Xbra*. Reporter gene expression was restricted to the marginal zone in approximately 80% (n>150) of transgenic embryos. (B) Expression of endogenous *Xbra* RNA at stage 9.0. Left panel: animal pole view; right panel: side view. Embryos were cleared to visualise internal staining. Nuclear staining indicates newly transcribed zygotic RNA. Non-transgenic embryos stained for GFP RNA for the same time showed no expression (not shown). Weaker staining in the vegetal pole may be due to poor probe diffusion, but see the sectioned in situ hybridisations of Panitz et al. (1998). (C) GFP fluorescence of an embryo transgenic for *Xbra*-4.1. Note slightly weaker expression in the dorsal marginal zone (top right).

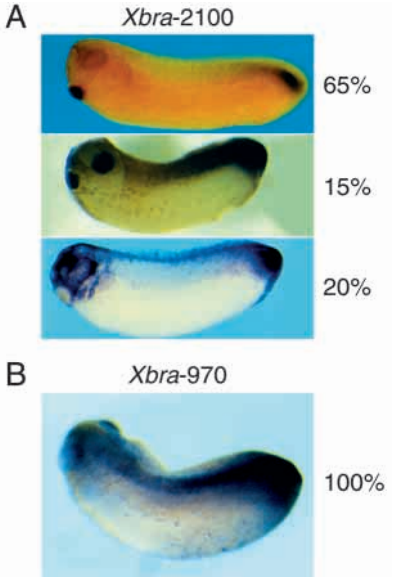

Fig. 2. Expression of *Xbra*-2.1 (A) at stage 28 compared with *Xbra*-970 (B). Percentages shown for *Xbra*-2.1 refer to numbers of transgenic embryos with the indicated expression patterns; 15% show expression in somites and eyes and 20% show expression in neural tissues and branchial arches. The *Xbra*-970 construct drives expression in neural tissues.

expression was weaker on the dorsal side of the embryo. Finally, deletion to 153 bp (*Xbra*-153) resulted in no specific expression (100%; n>40). Staining varied from patches of expression in different parts of the embryo (Fig. 3G) to weak ubiquitous activation (Fig. 3H).

Known transcription-factor-binding sites in the proximal *Xbra* promoter

Our deletion analysis shows that a 381 bp promoter fragment is sufficient to drive reporter gene expression in the marginal zone of the *Xenopus* embryo, and we note that this is also the smallest promoter fragment that can confer activation by activin and FGF (Latinkic et al., 1997). Fig. 4A shows a model based on our deletion analysis that predicts the positions of different activator or repressor motifs in this region of the *Xbra* promoter. The sequence of this region is shown in Fig. 4B, in which we highlight three putative transcription factor-binding sites, two of which are likely to be involved in repression of *Xbra* expression. The first is a TATA box located −25 to −31 bp from the transcription start site, and which overlaps with a putative serum response factor (SRF) site (Pollock and Treisman, 1990). Mouse embryos with a targeted mutation in SRF do not express *Brachyury* (Arsenian et al., 1998). The second region is located −175 to −153 bp from the transcription start site and contains a combination of homeodomain-binding sites that have been suggested to mediate repression of *Xbra* at high levels of activin (Latinkic et al., 1997). The final site, at base pairs −335 to −302, binds members of the δEF1 family

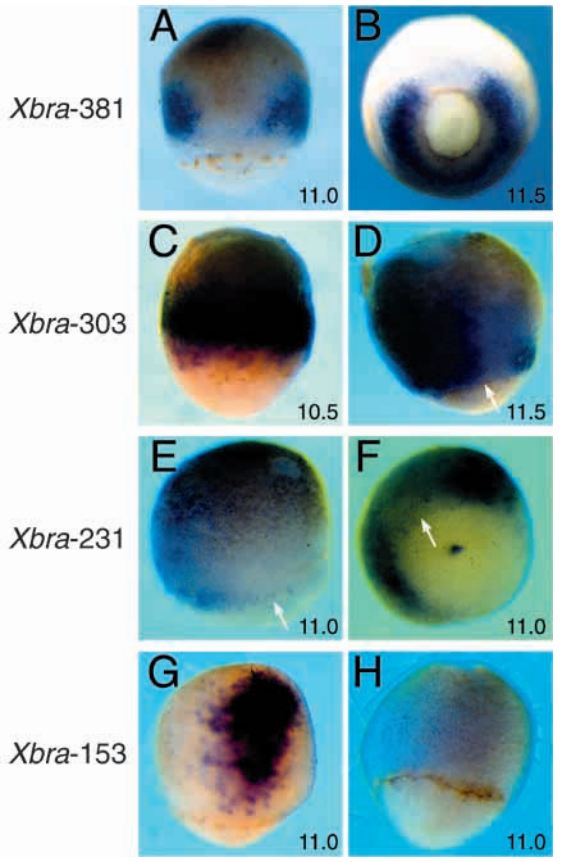

Fig. 3. Expression of a GFP reporter gene driven by the indicated constructs and analysed at the indicated stages. White arrows (D-F) mark the centre of the dorsal blastopore lip.

(Remacle et al., 1999). One member of this family, SIP1, is a Smad-binding protein that binds to this region of the *Xbra* promoter (Remacle et al., 1999) and, when overexpressed in the *Xenopus* embryo, can suppress transcription of the endogenous *Xbra* gene (Verschueren et al., 1999).

Additional motifs are also indicated in Fig. 4B; these are considered in the Discussion.

An SRF-binding site overlapping with the TATA-box is not necessary for *Xbra* promoter expression

The *Xbra* promoter has a putative TATA box located approximately 25 bp upstream of the transcription start site (Latinkic et al., 1997; Fig. 4B). The TATA box overlaps a putative Serum Response Factor (SRF)-binding site, an observation that is of interest because mice lacking functional SRF do not form mesoderm and do not express *Brachyury* (Arsenian et al., 1998). Although the putative SRF site in the *Xbra* promoter (CCTATTTATG) differs slightly from the consensus $CC(T/A)_6GG$ (Pollock and Treisman, 1990), electrophoretic mobility shift assays indicate that SRF binds this site in a specific manner, although it is unable to form a ternary complex with the ETS factor Elk-1 (data not shown).

To investigate the significance of the TATA/SRF site, we created frog embryos transgenic for modified versions of *Xbra*-2.1 or *Xbra*-381 that carried mutations designed to abolish binding to both SRF and TFIID (Taylor et al., 1989; Leibham et al., 1994). In neither context did either mutation have a significant effect on reporter gene expression in the marginal zone (data not shown). Thus, the SRF-binding site and the TATA box are not necessary for *Xbra* expression in the marginal zone, although it remains possible that they are required for transcription of *Xbra* in dorsal mesoderm and notochord.

A module of two homeobox-binding site confers repression on the dorsal side

The deletion analysis described in Figs 3 and 4 suggests that an element located between 153 and 231 bp 5′ of the transcription start site is necessary for suppression of *Xbra* expression on the dorsal side of the embryo. This region of the *Xbra* promoter contains one homeodomain-binding site of the Antennapedia class (A) and one of the Bicoid class (B) (Fig. 4; Wilson et al., 1996). These sites have previously been shown to bind Gsc, Mix.1 and Otx-1 in vitro, and are necessary for the suppression of *Xbra*-381 activity at high levels of activin (Latinkic et al., 1997).

To investigate whether these sites are required for the correct spatial regulation of *Xbra*, they were mutated singly or in combination (Fig. 5A), using the same mutations that resulted in relief of repression of *Xbra*-381 at high activin doses (Latinkic et al., 1997). Point mutations in the context of *Xbra*-2.1 had little or no effect on the spatial or temporal pattern of reporter gene expression (data not shown). It is likely that transcription factor-binding sites 5′ of the 381 bp region can compensate for mutation of the proximal sites. In the context of the –381 bp promoter, however, the effects of such mutations are striking.

At stage 10.5, when wild-type *Xbra*-381 becomes downregulated in dorsal mesoderm, constructs containing point mutations in both the A and B homeodomain sites are upregulated in this region (Fig. 5Ba; 100%, n=15). By stage 11 this expression domain extends into dorsal ectoderm (Fig. 5Bb; 100%, n>30) and during later stages it becomes narrower, stretching from posterior to anterior on the dorsal side (Fig. 5Bc; 100%, n=12). Staining in dorsal ectoderm and mesoderm is much stronger than in lateral and ventral mesoderm, but extended incubation does reveal expression in these tissues as well as in endoderm and the rest of the ectoderm at mid and late gastrula stages (data not shown).

To dissect the effects of the individual binding sides, we created transgenic embryos in which *Xbra*-381 contained point mutations in either the Antennapedia-binding site (A) alone or the Bicoid-binding site (B) alone. At the mid-gastrula stage, a point mutation in the A site caused an expression phenotype similar to that observed with combined mutations in both the A and B sites, but with less pronounced expression in dorsal ectoderm (Fig. 5Bd,e; 100%, n>30). Very weak expression was also visible in the endoderm (data not shown). At stage 11.5, expression in dorsal mesoderm and ectoderm receded (Fig. 5Bf; 100%, n>30).

Mutation of the Bicoid homeodomain-binding site (B) had a different effect, with expression of the reporter construct being upregulated on the ventral side of the embryo in both mesoderm and ectoderm. However, expression was also visible, albeit weakly, in dorsal mesoderm at stage 11 (Fig. 5Bg,h; 100%, n>30), at which time wild-type *Xbra*-381 was inactive in this region (Fig. 3A). At later stages of gastrulation

expression in the ectoderm was continuous, while there was a defined gap of expression in the dorsal mesoderm (Fig. 5Bi; 100%, *n*>25). At no time was expression detected in the endoderm.

Together these results suggest that the Antennapedia and Bicoid homeodomain-binding sites act in concert to repress expression in dorsal ectoderm at the late gastrula stage. The Antennapedia site alone mediates repression in dorsal ectoderm and dorsal mesoderm at the mid-gastrula stage and also mediates repression in the endoderm. In contrast, the Bicoid site appears to be necessary for enhancing repression in ventral tissues.

A bipartite δEF1-binding site restricts reporter gene expression to the mesoderm at the early gastrula stage

In contrast to *Xbra*-381, *Xbra*-303 drives strong expression in ectoderm and, to some extent, endoderm, suggesting that a repressor element responsible for establishing the borders between ectoderm, mesoderm and endoderm lies in the region between –303 and –381 nucleotides (Fig. 3C,D). The search for such an element was facilitated by the findings that SIP1, a novel Smad interacting protein of the δEF1 family, can bind to this region via two CACCT target sequences (see Fig. 4B) and that mis-expression of SIP1 in the *Xenopus* embryo eliminates endogenous *Xbra* expression (Verschueren et al., 1999). We recently demonstrated that binding of SIP1 to this domain requires an intact downstream CAGGTG sequence and an upstream CACCT (Remacle et al., 1999) and, in the course of this work, we created four point mutations, designated mut1 to mut4, designed to prevent such binding. Electrophoretic mobility shift assays showed that mut1, mut2 and mut4 completely abolish binding of full-length SIP1, while mut3 only decreases the affinity of binding. Preliminary experiments indicated that such mutations cause illegitimate activity of the *Xbra* promoter at late gastrula stages (Remacle et al., 1999). We now study the temporal and spatial consequences of these mutations on promoter activity in detail and find that their main effects are manifest at the early gastrula stage.

A point mutation in the downstream target half site (CAGGTG), in the context of *Xbra*-2.1, (Fig. 6A, mut1) results in loss of the mesoderm-ectoderm and mesoderm-endoderm boundaries early in gastrulation, with mis-expression being more extensive dorsally than ventrally (Fig. 6Be,f; 100%, *n*>30). As we have shown previously (Remacle et al., 1999), the mesoderm-endoderm boundary is re-established later in gastrulation, but the embryos continue to show reporter gene expression in the inner ectodermal layer, which is readily visible in cleared embryos (Fig. 6Bh; 100%, *n*>30).

Different mutations, either disrupting the same half site (mut2) or the upstream CACCT (mut4), have identical effects (Fig. 6Bi-l and data not shown). The deletion mutant, mut3, which changes the spacing between the two half-sites from 24 to 21 bp and results in a decreased affinity of SIP1 for the promoter, causes an intermediate expression phenotype. Most embryos show some mis-expression of the GFP reporter, but some have completely normal expression (data not shown; Remacle et al., 1999).

Together, these results suggest that a protein of the δEF1 family, possibly SIP1, plays an important role in confining *Xbra* expression to the mesoderm at the beginning of gastrulation, although additional factors may play a role at later stages, when the effects of mutating the SIP sites are much less dramatic (Fig. 6B; stage 11.5). Experiments studying the expression pattern of a *Xenopus* homologue of *SIP1* (*XSIP1*) are consistent with this interpretation (van Grunsven et al., 2000).

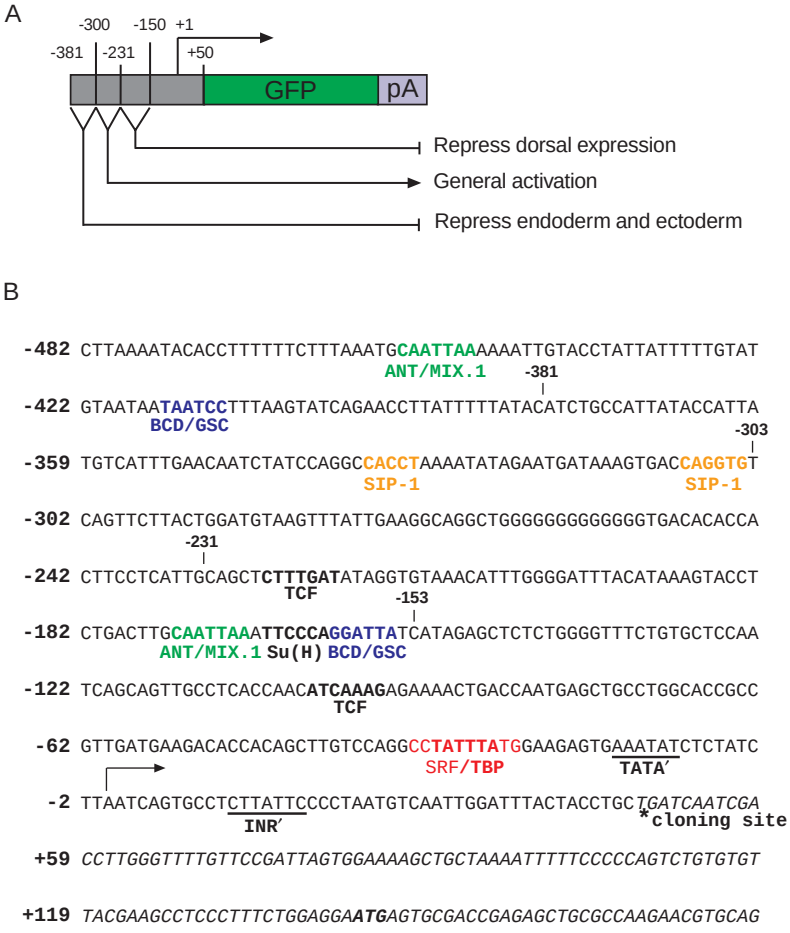

Fig. 4. Proximal 5′ flanking region of the *Xbra* promoter. (A) Positions of regulatory elements suggested by deletion analysis. FGF and activin response elements lie between nucleotides –381 and –231 (Latinkic et al., 1997). (B) Sequence of the proximal *Xbra* promoter region. Transcription-factor-binding sites addressed in this study are in colour and additional binding sites with putative regulatory function are in **bold**. SIP1, δEF1 and SIP1 half site; ANT/MIX.1, Antennapedia type homeodomain-binding site; BCD/GSC, Bicoid type homeodomain-binding site; SRF, serum response factor-binding site; TBP, putative TATA box; Su(H), putative Suppressor of Hairless-binding site. The transcription start site is marked with an arrow. Cloning site marking the fusion point of the GFP reporter gene (except for *Xbra*-4.1) is indicated with an asterisk (*). Potential alternative TATA box and initiator region (see text) are indicated TATA′ and INR′.

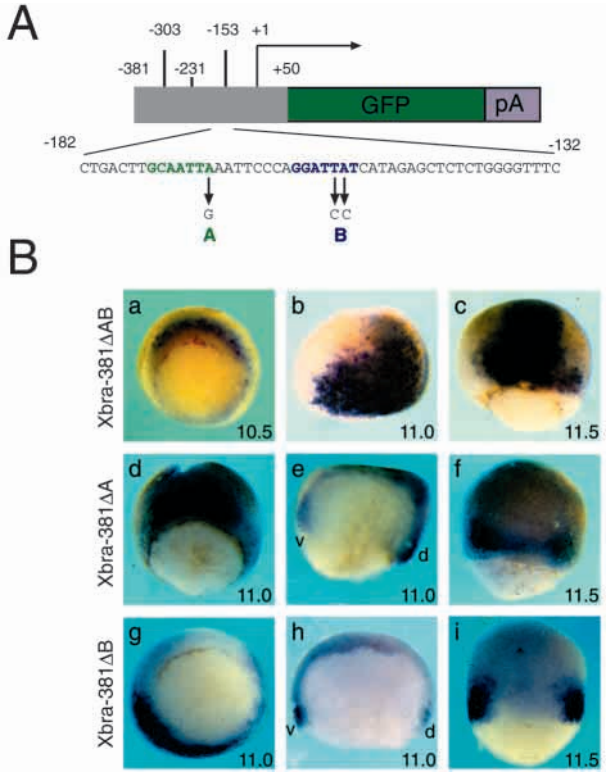

Fig. 5. Effects of point mutations disrupting two homeodomain-binding sites. (A) Representation of the *Xbra* promoter showing positions of mutations. (B) Expression patterns of *Xbra* promoter constructs with the indicated point mutations. In a,d and g, dorsal is to the top; in b,e and h, dorsal is to the right; in c,f and i, dorsal is to the front. Embryos in e and h are bisected along the dorsoventral axis to reveal internal expression. v, ventral; d, dorsal.

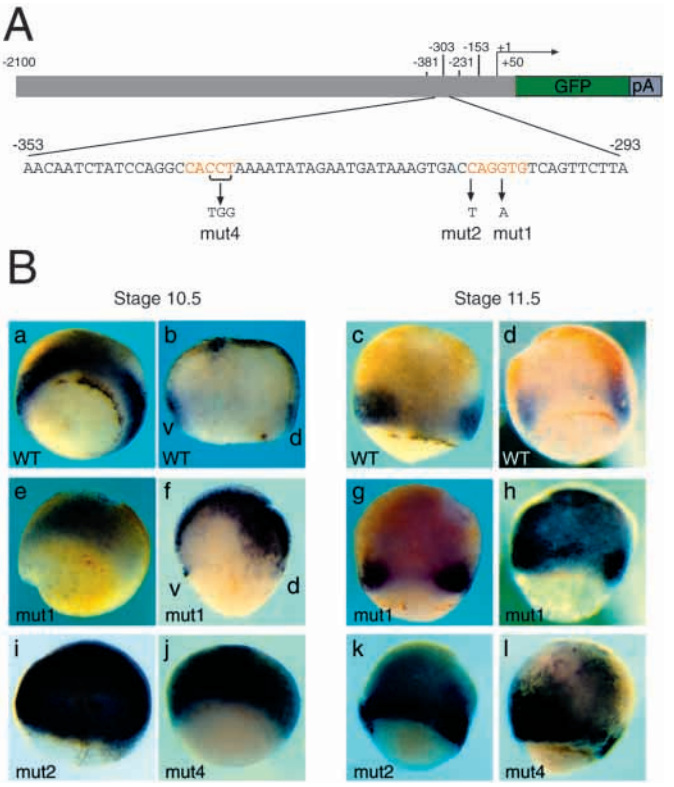

Fig. 6. Effects of point mutations disrupting δEF1 half sites in the context of *Xbra*-2.1. (A) Representation of the *Xbra* promoter indicating positions of mutations, with nomenclature according to Remacle et al. (1999). (B) Expression patterns of *Xbra* promoter constructs carrying the indicated mutations. WT, wild-type. Embryos are positioned animal pole upward, dorsal to the front, except b and f, where dorsal is to the right, and a, which is tilted such that the vegetal pole faces the viewer. Embryos in d, (h) and i are cleared, embryos in b and f were bisected along the dorsoventral axis to reveal internal staining.

DISCUSSION

In previous work, we investigated the ability of the mesoderm-inducing factors activin and FGF to activate the *Xbra* promoter (Latinkic et al., 1997). Our results suggested that 381 bp 5′ of the transcription start site, but not 231 bp, are sufficient to confer responsiveness to activin and FGF. In this paper, we address the spatial and temporal regulation of *Xbra* by making use of a recently developed technique for making transgenic *Xenopus* embryos (Kroll and Amaya, 1996). Our results are consistent with the previous work, in that 381 nucleotides, but not 303, prove to be sufficient to drive mesoderm-specific expression of reporter genes. However, our results lead us to the surprising conclusion that restriction of *Xbra* to the mesoderm occurs in part through two repressor modules.

Upregulation of the *Xbra* promoter before gastrulation

Xbra reporter constructs are first activated in transgenic embryos shortly before the onset of gastrulation in a widespread fashion, either throughout the embryo or in a half or in a quarter of it. Such expression was observed with all *Xbra* promoter constructs tested, including those with point mutations in repressor modules (not shown). Interestingly, we also observe a similar, albeit weak, activation of the endogenous *Xbra* gene at the late blastula stage (Fig. 1B; see also Panitz et al., 1998).

Early ubiquitous expression of endogenous *Xbra* and of *Xbra* reporter constructs is consistent with the idea derived from experiments in this paper that regulation of *Xbra* involves a general activation followed (or accompanied) by specific repression in those regions where it is not required. We note, however, that expression of MyoD also occurs in a widespread fashion before it becomes restricted to presumptive muscle tissue (Frank and Harland, 1991; Rupp and Weintraub, 1991), and that this phenomenon has been linked to changes in chromatin structure between the mid-blastula transition and the end of gastrulation (Dimitrov et al., 1993; Patterton and Wolffe, 1996). Further research is necessary to investigate the universality of this phenomenon with other promoters.

Absence of an element responsible for notochord expression

Unlike endogenous *Xbra*, reporter gene expression driven by 2.1 kb of *Xbra* 5′ flanking sequence is downregulated in the dorsal marginal zone at the mid-gastrula stage and is never expressed in the notochord (Fig. 1A). Our results indicate that

a combination of two homeodomain-binding sites is necessary for this downregulation; mutations that disrupt the sites show strong expression in dorsal ectoderm and mesoderm, including the presumptive notochord.

These observations suggest that there exist additional regulatory elements that can overcome repression by the homeodomain-binding sites and thereby maintain expression of *Xbra* in the notochord. Such elements were not present within a 4.1 kb *Xbra* promoter construct (data not shown) and, indeed, a 13 kb mouse *Brachyury* reporter construct also fails to drive expression in the notochord (Clements et al., 1996). A notochord enhancer has been identified in *CiBra*, the *Ciona intestinalis* homologue of *Brachyury* (Corbo et al., 1997), although *Ciona* differs from *Xenopus* and the mouse in that expression of *CiBra* occurs solely in the notochord (Yasuo and Satoh, 1994). Notochord-specific expression of *CiBra* requires two Suppressor of Hairless Su(H) sites (Corbo et al., 1998). Interestingly, the *Xbra* promoter also contains a single putative Su(H) site located between the two homeodomain sites that are necessary for dorsal repression (Fig. 4B). However, the *CiBra* promoter cannot drive expression of a reporter gene in the notochord of *Xenopus* and cannot rescue notochord expression when placed upstream of *Xbra*-970 (W. L., unpublished observations).

A TATA box and SRF-binding site are not required for expression of *Xbra*

The *Xbra* promoter contains a TATA box 25-31 bp upstream of the transcription start site (Latinkic et al., 1997), which overlaps with an SRF-binding site (Fig. 4B). Mutation of this region of the promoter does not affect expression of *Xbra* reporter constructs. This result should be viewed in the context of work showing that mouse embryos lacking functional SRF protein do not form mesoderm and do not express *Brachyury* (Arsenian et al., 1998). Our results in *Xenopus* would suggest that the requirement for SRF activity in expression of *Brachyury* is indirect, a conclusion consistent with the observation that a constitutively active form of SRF, SRF-VP16, does not induce expression of *Xbra* (Panitz et al., 1998).

The lack of effect of mutation of the TATA box may be due to the use of an alternative TATA box positioned 16 bp downstream of the mutated site (see Fig. 4B). Consistent with this suggestion, there is a consensus initiator region (Smale and Baltimore, 1989) 14 bp downstream of the original transcription start site (see Fig. 4B).

Homeodomain-binding sites necessary for dorsal repression

Disruption of a pair of homeodomain-binding sites located in the proximal *Xbra* promoter results in strong upregulation of expression in dorsal mesoderm and ectoderm, with additional weak expression in the endoderm and the rest of the ectoderm. The effect of this double mutation represents the sum of the effects of the single mutations, with the exception of expression at late gastrula stages, where disruption of neither of the individual sites caused the strong upregulation in dorsal ectoderm observed with the double mutation.

Several studies have implicated the homeobox gene *gsc* in negative regulation of *Xbra* (Artinger et al., 1997; Latinkic et al., 1997; Latinkic and Smith, 1999). *gsc* is expressed in the dorsal mesendoderm at the beginning of gastrulation and in anterior mesoderm later in development (Steinbeisser and De Robertis, 1993). Although this expression pattern overlaps in part with the regions of upregulation shown in Fig. 5B, the main area of ectopic expression in dorsal ectoderm does not coincide with *gsc* expression. Furthermore, although Gsc protein would be expected to bind to the Bicoid class of binding site (Wilson et al., 1993), disruption of this site causes upregulation in ventral ectoderm and not in dorsal mesoderm or endoderm. These results therefore suggest that, although overexpression of Gsc can suppress *Xbra* promoter activity, other homeodomain proteins regulate *Xbra* expression via the Bicoid site during normal development. This conclusion is in agreement with recent work suggesting that proteins other than Gsc are necessary for mediating the repression of *Xbra* at high doses of activin (Papin and Smith, 2000).

We know of no Bicoid or Antennapedia class homeodomain protein that functions as a transcriptional repressor whose expression pattern is consistent with the effects of disrupting the homeodomain-binding sites shown in Fig. 4B. The complexity of the expression phenotype when the sites are mutated suggests that multiple homeodomain proteins are involved.

A protein of the δEF1 family may confine *Xbra* expression to the mesoderm

Vertebrate members of the δEF1 family are large zinc finger/homeodomain-like DNA-binding proteins that act as transcriptional repressors (Sekido et al., 1997; Verschueren et al., 1999). Here we show that any point mutation that disrupts high affinity binding of δEF1 family members to the *Xbra* promoter (Remacle et al., 1999) results in widespread mis-expression of reporter constructs in ectoderm and, more weakly, endoderm at the early gastrula stage. Subsequently, during mid-gastrula stages, expression becomes confined to a ring in the marginal zone mesoderm, but it continues to be mis-expressed in the inner layer of the ectoderm (Fig. 6).

Of the δEF1 family members, SIP1 is of particular interest because it interacts with activated Smad proteins and interferes with transcription of endogenous *Xbra* when overexpressed in the embryo (Verschueren et al., 1999). Furthermore, we have recently found that a *Xenopus* homologue of *SIP1* is expressed during early gastrula stages (van Grunsven et al., 2000). Members of the δEF1 family might exert their repressive effects either by competing with activators for occupancy of E-box-binding sites or by active repression (Sekido et al., 1997). In the case of SIP1, which is unique among the δEF1 family in that it interacts with activated receptor-specific Smads (Verschueren et al., 1999), it is possible that SIP1 is bound to its binding site in the absence of Smad signalling, but changes its conformation and disassociates from DNA when associated with an activated Smad molecule. We are currently investigating this model.

Repression and region-specific expression in early *Xenopus* development

Together, our results suggest that the restriction of *Xbra* expression to the mesoderm of the early *Xenopus* embryo is achieved by a rather general activation followed by repression in ectoderm and endoderm. This repression is mediated at least in part by the homeodomain-binding sites located in the

proximal *Xbra* promoter (Fig. 5) and the SIP1 site discussed above (Fig. 6).

Repression is also involved in the regulation of other genes expressed during *Xenopus* gastrulation. For example, both *Xfkh1* and *goosecoid* are activated in the dorsal marginal zone by Smad2 (Watabe et al., 1995; Howell and Hill, 1997; Labbe et al., 1998) and appear to be repressed elsewhere by Xvent1 and Xom/Xvent2, respectively (Friedle et al., 1998; Trindade et al., 1999). Repression is mediated by elements in the 5′ flanking regions of *Xfkh1* and *goosecoid* (Friedle et al., 1998; Trindade et al., 1999), but the effects of mutating these sites on the spatial and temporal expression patterns of reporter constructs have not yet been analysed. We also note that sequences in the first intron of *Xlim-1*mediate repression of basal promoter activity and that this repression is relieved by activin signalling (Rebbert and Dawid, 1997).

Conservation of Brachyury regulation

Have *Brachyury* regulatory sequences been conserved during evolution? Comparison of the *Xenopus* (Latinkic et al., 1997) and mouse (Clements et al., 1996) *Brachyury* promoters reveals a region of homology corresponding to nucleotides −225 to −198 of the *Xbra* promoter. The 5′ end of this region contains a TCF-binding site (van der Wetering et al., 1991) adjacent to which is an E-box motif (Fig. 4B). A similar juxtaposition of TCF site and E-box, in the opposite orientation, is found between nucleotides −118 and −96 of *Xbra*, and is also present in the mouse *Brachyury* promoter (Yamaguchi et al., 1999). Interestingly, mouse embryos lacking functional Wnt3a do not express *Brachyury*, and mutation of the TCF sites (but not of the E-boxes) prevents activation of a reporter gene in transgenic embryos, suggesting that *Brachyury* is a direct target of the Wnt signalling pathway (Yamaguchi et al., 1999). It is possible that the TCF sites are also necessary for activation of *Xbra*, although we note that, while misexpression of Wnt RNA in *Xenopus* animal caps can activate expression of *Siamois*, whose promoter also contains TCF sites (Carnac et al., 1996; Brannon et al., 1997; Fan et al., 1998), Wnt signalling has not been reported to induce *Xbra*. This issue requires further investigation. Elsewhere within the mouse *Brachyury* promoter, we have detected neither Bicoid or Antennapedia class homeodomain-binding sites nor a SIP1 site.

We have detected no significant regions of homology between the *Xenopus* and *Ciona Brachyury* promoters although, as we note above, the *Xenopus* promoter contains one (Fig. 4B), and *Ciona* two (Corbo et al., 1998), Suppressor of Hairless Su(H) sites. In *Ciona* these sites are required for expression of *Brachyury* in the notochord, the only site where the gene is expressed (Corbo et al., 1998). It is not possible to address this question in *Xenopus*, because we do not observe expression of reporter genes in the notochord (Fig. 1).

Downstream of *Xbra*

Our results indicate that the regulation of *Xbra* is highly complex, with different activator and repressor modules acting in concert to ensure accurate restriction of expression to the marginal zone. This complexity may be responsible for the fact that, even when 2.1 kb of *Xbra* 5′ flanking sequence was used to drive reporter gene expression, GFP activity was detected in ectopic locations in a significant proportion of embryos (Fig. 2). This phenomenon might be due to recombination events that disrupt different repressor elements and thereby bring about ectopic activation (see Kroll and Amaya, 1996). Copy number and position effects are also likely to play a role.

Once the correct expression domain of *Xbra* has been established, however, the regulation of Xbra target genes may be much simpler. For example, we have recently demonstrated that T-box sites are necessary for the mesodermal expression of Xbra targets such as *eFGF* (Casey et al., 1998) and the *Bix* family (Tada et al., 1998; Casey et al., 1999), and indeed that a single Xbra-binding site placed upstream of a minimal promoter is sufficient to drive mesoderm-specific expression of a reporter gene during gastrulation (Casey et al., 1999). These observations stress the importance of *Xbra* and other T-box genes in defining mesodermal identity. They may also help explain why ectopic expression of reporter genes driven by the later-acting cardiac actin and neuro-tubulin promoters is rarely observed (Kroll and Amaya, 1996). These genes may be subject to a simpler, mainly positive form of regulation (Arnone and Davidson, 1997), where disruption of regulatory elements would cause absence of expression rather than mis-expression.

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