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Regulation of the early expression of the *Xenopus nodal-related 1* gene, *Xnr1*

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SUMMARY

The *Xenopus nodal related-1* (*Xnr1*) gene has a complex expression pattern in embryos, with two temporal phases. In the first phase, transcripts are first detected in perinuclear sites in the vegetal region of the blastula. During gastrulation, this expression disappears and transcripts become localised to the dorsal marginal zone. Expression stops and then restarts in a second phase at neurula and tailbud stages, firstly in two symmetric patches near the posterior end of the notochord, and then asymmetrically in a large domain in the left lateral plate mesoderm. In this study, we have investigated the regulation of the early phase of expression of *Xnr1*. We show that the T-box transcription factor VegT can induce *Xnr1*. It had previously been shown that Xnr1 can induce *VegT* in ectoderm cells and we show that the early expression of *Xnr1* is regulated by an autoregulatory loop. By inspection of the *Xnr1* promoter sequence, we have identified two non-palindromic T-box-binding sites, which are 10 bp apart. Using mutational analysis, we have shown that these elements are required for the VegT induction of *Xnr1*. The *Xnr1* promoter shows striking homologies with the *Xnr3* promoter. In particular, two elements that are required for Wnt signaling are conserved between these two promoters, but the two T-box sites are not conserved, and *Xnr3* is not induced by VegT. A region of the promoter containing the T-box sites and the Wnt sites is sufficient to drive expression of a reporter gene in a dorsal domain in transgenic *Xenopus* at the gastrula stage. We show that this pattern of expression of the transgene in gastrulae is not dependent on the T-box sites.

Key words: *Xenopus*, Nodal, Xnr1, Organizer, Endoderm, Transgenesis, T-box, VegT

INTRODUCTION

Much progress has been made in identifying signaling molecules that are expressed in early stages of *Xenopus* development, and which are therefore candidates for having significant roles in patterning the embryo. More recently, greater attention has been focused on the regulatory circuits that control the regionally specific localisation and activity of these molecules.

Among these early signaling molecules are the four known *Xenopus* NODAL-related molecules, which are members of the TGFβ family of secreted signaling molecules. The four *Xnr* genes are expressed in different layers of the Spemann organizer during gastrulation. From whole-mount in situ hybridization, *Xnr3* expression was thought to be restricted to the epithelial layers of the organizer (Ecochard et al., 1995; Smith et al., 1995), but hybridizations to sections showed that expression extends into the deep marginal and vegetal cells (Darras et al., 1997). *Xnr4* is first expressed in the deep cells of the organizer and then continues to be expressed during neurula stages in the notochord and neural tube (Joseph and Melton, 1997). *Xnr1* and *Xnr2*, on the contrary, are first expressed earlier, in punctate regions over the entire vegetal region of the blastula (Jones et al., 1995). During gastrulation, this expression disappears and transcripts become localised to the dorsal marginal zone, with enrichment in the organizer (Jones et al., 1995). However, the apparent low abundance of *Xnr1* transcripts during gastrula stages has made it difficult to use whole-mount in situ hybridization to determine the precise spatial distribution of *Xnr1* transcripts within the organizer (Lustig et al., 1996a; and our unpublished observations).

Xnr1 is unique among the *nodal-related* genes, in that its expression also reappears during tailbud stages. This later expression is first detected in two symmetric patches near the posterior end of the notochord and then in a large asymmetric domain in the left lateral plate mesoderm (Lustig et al., 1996a). The asymmetry is highly conserved among vertebrates (Ramsdell and Yost, 1998) and studies in *Xenopus*, mice and chicks have indicated that NODAL is a key player in the development of the left-right axis (Sampath et al., 1997; Collignon et al., 1996; Levin et al., 1995). Less is known about the role of Xnrl at the earlier stages. Loss-of-function experiments by Osada and Wright (1999) involved creation and expression of a dominant negative cleavage mutant form of Xnr2, which specifically blocked the mesoderm-inducing effects of *Xnr1*, *Xnr2* and *Xnr4* mRNA in animal caps. In whole embryos, expression of the dominant negative Xnr2 resulted in embryos with delayed dorsal lip formation and subsequent anterior truncations. Further investigation implicated an effect upon anterior endoderm/head organizer specification. A role for Xnr1 in the induction of head development by the anterior endomesoderm has recently been described (Piccolo et al.,

1999). An important molecule in *Xenopus* head development is the secreted protein Cerberus. When overexpresssed in early *Xenopus* embryos, Cerberus induces the formation of ectopic head structures (Bouwmeester et al., 1996). The *cerberus* gene is transiently expressed in the anterior endomesoderm of the gastrula. The anterior endomesoderm (Vodicka and Gerhart, 1995) overlaps the deep cells of the organizer, but is distinct from the classical organizer region (Zorn et al., 1999). Signals from the organizer, including Xnr1, are thought to be involved in the initial establishment of *cerberus* expression in the anterior endomesoderm. At later stages, Cerberus is then thought to bind to and antagonise Nodal, BMP and Wnt signals, in a mechanism that prevents trunk mesoderm from forming in the head field (Piccolo et al., 1999).

A model for the development of the anterior endomesoderm with reference to expression of *cerberus* and another anterior endomesodermal marker, *Xhex* (Newman et al., 1997), was recently described (Zorn et al., 1999). This model has three phases: a maternal phase, when the anterior endomesoderm is initially specified, a blastula phase, when zygotic signaling amplifies *cerberus* and *Xhex* expression in this region, and then a gastrula phase, when expression of *cerberus* and *Xhex* in the anterior endomesoderm is maintained. In the first phase, the combined action of dorsalising Wnt/β-catenin signals and endoderm-specific factors commit the dorsovegetal region of the early embryo to form the anterior endomesoderm. A candidate for the endoderm-specific factor is the TGFβ molecule Vg1, which is a potent endoderm inducer and is required for normal endoderm development (Henry et al., 1996; Joseph and Melton, 1998). Another candidate is the T-box transcription factor VegT (also known as Antipodean, Xombi and BraT) (Zhang and King, 1996, Stennard et al., 1996; Lustig et al., 1996b; Horb and Thomsen, 1997), which is essential for endoderm formation (Zhang et al., 1998). Depletion of VegT mRNA causes embryos to develop without endoderm and the vegetal region of such embryos does not develop the ability to induce mesoderm. Targets of VegT are therefore expected to include genes necessary for the specification of endoderm and the production of mesoderm-inducing signals from endoderm. Among the known targets of VegT is the *Bix4*, a Mix-like homeobox gene, which contains multiple T-box-binding sites in its promoter, and which has been shown to act directly downstream of VegT in specifying endodermal differentiation (Tada et al., 1998; Casey et al., 1999). Other endoderm-specific zygotic factors include the HMG box proteins Xsox17α/β and Mixer, and other Mix-like homeobox proteins (Hudson et al., 1997; Henry and Melton, 1998; Ecochard et al., 1998; Lemaire et al., 1998; Rosa, 1989), Xnr1 and Xnr2 (Clements et al., 1999). Experiments in which cycloheximide was used to inhibit protein synthesis in embryos have demonstrated that *Xnr1* expression is activated cell-autonomously around the mid-blastula transition by maternal determinants (Yasuo and Lemaire, 1999). With the onset of zygotic transcription in the second phase, endoderm-specific zygotic factors such as Xnr1 and Xnr2, are proposed then to induce *cerberus* and *Xhex*. In the third phase, signals from the adjacent organizer, such as noggin and chordin, help to maintain *cerberus* and *Xhex* expression in the anterior endomesoderm during gastrulation, by repression of ventral BMP signals.

In this study, we have investigated the early transcriptional regulation of *Xnr1* so as to further understand the cascade of events leading to the formation of the endoderm and mesoderm. Our approach was to isolate the *Xnr1* gene and its promoter. Inspection of the promoter sequence revealed two half T-box-binding sites. We therefore explored the possible direct induction of *Xnr1* by VegT. Our results lead us to propose a model for the regulation of the early, symmetrical expression of *Xnr1*, which also explains the different patterns of *Xnr1* and *Xnr3* expression. In parallel with this work, we have investigated the ability of an *Xnr1* promoter region to drive reporter gene expression with appropriate regional specificity.

MATERIALS AND METHODS

Library screening and cloning

A genomic library in λGEM-11 (a gift from M. L. King) was screened at high stringency with a full-length *Xnr1* cDNA probe. *Bam*HI fragments were subcloned and the 5.5 kb promoter fragment was identified by Southern blotting.

Primer extension

Total RNA was extracted from stage 10.5 and stage 24 whole embryos essentially as previously described (Barnett et al., 1998). Primer extension was performed as previously described (Sambrook et al., 1989). The primer used at stage 10.5 was 5′-AGA GGA TTC CTG CTC TCT AC-3′ and at stage 24 was 5′-GCC ATG CTT GCA CTG CTG AT-3′.

Embryos and injections

Embryos were obtained by standard procedures and staged according to Nieuwkoop and Faber (1967). Synthetic mRNA was injected using a Drummond microinjector. Animal caps were dissected at stage 9.

Preparation of synthetic mRNA

Xnrl(ΔUTR)-pSPJC2L and VegT(ΔUTR)-pSPJC2L were constructed by removing the untranslated regions from Xnrl-pSP64T (Jones et al., 1995) and VegT-pSP64T (Horb and Thomsen, 1997), and inserting the coding regions into the pSPJC2L vector (Cook et al., 1993). Both plasmids were generous gifts from D. Clements. *Xwnt8* mRNA was prepared as described (Christian et al., 1991). Plasmids were linearised with the appropriate restriction enzyme and transcribed using the Ambion mMESSAGE mMACHINE^{TM} in vitro Transcription Kit.

RT-PCR analysis

Total RNA was isolated from animal caps and whole embryos as previously described (Barnett et al., 1998). 0.5-1 μg of total RNA was reverse transcribed using Superscript^{TM} RT (Gibco BRL). *EF1α* was amplified over 25 cycles of PCR, *VegT* was amplified over 30 cycles, and *Xnr1* and *Xnr3* were amplified over 35 cycles. In all four amplifications, denaturation was at 94°C for 30 seconds, annealing was at 55°C for 30 seconds and extension was at 72°C for 45 seconds. The primer pairs were as follows: *EF1α* F 5′-CAG ATT GGT GCT GGA TAT GC-3′ and R 5′-CAC TGC CTT GAT GAC TCC TA-3′; *VegT* F 5′-CCT GAC ATT GCT ACA GTG CC-3′ and R 5′-CGA TAT TGA CCT TGA GCT GG-3′; *Xnr1* F 5′-ATA GCT GCC ATT TAT ATC-3′ and R 5′-CAA CAA AGC CAA GGC ATA AC-3′; *Xnr3* F 5′-GTG CAG TTC CAC AGA ATG AG-3′ and R 5′-CCA TGG ATC GGC ACA ACA GA-3′.

PCR mutagenesis by hybrid overlap extension

616 bp of sequence immediately upstream from the translation start codon was amplified by PCR using Expand High Fidelity DNA

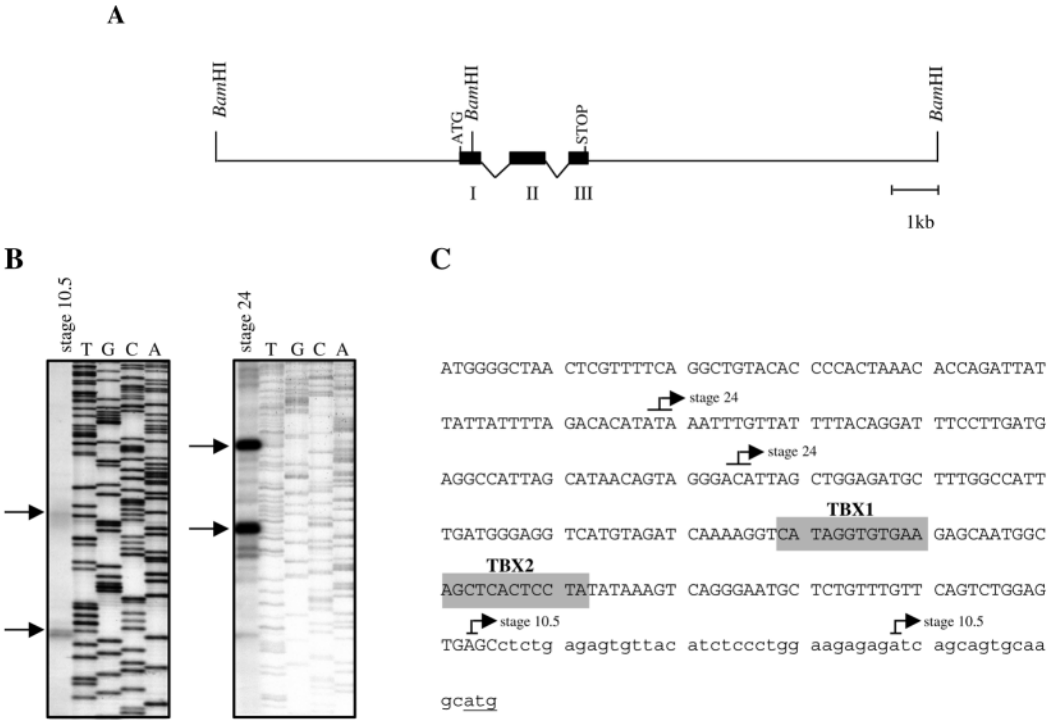

Fig. 1. The *Xnr1* gene. (A) *Xnr1* is composed of three exons (I, II and III), separated by two introns of 627 bp and 527 bp. The translation start and stop codons are indicated in the first and third exons, respectively. (B) Transcription initiation of *Xnr1*. Primer extension assays were performed using stage 10.5 and stage 24 total RNA. The nucleotide ladder was created by sequencing with the extension primer. (C) Sequence of the first 255 nucleotides of the *Xnr1* promoter. An overlap with the published cDNA sequence is shown in lower case (GenBank accession number U29447). The translational start codon is underlined. The major transcription initiation sites at stage 10.5 and stage 24 are indicated by arrows. The two half T-box sites (TBX1 and TBX2) are shaded.

polymerase (Roche) and the following external primer pair (restriction sites underlined): F 5′-GTC CGG TAC CAC CGA TTG TAA GCT CTA-3′ and R 5′-GAA TGC TAG CGC TTG CAC TGC TGA TCT-3′. This DNA was used as the template in PCR hybrid overlap extension to create Ml and M2. The internal primer pairs were as follows: Ml F 5′-AAA GGT CAT AAG CTT GAA GAG CAA TGG CAG-3′ and R 5′-CTG CCA TTG CTC TTC AAG CTT ATG ACC TTT-3′; M2 F 5′-GCA ATG GCA GCT GGA TCC TAT ATA AAG TC-3′ and R 5′-GAC TTT ATA TAG GAT CCA GCT GCC ATT G-3′. M3 was created using the Ml DNA as the template in the PCR and the M2 primer pairs.

Electrophoretic mobility shift assays

VegT protein was prepared from the VegT(ΔUTR)-pSPJC2L plasmid using the TNT® Coupled Reticulocyte Lysate Extract System (Promega). Electrophoretic mobility shift assays were performed as previously described (Casey et al., 1998). Radiolabelled probes were prepared by digestion of either the wild-type or mutant DNAs with *Hae*III and *Dde*I, and then end-filling the *Dde*I 5′ overhang with α^{32}P-dGTP and Klenow DNA polymerase.

Luciferase reporter gene assays

The 616 bp wild-type or mutant DNAs were cloned into the *Kpn*I-*Nhe*I sites of the luciferase reporter vector pGL3Basic (Promega). To assay for promoter activity, 2-cell *Xenopus* embryos were injected with approximately 10-50 pg of test plasmid. Where indicated, 100 pg of synthetic mRNA was also injected with the plasmid. Embryos were harvested at stage 11 and analysed for luciferase activity as previously described (Metz et al., 1998).

Transgenic embryos

Approximately 3.5 kb of sequence immediately upstream from the translation start codon was amplified by PCR using Expand High Fidelity DNA polymerase (Roche). An analogous fragment containing mutations in both T-box sites was also amplified. These promoter fragments were inserted into the *Nhe*I site of a β-galactosidase reporter vector (adapted from pGL3Basic, Promega). Embryos containing the *Xnr1::lacZ* transgenes were created as previously described (Kroll and Amaya, 1996). Transgenic embryos were harvested at stages 9.5, 10.25 and 24 and analysed for β-galactosidase activity as previously described (Zakany et al., 1988).

RESULTS

Differential transcription initiation of the *Xnr1* gene at stage 10.5 versus stage 24

A genomic *Xnr1* clone was isolated by screening a genomic library in λGEM11 at high stringency with a full-length cDNA probe. A 5.5 kb *Bam*HI fragment containing approximately 5.3 kb of sequence upstream from the start of the cDNA was identified by Southern blotting (data not shown).

The organisation of the *Xnr1* gene was determined by restriction digestion and PCR. *Xnr1* is composed of three exons separated by two introns of 627 bp and 527 bp, respectively (Fig. 1A). The translation start codon is located in the first exon and the cysteine knot motif (Jones et al., 1995) is located in the third exon.

To identify the transcription initiation site(s), primer extension assays were performed using stage 10.5 and stage 24 total RNA (Fig. 1B). Different transcription initiation sites were identified at the earlier stage by comparison with the late stage, where expression is asymmetric. Two prominent primer extension products were identified with the stage 24 RNA, corresponding to two major transcription start sites which are centred 180 bp and 236 bp upstream from the translation start codon (Fig. 1C). With the stage 10.5 RNA several minor bands were identified (Fig. 1B); these primer extension products gave considerably fainter bands than those at stage 24, reflecting the differences in abundances of the transcripts at the two stages. The larger of the primer extension products is centred 52 bp upstream from the translation start codon and is approximately

35 bp downstream from a TATA box (Fig. 1C). The position of this transcription initiation site corresponds to the transcription initiation site previously identified in the related *Xnr3* gene: see Discussion (McKendry et al., 1997).

Two half T-box sites are located close to the TATA box in the *Xnr1* promoter

The consensus Brachyury-binding site (Fig. 3A) was determined by Kispert and Herrmann (1993) using a PCR-immunoprecipitation approach. Originally it was thought that Brachyury binds to this site as a dimer (Muller and Herrmann, 1997); however, recently, it was shown that it can also bind to a half site as a monomer (Casey et al., 1998). Other T-box proteins, such as Tbx2 and VegT, have also been shown to bind to half sites (Carreira et al., 1998; Tada et al., 1998; for review see Smith, 1999).

Inspection of the *Xnr1* promoter sequence revealed the presence of two non-palindromic T-box sites, called TBX1 and TBX2, which are 10 bp apart (Fig. 1C). TBX1, CATAGGTGTGAA, matches 11/12 nucleotides with the consensus sequence. TBX2, AGCTCACTCCTA, which is in the opposite orientation, is less well conserved and matches 9/12 consensus nucleotides. Like the T-box sites in the *eFGF* (Casey et al., 1998) and *Bix4* (Tada et al., 1998) promoters, both TBX1 and TBX2 contain the highly conserved non-palindromic region of the consensus sequence (Fig. 3A,B).

The early zygotic *Xnr1* expression, which is activated around mid-blastula transition by maternal determinants (Yasuo and Lemaire, 1999), occurs in a similar domain to that of maternal VegT in the blastula (Jones et al., 1995; Zhang and King, 1996; Stennard et al., 1996; Lustig et al., 1996b; Horb and Thomsen, 1997). In the gastrula, expression of both zygotic *VegT* and *Xbra* overlaps with that of *Xnr1* in the dorsal marginal zone (Smith et al., 1991). *Eomesodermin* expression overlaps the deep region of the organizer (Ryan et al., 1996). Of the T-box proteins identified so far in *Xenopus*, VegT is therefore the best candidate for directly regulating the endodermal expression of *Xnr1*, whereas other T-box proteins are candidates for activating later expression.

VegT directly induces *Xnr1* expression through the T-box sites

The ability of VegT to induce *Xnr1* was analysed using the animal cap assay. 1-cell *Xenopus* embryos were injected at the animal pole with synthetic *VegT* mRNA. Animal caps were dissected at the blastula stage and harvested at midgastrulation, at a stage when both *VegT* and *Xnr1* are normally expressed. The induction of *Xnr1* transcripts was analysed by RT-PCR. As shown in Fig. 2A, VegT induced *Xnr1* at both amounts of mRNA injected.

In order to determine whether VegT can bind to either TBX1 or TBX2, electrophoretic mobility shift assays were performed using a radiolabelled probe containing both T-box sites. Probes containing mutations in either TBX1 (M1) or TBX2 (M2) or both TBX1 and TBX2 (M3) were also used (Fig. 3B). VegT produced a shift with the wild-type DNA, confirming that the promoter region contains at least one binding site (Fig. 3C). The M2 mutated probe showed a shifted band of equal intensity to the wild-type probe, whereas the M1 and M3 mutated probes showed faint shifts. This indicates that VegT can bind to TBX1, but not TBX2, in this in vitro assay. A similar result was observed with VegT binding to two half sites in the *Bix4* promoter (Tada et al., 1998).

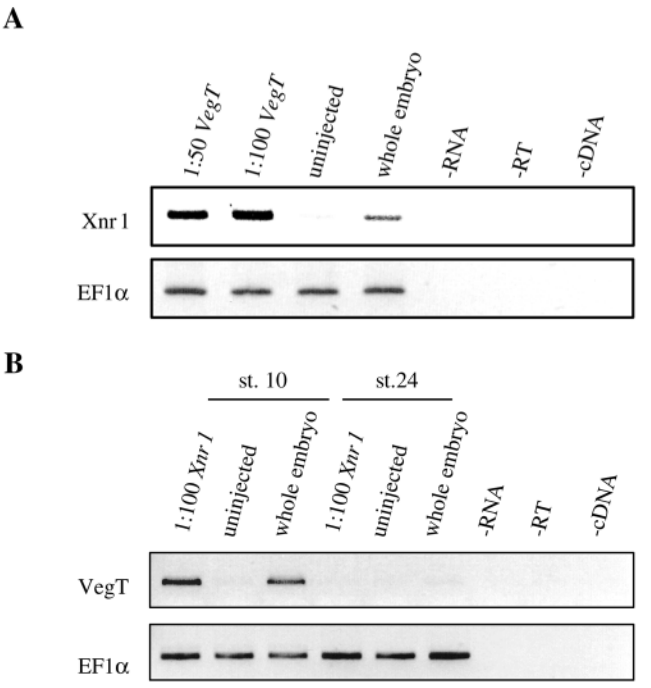

Fig. 2. Mutual induction of Xnr1 and VegT. (A) VegT can induce expression of *Xnr1* in ectodermal cells. (B) Xnr1 can induce expression of *VegT* in ectodermal cells. Approximately 200 pg (1:50 dilution) and 100 pg (1:100 dilution) of *VegT* mRNA or *Xnr1* mRNA was injected into the animal pole of 1-cell *Xenopus* embryos. Animal caps were dissected at stage 9, and harvested at stage 11 for the *VegT*-injected embryos, and at stages 10 and 24 for the *Xnr1*-injected embryos. Expression of *Xnr1* and *VegT* was analysed by RT-PCR. The housekeeping gene *EF1α* is a loading control.

To investigate whether VegT can activate transcription of *Xnr1*, a 616 bp region containing either the wild-type or mutant TBX sites was cloned into the luciferase reporter vector pGL3Basic. Circular DNA was injected unilaterally into 2-cell embryos, either alone or together with in vitro-synthesised *VegT* mRNA. Embryos were harvested at midgastrulation, at a stage when *Xnr1* is normally expressed, and analysed for luciferase activity. As shown in Fig. 3D, VegT induced luciferase reporter activity approximately eight-fold. This induction was reduced to approximately two-fold with either the Ml or M2 mutations individually and was abolished with the double mutant (M3).

Xnr1 induction via an autoregulatory loop

In this study, we have shown that VegT directly induces expression of *Xnr1*. It has previously been shown that Xnr1 can induce expression of *VegT* in ectoderm cells (Lustig et al., 1996b). This was confirmed by the results in Fig. 2B. Taken together, these observations suggest that *Xnr1* may participate in an autoregulatory loop with VegT. This possibility was investigated using the animal cap assay. In vitro-synthesised *Xnr1* mRNA, devoid of its 3′ and 5′ untranslated regions, was injected into the animal pole of 1-cell *Xenopus* embryos. Animal caps were then dissected at the blastula stage and harvested at midgastrulation. Induction of endogenous *Xnr1*

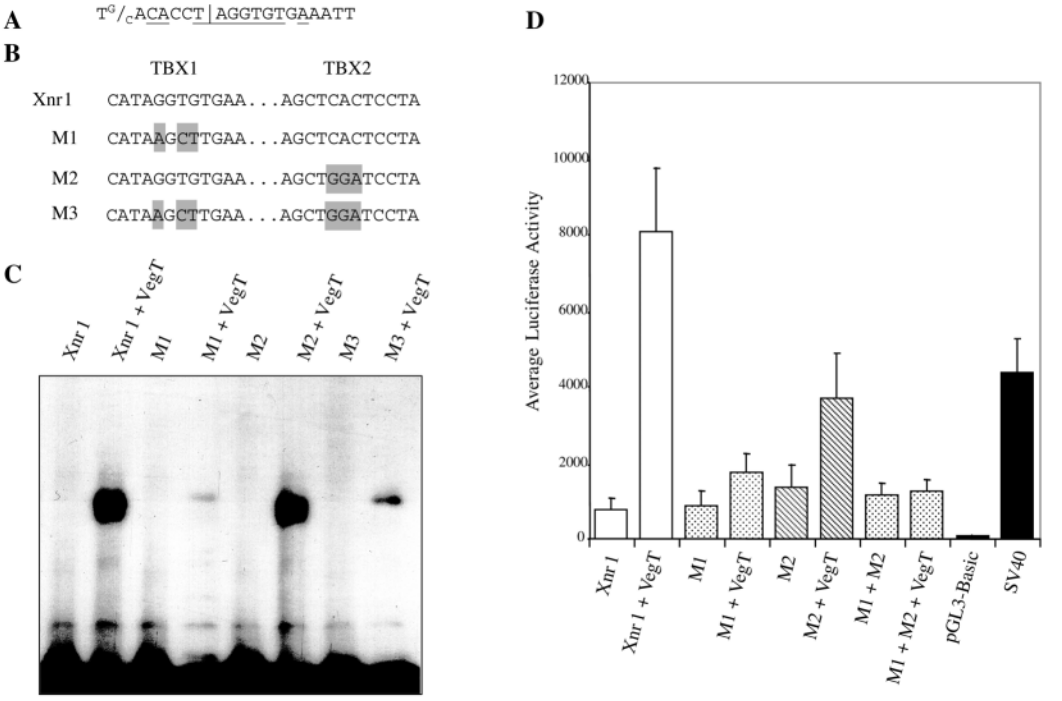

Fig. 3. VegT directly induces *Xnr1* expression. (A) The Brachyury consensus binding site (Kispert and Herrmann, 1993). The most conserved nucleotides (>95% in Kispert and Herrmann's analysis) are underlined. The vertical line indicates the axis of the approximate palindrome. Note that the right half site (12 bases) is more conserved than the left half site (8 bases). (B) Sequences of the wild-type and mutant T-box sites. Mutations were introduced into TBX1 (Ml), TBX2 (M2) and both TBX1 and TBX2 (M3) using PCR mutagenesis by hybrid overlap extension. The shaded boxes indicate the nucleotides which have been mutated. (C) Electrophoretic mobility shift assay of VegT binding to wild-type and mutant *Xnr1* promoter T-box sites. The radiolabelled DNA probe containing both of the T-box sites (wild type; single mutations, M1 and M2; or double mutation M3) was incubated either with or without in vitro-synthesized VegT before gel electrophoresis. Mutations in TBX1, but not TBX2, greatly reduced VegT binding in this in vitro assay. (D) VegT directly induces *Xnr1* expression in a reporter gene assay. 50-l00 pg of circular DNA was injected unilaterally into 2-cell *Xenopus* embryos. Embryos were harvested at stage 11 and analysed for luciferase activity. Samples of ten embryos were analysed and each bar represents the average of triplicate samples. The induction by VegT was confirmed with multiple independent experiments, although the extent of the induction varied between individual experiments. The positive control contains luciferase expression driven by the SV40 promoter, and the negative control contains the reporter vector only. Error bars represent standard deviations.

was analysed by RT-PCR using primers specific to the 5′ untranslated region. As shown in Fig. 4A, the injected *Xnr1* mRNA induced expression of the endogenous *Xnr1* gene, confirming autoregulation of *Xnr1*.

3.5 kb of *Xnr1* promoter sequence can drive expression of β-galactosidase in the organizer and anterior endomesoderm of transgenic embryos

In order to investigate the transcriptional activity of the cloned *Xnr1* promoter in intact embryos, we employed a transgenic approach using the technique of Kroll and Amaya (1996). Approximately 3.5 kb of *Xnr1* promoter sequence was cloned into a reporter vector so as to drive expression of nuclear-localised β-galactosidase (Fig. 5A; Table 1). Transgenic embryos were harvested at various stages, fixed and stained for β-galactosidase expression. No pan-vegetal expression was detectable in blastulae. Clear expression was first detected just before blastopore formation at stage 9.5 on the dorsal side of the embryo. At stage 10.25 this nuclear β-galactosidase activity was externally visible in a region extending dorsally from the blastopore to the animal pole of the embryo (Fig. 5C). Sectioning of stained embryos revealed nuclear β-galactosidase activity in all layers of the organizer, including the surface layer and deep cells, and extending into the anterior endomesoderm (Fig. 5D). This region corresponds with the endogenous expression domain of *Xnr1* in the dorsal marginal zone, as revealed by whole-mount in situ hybidization (Jones et al., 1995). However, expression of *Xnr1* in the anterior

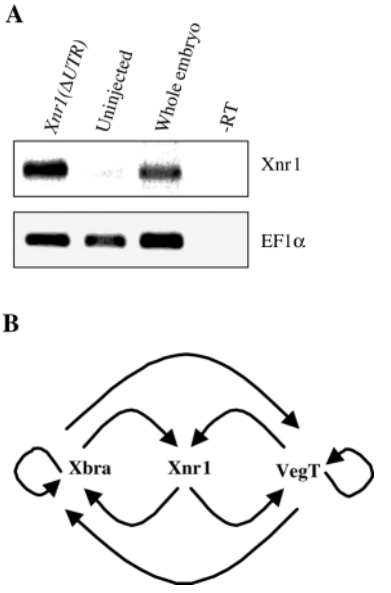

Fig. 4. Autoregulation of *Xnr1*. (A) The injected *Xnr1* mRNA can induce the endogenous *Xnr1* transcript in ectodermal cells. Approximately 100 pg of *Xnr1* mRNA, devoid of its own untranslated regions, was injected into the animal pole of one-cell *Xenopus* embryos. Animal caps were dissected at stage 9 and harvested at stage 11. Expression of the endogenous *Xnr1* transcript was analysed by RT-PCR using primers specific to the 5′ UTR. The housekeeping gene *EF1α* is a loading control. (B) Interactions between T-box genes and *Xnr1* in *Xenopus*. Known regulatory interactions include the autoregulation of *Xbra* (Isaacs et al., 1994, Schulte-Merker and Smith, 1995; Casey et al., 1998), Xbra induction of *VegT* (Stennard et al., 1996), VegT induction of *Xbra* (Stennard et al., 1996; Horb and Thomsen, 1997), Xnr1 induction of *Xbra* (Jones et al., 1995); Xbra induction of *Xnr1*, *VegT* autoinduction (Zhang and King, 1996), Xnr1 induction of *VegT* (Lustig et al., 1996b), VegT induction of *Xnr1* (this work) and *Xnr1* autoinduction (this work).

Table 1. Expression of *Xnr1* reporter gene constructs in transgenic embryos

Construct	Number of experiments	Number of embryos examined at gastrula stages	Number showing expression of transgene localised to dorsal domain	Number showing expression of transgene elsewhere*
3.5 kb *Xnr1* promoter::βgal	4	127	33	0
As above with both T-box sites mutated	3	118	29	0

*Excludes a small proportion of embryos showing intense staining of groups of a few ectodermal cells

endomesoderm has not been documented previously. Mapping the precise domain of *Xnr1* expression in the dorsal marginal zone has proven difficult, owing to the weak signal (Jones et al., 1995; Lustig et al, 1996a). Therefore, on the basis of the expression of the transgene, we predict that the expression of *Xnr1* probably extends from the surface of the organizer into deeper cells constituting the anterior endomesoderm.

Embryos analysed at stage 24 showed bilateral expression in a broad dorsal domain (data not shown), which did not match the endogenous left-sided expression of *Xnr1* in the lateral plate mesoderm. This suggests that the elements that control the later asymmetric expression of *Xnr1* are not in this 3.5 kb promoter fragment.

In order to investigate the role of T-box proteins in directing expression of the transgene, analogous experiments were carried out with the 3.5 kb promoter fragment containing the two T-box mutations. At stage 10.25, the pattern of mutant transgene expression was indistinguishable from that of the wild type (Fig. 5E).

Conserved elements in the *Xnrl* and *Xnr3* promoters

The promoter of the related gene *Xnr3* has been described (McKendry et al., 1997). By mutational analysis of the promoter, McKendry et al. (1997) identified two distinct sequence elements that are required for the response to Wnt signals, called Wnt Response Elements 1 and 2 (WRE1 and WRE2). WRE1 was shown to interact with an unknown protein from *Xenopus* gastrulae and WRE2 was shown to interact with proteins from the LEF1/TCF family of transcription factors.

Alignment of the *Xnr1* and *Xnr3* promoters reveals striking homologies between the two sequences (Fig. 6A). There are three regions of very strong homology: (1) WREl is entirely conserved between the two promoters, (2) the left half of WRE2 is conserved and (3) there is also a conserved region of 15 nucleotides around the TATA box.

Whereas these identified elements are conserved between the two promoters, the 269 bp *Xnr3* promoter sequence does

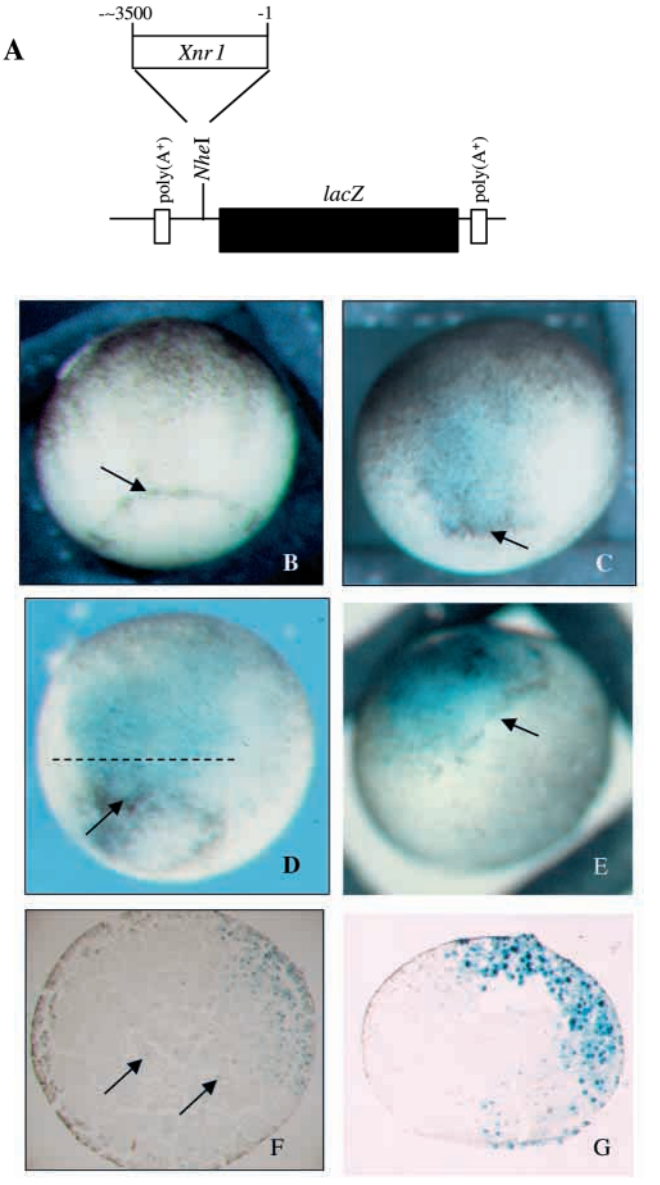

Fig. 5. *Xnr1* transgenic embryos. (A) The reporter construct used in the transgenic experiments contains the open reading frame of the nuclear β-galactosidase gene flanked by transcriptional pause sites. Approximately 3.5 kb of *Xnr1* promoter sequence was inserted into the *Nhe*I restriction site (where −1 indicates the nucleotide immediately 5′ to the ATG translation start codon). (B) Stage 10.25 control embryo (fertilised in vitro using conventional methods), stained for β-galactosidase activiy. Arrow indicates blastopore lip. (C,D) Stage 10.25 embryos expressing the *Xnr1::lacZ* transgene, stained for β-galactosidase activity, showing expression in the dorsal marginal zone. Arrows indicate blastopore lip. The dotted line in D indicates the plane of sectioning in F. (E) Stage 10.25 embryo expressing the transgene having both T-box sites mutated. Expression is in the dorsal marginal zone. The arrow indicates the blastopore lip. (F) Section through embryo shown in D, showing nuclear expression of the transgene in all layers of the organizer. Deeper expression in the anterior endomesoderm is indicated by arrows. (G) Vertical section in the dorsal-ventral plane of a stage 10 transgenic embryo expressing the *Xnr1::lacZ* transgene, stained for β-galactosidase activity. Dorsal is at the right. Expression extends to the animal pole and into deep cells of the anterior endomesoderm.

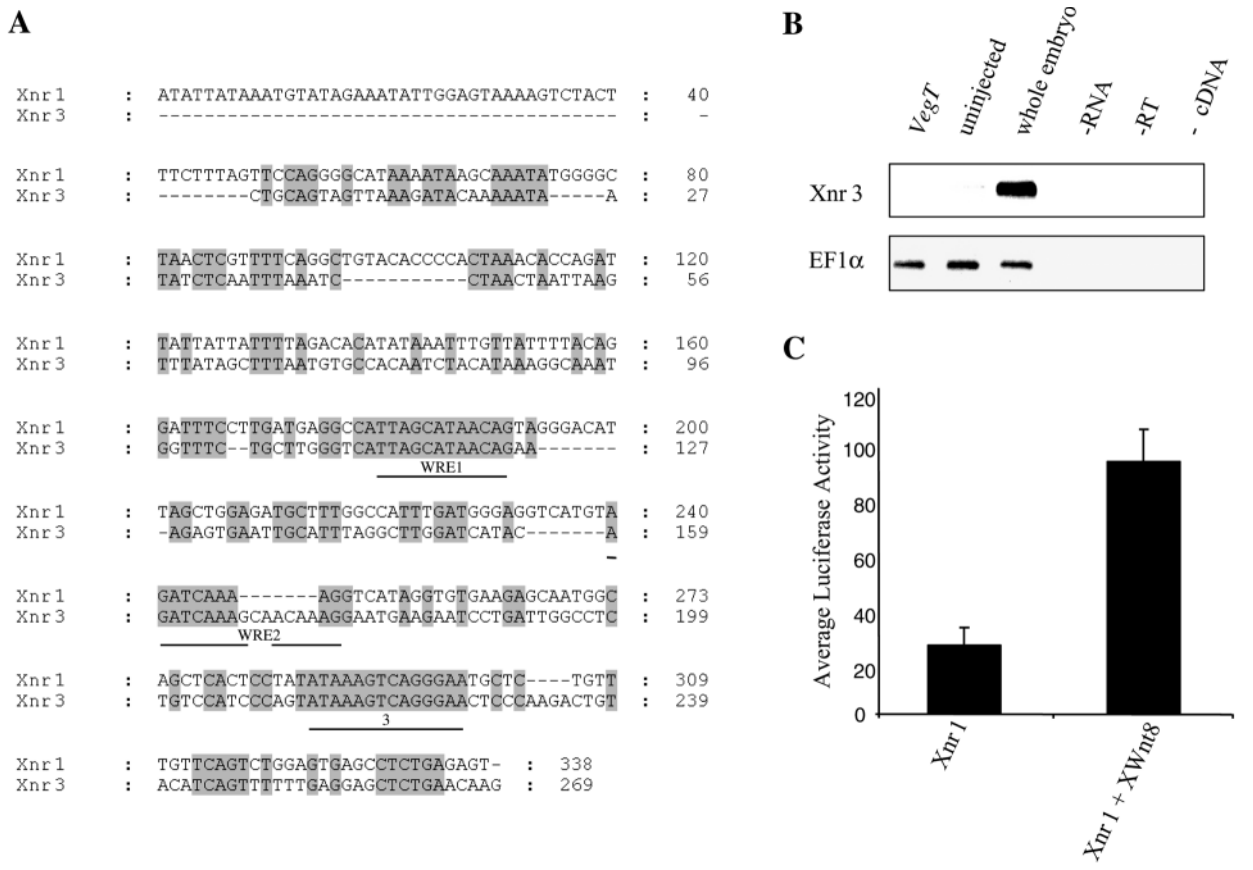

Fig. 6. Comparison of the *Xnr1* and *Xnr3* promoter sequences. (A) The *Xnr1* and *Xnr3* promoter sequences were aligned using GCG software. Three regions of strong homology have been indicated: Wnt Response Element 1 (WREl) is entirely conserved between the two promoters, the left half of Wnt Response Element 2 (WRE2) is conserved, and the region around the TATA box (3) is also highly conserved. (B) VegT does not induce *Xnr3* in ectodermal cells. Approximately 100 pg of *VegT* mRNA was injected into the animal pole of 1-cell *Xenopus* embryos. Animal caps were dissected at stage 9 and harvested at stage 11. Expression of *Xnr3* was analysed by RT-PCR. The housekeeping gene *EF1α* is a loading control. (C) Xwnt8 induces the 616 bp promoter::luciferase construct. Embryos were co-injected with *Xwnt8* mRNA and the DNA construct consisting of 616 bp of the *Xnr1* promoter upstream of the luciferase reporter gene. Luciferase activity was assayed at stage 10.5.

not contain any T-box consensus sequences, and the particular T-box locations within the *Xnr1* promoter are not represented in the *Xnr3* promoter, despite the surrounding homology. On this basis, we predicted: (1) that the *Xnr3* gene would not be inducible by VegT, in contrast to the *Xnr1* gene, and (2) that the *Xnr1* promoter would be inducible by Wnt. In order to see whether VegT could induce *Xnr3*, we employed the animal cap assay, as before. As shown in Fig. 6B, VegT does not induce *Xnr3* in ectodermal cells (compare with the induction of *Xnr1* by VegT in Fig. 2A). In order to see whether the *Xnr1* promoter is inducible by Wnt, we co-injected 2-cell embryos with *Xwnt8* mRNA and the *Xnr1* promoter::luciferase reporter construct. When assayed at stage 10.5, there was a stimulation of luciferase activity in response to Wnt (Fig. 6C).

Xnr1 and *Xnr3* are expressed in overlapping domains in the Spemann organizer, and *Xnr1* alone is also expressed vegetally at blastula stages (Jones et al., 1995; Ecochard et al., 1995; Smith et al., 1995). We propose that the similarity of the two expression patterns is due to the Wnt/β-catenin pathway and that T-box proteins may be required for the vegetal expression of *Xnr1*, in combination with other maternal determinants.

DISCUSSION

The *Xnr1* gene has a dynamic pattern of expression. There is no evidence for maternal transcripts. By whole-mount in situ hybridization, expression is first evident at stage 9 as very weak perinuclear staining in vegetal cells over the bottom third of the late blastula embryo (Jones et al., 1995). This disappears as development proceeds and, in the stage 10.5 gastrula, expression of *Xnr1* is localised in the dorsal marginal zone. Again the staining is weak (Jones et al., 1995). Later, expression is found in tailbud embryos in symmetrical patches near the posterior end of the notochord, followed by the characteristic asymmetric expression in the left lateral plate mesoderm at stage 24 (Jones et al., 1995; Lustig et al., 1996a).

The roles of this TGFβ family member are not fully documented. It is likely that the earliest vegetal expression of *Xnr1* signifies a role in endoderm formation, and it has recently been proposed that the differentiation of anterior endomesoderm involves Xnr1 and/or Xnr2 as inducers of *cerberus* and *Xhex* (Zorn et al., 1999). A role in continued formation and differentiation of axial mesoderm has also been proposed for *Xnr1* expression in the organizer (Jones et al., 1995). The later,

asymmetric expression has a role in a conserved vertebrate signaling cascade, involving TGFβ family members and the transcription factor Pitx2 (Ramsdell and Yost, 1998).

In this study, we have identified two consensus T-box half sites (Kispert and Herrmann, 1993) in the *Xnr1* promoter, only one of which appears to be a high affinity site by the electrophoretic mobility shift assay although both T-box sites are required for full inducibility. To date, the DNA-binding specificities of the characterised T-box proteins appear similar. Therefore it is probable that other T-box proteins will also bind to this region, but among these only VegT is a maternal determinant and therefore the only candidate for activating the earliest vegetal *Xnr1* expression (Zhang and King, 1996; Stennard et al., 1996; Lustig et al., 1996b; Horb and Thomsen, 1997).

We have also found that, at least in an ectodermal cell assay, *Xnr1* can positively autoregulate its own expression and, given the mutual induction of *Xnr1* and *VegT*, a relatively simple positive feedback loop can be envisaged as the mechanism for this (Fig. 4B). Whether such an autoregulatory loop has a significant role in embryogenesis is presently unclear. It is possible that the autoregulation may temporarily reinforce expression in the vegetal blastula cells and organizer, where VegT is present (Zhang et al., 1998). Among the four known Xnr molecules, the diffusibilty of only Xnr2 has been studied (Jones et al., 1996). If Xnr1 resembles Xnr2 in its low diffusibility through tissue (the two ligands are 87% similar; Jones et al., 1995), then this would restrict the extracellular signaling to short ranges within the embryo, hence predicting that the autoregulatory loop would act as a local reinforcement rather than as a signaling relay mechanism. As with other proposed autoregulatory loops, such as that involving Xbra and eFGF (Isaacs et al., 1994; Schulte-Merker and Smith, 1995), a mechanism for eventually terminating expression must involve additional factors.

We have initiated a transgenic approach to studying the *Xnr1* promoter and have shown that a 3.5 kb promoter fragment is sufficient to drive expression of a reporter gene in the anterior endomesoderm and organizer of gastrula stage embryos, in a domain that resembles the endogenous *Xnr1* expression at this stage. This promoter fragment did not drive detectable expression in the vegetal region of the blastula, where the earliest endogenous *Xnr1* expression is detected by in situ hybridization. At present we cannot distinguish whether this finding is due to lack of sensitivity of β-galactosidase detection in the vegetal region, or whether the promoter fragment simply fails to drive expression appropriately at the blastula stage. In other experiments (not shown here), this construct did not display the characteristic asymmetric expression at the tailbud stage, but rather showed symmetric expression in a broad dorsal region. Our finding that the transcriptional initiation sites employed at the later stage of *Xnr1* gene expression are different from those employed at gastrulation, is an indication that different promoter elements and transcriptional regulators control these two modes of expression.

It is unlikely that VegT alone is sufficient to activate expression of *Xnr1* in the vegetal region because vegetal expression of the transgene containing the T-box sites was not detected. Endodermal expression of the mouse *nodal* gene is under the control of sequences located in the first intron (Adachi et al., 1999; Norris and Robertson, 1999). We have found sequences conserved between the mouse *nodal* and *Xnr1* intron I, including a completely conserved 10 bp FAST site (unpublished), indicating that the intronic sequences probably have a role in *Xnr1* regulation.

The obvious conservation of Wnt response elements in the promoters of *Xnr1* and *Xnr3* suggests a common mechanism of regulation by the Wnt/β-catenin pathway, and we have shown that the *Xnr1* promoter is inducible by Xwnt8. However, inspection of the proximal 269 bp of the *Xnr3* promoter (McKendry et al., 1997) does not reveal any T-box sites, and the particular T-box regions in the *Xnr1* promoter are absent in the *Xnr3* promoter despite the conservation of adjacent sequences. This led us to ask whether *Xnr3* is inducible by VegT, and our experiments confirmed that *Xnr3* is not induced by VegT under circumstances where *Xnr1* is inducible. These observations suggest a model to account for the similarities in expression patterns between *Xnr1* and *Xnr3* in early embryos. We propose that the early vegetal expression of *Xnr1* in blastula stage embryos depends upon activiation by VegT in conjunction with another maternal determinant, probably a TGFβ family member. We find that *Xnr3* is not induced by VegT and is therefore not expressed in this region of the blastula. Later, both *Xnr1* and *Xnr3* are expressed in the organizer. We propose that dorsal expression is mediated by activation of the Wnt/β-catenin pathway, through the Wnt response elements, as shown previously for *Xnr3* (McKendry et al., 1997). This pattern is consistent with the extensive dorsal domain of nuclear β-catenin (Larabell et al., 1997). The T-box sites are not required for this dorsal expression in early gastrulae, since mutating the T-box sites did not perturb the expression of the transgene. These basic dorsal expression domains of both *Xnr1* and *Xnr*3 may be refined by other transcriptional regulators.

Recent work by others has shown that the *Xnr1* promoter contains functional T-box sites (Kofron et al., 1999). Our data confirm and extend those findings. The likely roles of other regulatory pathways in controlling *Xnr1* expression, such as Wnt pathways and TGFβ signaling, and their combinatorial effects, await further investigation.

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