

## Activin/Nodal responsiveness and asymmetric expression of a *Xenopus* *nodal*-related gene converge on a FAST-regulated module in intron 1

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### SUMMARY

Vertebrate Nodal-related factors play central roles in mesendoderm induction and left-right axis specification, but the mechanisms regulating their expression are largely unknown. We identify an element in *Xnr1* intron 1 that is activated by activin and Vg1, autoactivated by Xnrs, and suppressed by ventral inducers like BMP4. Intron 1 contains three FAST binding sites on which FAST/Smad transcriptional complexes can assemble; these sites are differentially involved in intron 1-mediated reporter gene expression. Interference with FAST function abolishes intron 1 activity, and transcriptional activation of Xnrs by activin in embryonic tissue explant assays, identifying FAST as an essential mediator of *Xnr* autoregulation and/or 'signal relay' from activin-like molecules. Furthermore, the mapping of endogenous activators of the

*Xnr1* intronic enhancer within *Xenopus* embryos agrees well with the pattern of *Xnr1* transcription during embryogenesis. In transgenic mice, *Xnr1* intron 1 mimics a similarly located enhancer in the mouse *nodal* gene, and directs FAST site-dependent expression in the primitive streak during gastrulation, and unilateral expression during early somitogenesis. The FAST cassette is similar in an ascidian *nodal*-related gene, suggesting an ancient origin for this regulatory module. Thus, an evolutionarily conserved intronic enhancer in *Xnr1* is involved in both mesendoderm induction and asymmetric expression during left-right axis formation.

Key words: Nodal, activin, FAST-1, Left-right asymmetry, *Xenopus*, TGF $\beta$

### INTRODUCTION

The process of mesendoderm induction during *Xenopus* embryogenesis is a good model system for understanding how tissues are patterned and specified (Harland and Gerhart, 1997). Members of the transforming growth factor  $\beta$  (TGF $\beta$ ) superfamily of secreted factors – activin, Vg1, and *Xenopus* nodal-related factors (Xnrs) – have been implicated in mesendoderm induction (Harland and Gerhart, 1997). Activin has been intensely studied as a candidate morphogen, since it activates different mesodermal markers in a concentration-dependent manner in whole (Gurdon et al., 1994; Gurdon et al., 1995) and dissociated animal caps (Green et al., 1992; Green and Smith, 1990).

Consistent with a crucial role of activin-like molecules in embryogenesis, activin response elements (AREs) have been reported in several activin-inducible transcription factor genes, such as the homeobox genes *gooseoid* (*gsc*; Watabe et al., 1995), *Mix.2* (Huang et al., 1995), *HNF1 $\alpha$*  (Weber et al., 1996), and *Xlim-1* (Rebbert and Dawid, 1997), a T-box gene *Xbrachyury* (*Xbra*; Latinkic et al., 1997), and a forkhead gene

*XFD-1'* (Kaufmann et al., 1996). Although the mechanisms regulating transcription of these genes remain poorly understood, identification of activin response factor (ARF) provides an entry point. ARF was first identified as a factor binding to an ARE in the *Mix.2* promoter in response to Vg1, TGF $\beta$  and activin (Huang et al., 1995). Subsequently, forkhead activin signal transducer-1 (FAST-1), Smad2, Smad3, and Smad4 were identified as components of ARF (Chen et al., 1996, 1997; Labbe et al., 1998; Liu et al., 1997; Yeo et al., 1999). The mammalian FAST-1 homolog, FAST-2/Fast1, possesses similar biochemical properties (we hereafter refer to *Xenopus* FAST-1 and mouse FAST-2/Fast1 as xFAST and mFAST, respectively, and use 'FAST site(s)' collectively, since both recognize the same target sites) (Labbe et al., 1998; Liu et al., 1999; Weisberg et al., 1998; Zhou et al., 1998). Studies with a dominant-negative form of xFAST and xFAST antibody are consistent with the view that xFAST is an endogenous mediator of mesendoderm induction (Watanabe and Whitman, 1999). However, the contribution of FAST target sites to the endogenous regulation of mesendodermal genes has not been directly investigated.

Despite the progress in understanding activin signaling pathways, several lines of evidence argue against a role for activin in mesendoderm induction. In frog, neither follistatin nor the extracellular domain of the activin type II receptor, both of which block activin signaling, act to prevent mesoderm formation (Dyson and Gurdon, 1997; Schulte-Merker et al., 1994). Furthermore, mesoderm forms in mouse activin null mutants (Matzuk et al., 1995; Smith, 1995). In contrast, much data suggests that Nodal-related factors play essential conserved roles in early vertebrate embryogenesis. In mouse and zebrafish, Nodal signaling is genetically essential for mesendoderm formation (Conlon et al., 1994; Erter et al., 1998; Feldman et al., 1998; Rebagliati et al., 1998a,b; Sampath et al., 1998; Zhou et al., 1993). Recent misexpression and interference experiments imply that *nodal*-related (*Xnr*) genes perform similar functions in *Xenopus* (Jones et al., 1995; Joseph and Melton, 1997; Osada and Wright, 1999; Piccolo et al., 1999). For example, the Nodal-specific antagonist Cerberus blocks mesoderm induction in embryos (Piccolo et al., 1999) and Nieuwkoop-type induction of mesoderm in animal caps by vegetal explants (Agius et al., 2000) and blocking Nodal signals in the vegetal region inhibits endoderm specification (Osada and Wright, 1999).

However, our previous findings that *Xnr1* and *Xnr2* are induced in ectodermal explants treated with activin protein (Jones et al., 1995), and that mesendoderm induction by activin protein can be suppressed by a dominant-negative cleavage mutant form of *Xnr2* (Osada and Wright, 1999), suggested that *Xnrs* act downstream of activin-like signals (which might include *Xnrs* themselves). Thus, studies of the molecular circuitry involved in the transcriptional regulation of *Xnrs*, and reconsideration of the meaning of 'activin responsiveness', will provide new clues towards understanding mesendodermal induction and patterning.

Recent evidence indicates that mesoderm induction in *Xenopus* begins at the blastula stage (Wylie et al., 1996; Yasuo and Lemaire, 1999), with a maternal, vegetally localized transcript, *VegT* (Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996), which encodes a T-box transcription factor, playing a central role (Zhang et al., 1998). *VegT*-depleted endoderm cannot induce mesoderm (Zhang et al., 1998), and a two-step model for mesoderm induction has recently been proposed (Clements et al., 1999; Kimelman and Griffin, 1998; Kofron et al., 1999; Yasuo and Lemaire, 1999; Zorn et al., 1999). First, vegetally located maternal *VegT* activates, cell-autonomously, the blastula-stage expression of *TGFβ*-related mesoderm inducers. Subsequently, *TGFβ*-related intercellular signaling leads to the maintenance and upregulation of these signals, and the establishment of mesendodermal fates. *Xenopus* Nodal-related factors are excellent candidates for these *TGFβ* factors. *VegT* induces *Xnr* expression (Agius et al., 2000; Clements et al., 1999; Hyde and Old, 2000; Yasuo and Lemaire, 1999), which is, conversely, downregulated in *VegT*-depleted embryos (Kofron et al., 1999). Molecular epistasis experiments show a more complete rescue of the *VegT*-depleted embryonic phenotype by *Xnrs* than other ligands (Kofron et al., 1999). In addition, *Xnr1* is *VegT*-responsive via T-box binding sites in the promoter region (Hyde and Old, 2000; Kofron et al., 1999).

Nodal-related factors are also involved in establishing left-right (L-R) asymmetry. In all vertebrates examined, *nodal*

homologs [mouse *nodal* (Conlon et al., 1994), chick *cNR* (Levin et al., 1995), frog *Xnr1* (Lowe et al., 1996), and zebrafish *cyclops* (Rebagliati et al., 1998a; Sampath et al., 1998)] are expressed in the left lateral plate mesoderm (LPM), preceding overt asymmetric organ morphogenesis. *nodal* expression is regulated by *lefty1* and *lefty2*, which are diverged members of the *TGFβ* superfamily also expressed in a left-sided manner (Meno et al., 1997, 1996). *Lefty1* acts as a 'midline barrier' to maintain asymmetric expression of *nodal* (Meno et al., 1998), whereas *lefty2* antagonizes *nodal* functions (Meno et al., 1999), activities that are apparently conserved in zebrafish and frog embryos (Bisgrove et al., 1999; Cheng et al., 2000; Thisse and Thisse, 1999).

Regulatory elements driving asymmetric expression of *nodal* and *lefty* have been identified. Left-side expression of *nodal* and *lefty2* is achieved via asymmetric enhancers (Adachi et al., 1999; Norris and Robertson, 1999; Saijoh et al., 1999), and mFAST sites in these enhancers are essential for their asymmetric expression (Saijoh et al., 2000), while *lefty1* is regulated by a combination of bilateral enhancers and a right side-specific silencer (Saijoh et al., 1999). Preceding asymmetric *nodal* expression during early somitogenesis, its expression in posterior epiblast and anterior extraembryonic visceral endoderm is essential for primitive streak formation and patterning of anterior central nervous system, respectively (Conlon et al., 1994; Varlet et al., 1997). Targeted deletion of the *nodal* asymmetric enhancer disrupts both the asymmetric expression and the epiblast/visceral endoderm expression (Norris and Robertson, 1999).

In this study, we addressed the molecular basis of two aspects of *Xnr1* expression: its activation and maintenance/upregulation associated with its role as a mesendoderm inducer, and its later unilateral expression in LPM. We mapped a strong ARE to an intronic enhancer, providing a link to the previous observation of *Xnr* induction by activin-like molecules, and suggesting *Xnr* signaling as a relay mechanism in mesendoderm induction. The enhancer can be used to map endogenous activators in the early *Xenopus* embryo. Three FAST sites mediate the response of *Xnr1* to activin-like signals, and the asymmetric expression of *Xnr1* as assessed by cross-species experiments in transgenic mice. Thus, an intronic FAST-regulated enhancer probably regulates both phases of *Xnr1* expression during embryogenesis. Evolutionary conservation of the regulatory module in *nodal*-related genes is also discussed.

## MATERIALS AND METHODS

### Isolation of *Xnr1* genomic DNA

An *EcoRI* cDNA fragment (approx. 400 bp) probe covering the *Xnr1* pro region allowed the isolation of 3 clones from a *Xenopus laevis* genomic library in  $\lambda$ DASH (gift from Eddy De Robertis);  $\lambda$ D2 was analyzed further. The transcription start site was determined using 5' RACE (Gibco BRL) on total RNA (500 ng) from stage 25 embryos. Identical 5' ends were obtained from multiple independent clones.

### *Xenopus* embryo manipulation

Artificial fertilization and culture were as described previously (Kay and Peng, 1991), and embryos staged according to Nieuwkoop and Faber (1967). RNAs were synthesized with the mMESSAGE mMACHINE kit (Ambion). For animal cap assays, RNAs (10 nl) were

injected animally into 1-cell embryos. Animal caps were explanted at stage 8-9, cultured until stage 10.5, and subjected to RT-PCR. *FGFR*, *Xbra*, *Xnr1*, *Xnr2* and *Xsox17β* primers were described previously (Hudson et al., 1997; Osada and Wright, 1999). For 32-cell stage injections, dorsal and ventral sides were discriminated by pigmentation differences (injection volume: 5 nl/blastomere).

### Luciferase assay

Firefly luciferase reporter constructs (100 pg) were coinjected with control *Renilla* luciferase plasmid (pRL-TK; 2 pg) into the animal region of 1-cell embryos. Pools of 3 embryos were collected in triplicate for each injection mixture at stage 10.5 (early gastrula). Luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega). Embryos were homogenized in 100 μl of 1× Passive Lysis Buffer (kit reagent) by vigorous vortexing, and cleared by microcentrifugation (1 minute). The supernatant (5 μl) was assayed in 50 μl of assay mixture, and luciferase activity measured for 10 seconds with a Berhold luminometer. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Each experiment was repeated at least 3 times. Absolute values varied with egg batch, but relative values were similar. Thus, single representative experiments are shown here.

### Expression plasmids

Prox/Luc: An approx. 1 kb sequence lying 5' of the initiation codon was PCR-amplified from a 1.1 kb *EcoRI* genomic fragment (Fig. 1). The upstream primer (5'-ATTCAGAAGCTTCTAGAGCGGCCGCTGCAGGAATTCCTGCTGGAGCAGCACTATTAAC-3') contained *HindIII*, *NotI*, *PstI*, and *EcoRI* sites (underlined). Downstream primer: 5'-ATTCGAAAGCTTCTGCTGCACTGCTGATCTCTCTTCCA-3' (*HindIII* site underlined). Sequencing demonstrated that PCR amplification (with *Pfu* polymerase) was accurate except for the elimination of 17 of 24 TA repeats in the starting genomic DNA template. The resulting *HindIII* fragment was inserted into the pGL3-basic vector (Promega). For -6 kb/Luc, Int 1-Prox/Luc, and Int 2-Prox/Luc, respectively, approx. 5 kb, approx. 0.9 kb *EcoRI* fragments containing the first intron, or a approx. 0.9 kb *PstI*-*AccI* fragment containing the second intron (Fig. 1), were inserted upstream of the 1 kb fragment of Prox/Luc. ΔProx/Luc: a approx. 800 bp *EcoRI*-*MscI* fragment was removed from Prox/Luc. Insertion of the approx. 0.9 kb first intron *EcoRI* fragment into ΔProx/Luc lead to I1-ΔProx.

Reporter plasmids with FAST site mutations were made by overlap PCR. Mutations were introduced as described previously (Labbe et al., 1998; Zhou et al., 1998), using the following oligonucleotides: mtA, 5'-CTGTTTCATTTTAAGGTTTCTGTATCGGTATATGTTTTCTG; mtB, 5'-CCAACCTCAAGTCTAATATAAATAGTCGAGTGTTTTG-3'; mtC, 5'-CTATATAACACTTCAATCTAAATTGCTGAGAGGTAAC; mtP 5'-CATGACTCACTATAACTTCTGTATCATAATAAATGAAGTACC-3'. The oligonucleotides were used successively to eliminate multiple sites in mtAB, mtAC, mtBC, mtABC, and mt (ALL). Primers used were: upstream, 5'-ACAACTAGCAAAATAGGCT-3'; downstream, 5'-CGGAATTCAGACTTGAGGTTGGTGG-3' (for ΔBC), 5'-CGGAATTCAAGTGTATATAGATAC-3' (for ΔC), and 5'-CGGAATTCAACCTTAAATGAACAGT-3' (for ΔABC). PCR products were *EcoRI*-digested and inserted into Prox/Luc. Construction of activated (FV) and dominant-negative (FE) forms of xFAST, was described elsewhere (Watanabe and Whitman, 1999).

### Electrophoretic mobility shift assays

Electrophoretic mobility shift assays were carried out as previously described with modification (Huang et al., 1995). *Xenopus* embryos were injected with RNA encoding activin βB (50 pg/embryo), Flag-Smad2 (250 pg/embryo), and 6Myc-Smad4 (250 pg/embryo) at the 2-cell stage. Injected and uninjected sibling embryos were harvested at stage 9-9.5. For antibody supershift/interference assays, rabbit preimmune serum, rabbit polyclonal anti-xFAST

antibody, M2 anti-Flag monoclonal antibody, and 9E10 anti-Myc monoclonal antibody were used. Wild-type and mutant forms of *Xnr1* ARE probes were generated by PCR using Int 1-Prox/Luc (for wild type) and mtABC/Luc (for mutant) as templates, and <sup>32</sup>P end-labeled with T4 polynucleotide kinase. PCR primers: for *Xnr1*-A and *Xnr1*-mA, 5'-TAAATAACAACCACCAACCTC-3' and 5'-ATATAGATACAGATAGACTAAACA-3'; for *Xnr1*-B and *Xnr1*-mB, 5'-AACGTTTCTGTTTAGTCTATCT-3' and 5'-TCACTTCTGTGCACTCTGTG-3'; for *Xnr1*-C and *Xnr1*-mC, 5'-TAAAGACAAATTACTGCT-3' and 5'-TTGAGGTTGGTGGTTGTTATT-3'.

### Transgenic mice

*EcoRI* fragments containing intron 1 were isolated from Int 1-Prox/Luc, mtC, mtBC, and mtABC and blunt-end inserted into the *SmaI* site in the hsp68/lacZ vector (Kothary et al., 1989). The expression cassette (WT or mutant *Xnr1* intron1, hsp68 minimal promoter, and *lacZ*) was released by *NotI* digestion, gel-purified, and dissolved in injection buffer (5 mM Tris pH 7.5, 0.1 mM EDTA). DNA (3-4 ng/μl) was injected into the pronuclei of (C57BL/6 × C3H) F<sub>1</sub> fertilized embryos using standard procedures (Hogan et al., 1994). Injected embryos were transferred to pseudopregnant females (ICR). Embryos recovered at 8.2 days post coitum were examined using X-gal staining following a standard protocol (Hogan et al., 1994). To roughly quantitate β-galactosidase activity, X-gal staining intensity was monitored at various time points (2, 8, 18 hours) during the color reaction. Unstained embryos were lysed and tested for the transgene using *lacZ* primers: upstream, 5'-CTCAAAGTGGCAGATGCACGGT-3'; downstream, 5'-CGTTGCACCACAGATGAAACGC.

### Ascidian *nodal*-related gene intron isolation

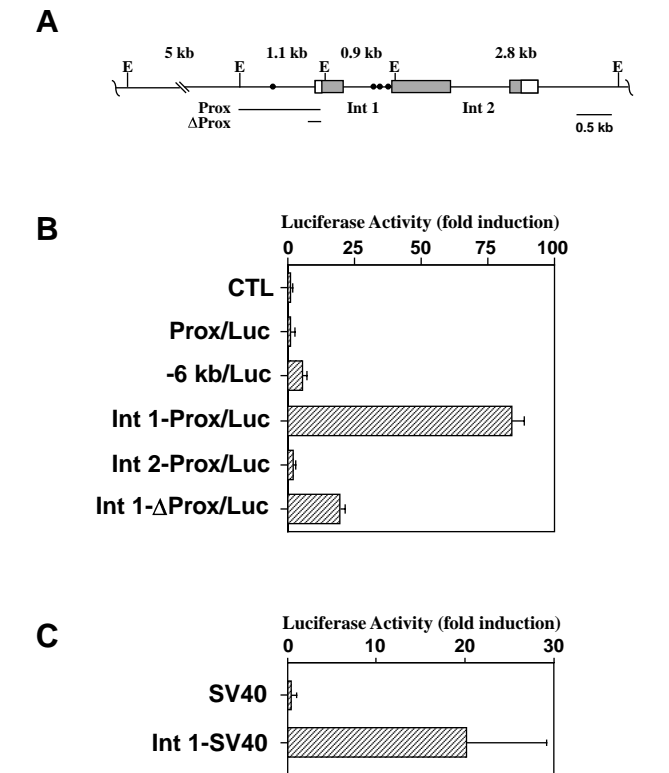
Based on the conserved intron 1 position in *nodal*-related genes from *Xenopus*, mouse and zebrafish (data not shown), primers were used to PCR amplify a fragment containing intron 1 from an amplified *Molgula oculata* genomic library, using 2 μl of heat-denatured, undiluted phage stock. Upstream primer, ATTGGTGCGCATGTGAC; downstream primer, ATTCTGATTTAGCCAATCG.

## RESULTS

### Strong activin response element in *Xnr1* intron 1

We previously showed that activin protein induces *Xnr* expression in animal cap explants (Jones et al., 1995), and that a dominant-negative *Xnr* reagent suppresses mesendoderm induction by activin protein in this situation (Osada and Wright, 1999). To explore the molecular mechanisms regulating *Xnr* activation by activin-like molecules, we have studied a approx. 10 kb *Xnr1* genomic DNA fragment (Fig. 1A).

We first tested whether activin response elements (AREs) are present in the 5' 'promoter' region. The approx. 1 kb 5' proximal flanking region (part of the approx. 1.1 kb *EcoRI* fragment; Fig. 1A and methods) linked to a luciferase reporter vector (Prox/Luc) did not respond to activin treatment (Fig. 1B). Thus, we inserted various genomic regions of *Xnr1* – a region 5 kb further upstream, intron 1 or intron 2 – upstream of the 1 kb proximal region, generating -6 kb/Luc, Int 1-Prox/Luc, and Int 2-Prox/Luc, respectively. Although activin significantly induced -6 kb/Luc transcription (approx. 8 fold), a much stronger ARE was detected in intron 1 (Fig. 1B); enhancement of Int 1-Prox/Luc was consistently increased 50-100 fold in multiple embryo batches. Reverse-oriented intron 1 (Int 1Rv-Prox/Luc) showed similar levels of activin inducibility to Int 1-Prox/Luc (data not shown), one criterion defining intron 1 as an enhancer. In contrast, Int 2-Prox/Luc

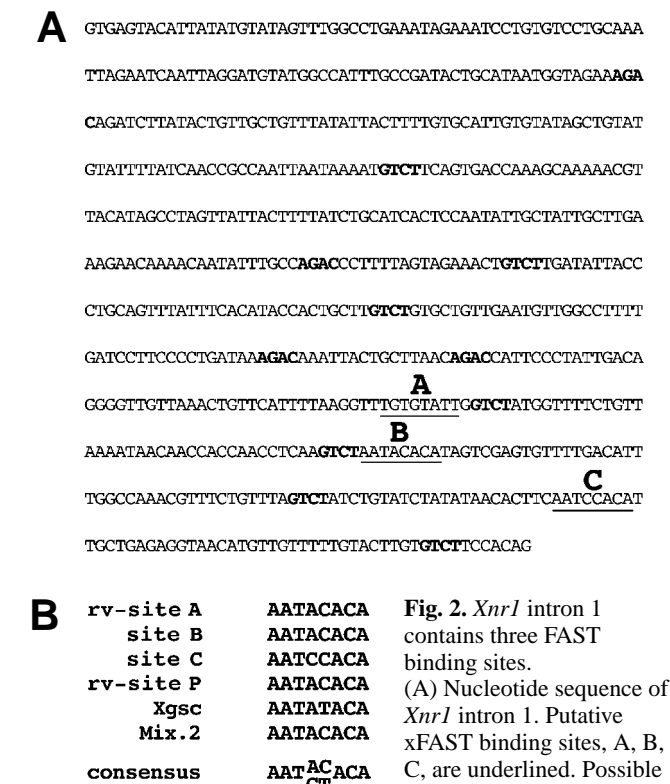


**Fig. 1.** A strong activin response element (ARE) in intron 1 of *Xnr1*. (A) *Xnr1* gene structure. Shaded and white boxes, protein-coding and non-coding regions, respectively; Prox, approx. 1 kb 5' proximal flanking region; Int, introns; black circles, FAST sites. ΔProx is a 5' deletion of the Prox fragment. *EcoRI* (E) fragment sizes are indicated. (B) A strong ARE in *Xnr1* intron 1. Indicated firefly luciferase constructs (100 pg) and control *Renilla* luciferase plasmid (pRL-TK; 2 pg) were coinjected with or without *activinβB* RNA (10 pg) into animal regions of 1 cell embryos. Fold induction represents the ratio of normalized luciferase activity of activin-injected compared to uninjected embryos (error bars: standard deviation). In -6kb/Luc, Int 1-Prox/Luc, and Int 2-Prox/Luc, the 5 kb, intron 1, and intron 2 sequences were inserted upstream of the Prox/Luc construct. For Int 1-ΔProx, intron 1 was placed upstream of the ΔProx region. (C) *Xnr1* intron 1 confers activin responsiveness to a heterologous SV40 minimal promoter (fold induction calculated as above).

showed no significant activity. Coupling of intron 1 to the 5'-deleted 1 kb promoter region, in the construct Int 1-ΔProx/Luc, substantially reduced luciferase activity. This result suggests that cooperative interactions between the upstream parts of this promoter region and intron 1 enable full enhancer activity. The activin responsiveness of intron 1 was transferable to the SV40 promoter (Fig. 1C). This SV40 promoter showed increased basal luciferase expression compared to the *Xnr1* 5' proximal region (data not shown), but did not respond to activin. In contrast, insertion of *Xnr1* intron 1 (Int 1-SV40) led to activin responsiveness. Thus, the intron 1 ARE behaves similarly with respect to the *Xnr1* 'promoter' and a heterologous promoter, and we conclude that a strong ARE-responsive enhancer resides in intron 1 of *Xnr1*.

***Xnr1* intron 1 contains FAST sites**

Since both the -1.0 kb 5' proximal region and intron 1 are necessary for the full activin responsiveness of *Xnr1*, we



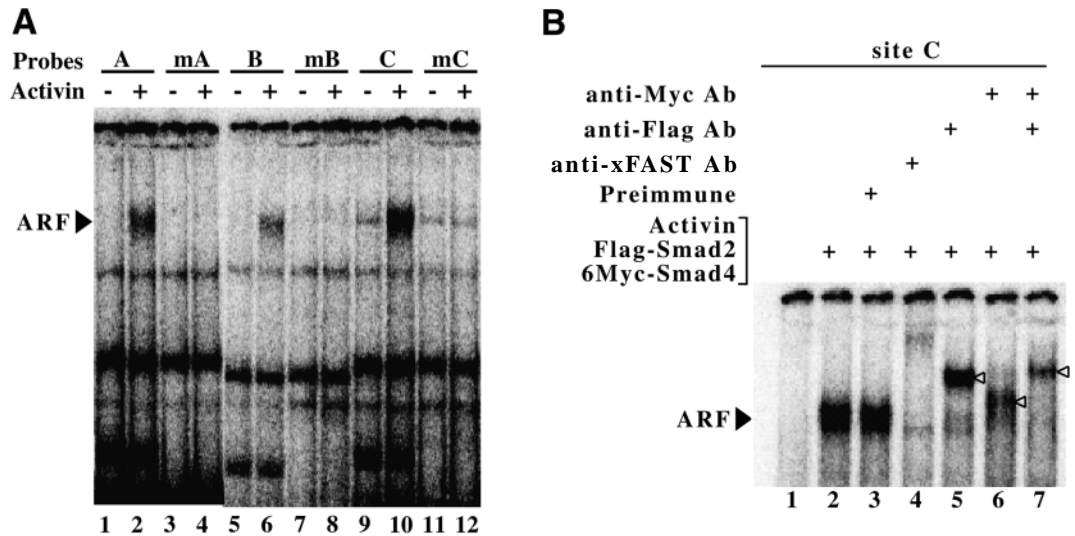
**Fig. 2.** *Xnr1* intron 1 contains three FAST binding sites. (A) Nucleotide sequence of *Xnr1* intron 1. Putative xFAST binding sites, A, B, C, are underlined. Possible Smad binding sites (GTCT or AGAC) are in bold. (B) The four xFAST binding sites (P site in the 5' flanking region, plus the A, B, C sites) in *Xnr1* are compared to related sites in the *Xenopus goosecoid* and *Mix.2* genes. A predicted consensus fits the FAST motif consensus. Orientation of A and P sites are reversed (rv) with respect to B and C.

determined their nucleotide sequences (Fig. 2A shows intron 1 sequence). Four putative binding sites for xFAST were found: one in the 5' half of the approx. 1 kb proximal region (site P, not shown) and three in intron 1 (sites A, B and C). The sequences identified in the promoter and intron 1 (Fig. 2B) perfectly match the consensus for FASTs, AAT(A/C)(A/C)ACA, as found by oligonucleotide selection methods (Zhou et al., 1998), and are consistent with those in the *Mix.2* and *goosecoid* (*gsc*) promoters (Chen et al., 1996; Labbe et al., 1998). FAST makes a protein complex, activin response factor (ARF), together with Smad factors (Chen et al., 1996, 1997; Liu et al., 1997). Consistent with this, several Smad binding sites (core sequence GTCT or AGAC; Zawel et al., 1998), which are sometimes abutted by degenerate GTCT sequences (Johnson et al., 1999), are scattered within the 5' flanking and intron 1 sequences near the xFAST sites. Besides xFAST sites, we also identified putative VegT/Xbra binding sites in the approx. 1 kb proximal region. The involvement of these sites in *Xnr1* activation, and the significance of the VegT-Xnr connection in mesendodermal induction is reported elsewhere (see Discussion; Hyde and Old, 2000; Kofron et al., 1999).

To determine whether ARF assembles on each xFAST site in intron 1, we performed electrophoretic mobility shift assays. Extracts prepared from embryos injected with *activin*, *Flag-Smad2*, and *Myc-Smad4* RNAs were used as a source of ARF. Double-stranded oligonucleotides containing each xFAST site

**Fig. 3.** ARF binds to the activin responsive elements of *Xnr1* intron 1.

(A) Electrophoretic mobility shift assays (EMSA) used extracts from embryos injected with *activin*  $\beta$ B RNA, or sibling uninjected embryos as control. Probes are for each of the putative xFAST binding sites from *Xnr1* intron 1, with controls being equivalent cpm of corresponding probes with specific mutations in the xFAST sites (mA, mB or mC). (B) Composition of activin-stimulated ARF activity assembling on *Xnr1* intron FAST sites. EMSA was performed with wild-type probe C and extracts from *Xenopus* embryos injected with *activin*  $\beta$ B, Flag-Smad2 and 6Myc-Smad4 RNAs, or from sibling uninjected embryos as control.



flanked by putative Smad sites were used as probes. In the frog embryo, Smad binding sites are not essential for ARF binding to the Mix.2 ARE, but enhance this recognition (Yeo et al., 1999). As shown in Fig. 3A, ARF/DNA complexes formed on each xFAST site, but not on oligonucleotides with mutated FAST sites (mA, mB, or mC). Oligonucleotide probes representing site C (which differs by one nucleotide from sites A/B) repeatedly recruited most ARF under these conditions. The specificity of ARF binding was shown by antibody interference (Fig. 3B). Preincubation of ARF extract with epitope tag antibodies caused an appropriate super-shift in the protein/DNA complexes, and in the case of xFAST antibody, abolished ARF assembly.

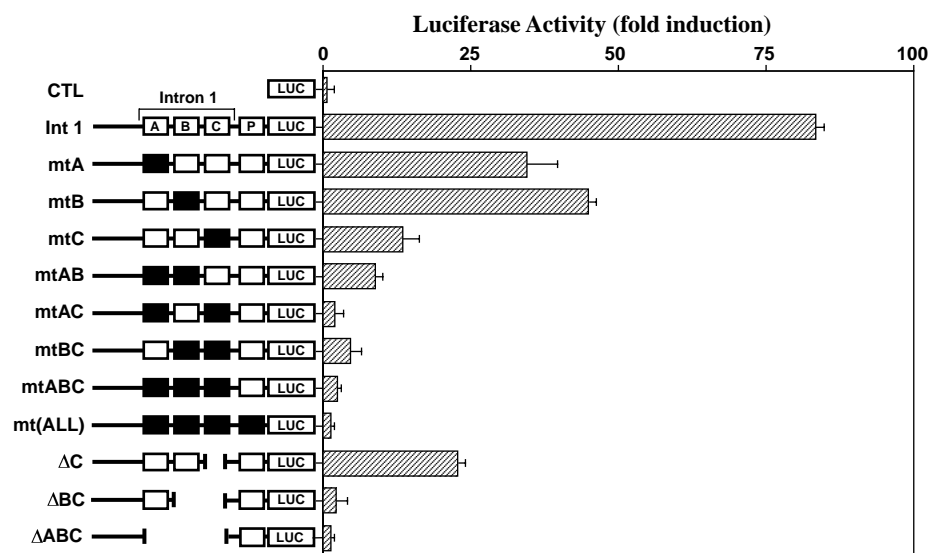
### Contribution of FAST sites to *Xnr1* intron 1 enhancer activity

To evaluate the contribution of the FAST sites to the activation of *Xnr1* expression by activin, we analyzed single or combined site-directed mutants, and deletion variants, within the Int 1-Prox/Luc context (Fig. 4). The less responsive Int 1- $\Delta$ Prox construct described above (Fig. 1B) lacks the 'P' FAST site, suggesting that, while the entire 1 kb promoter region itself is not activin-responsive, this site is involved in the full activin responsiveness of intron 1 in the context of the 1 kb 5' promoter region. Consistent with this possibility, mtP, a specific P site mutation within the Int 1-Prox/Luc context, showed similar activity to Int 1- $\Delta$ Prox (data not shown). Single site mutation or deletion (mtA, mtB, mtC, and  $\Delta$ C) caused activin responsiveness to be reduced to one-half or one-third of that of Int 1-Prox/Luc. Elimination of

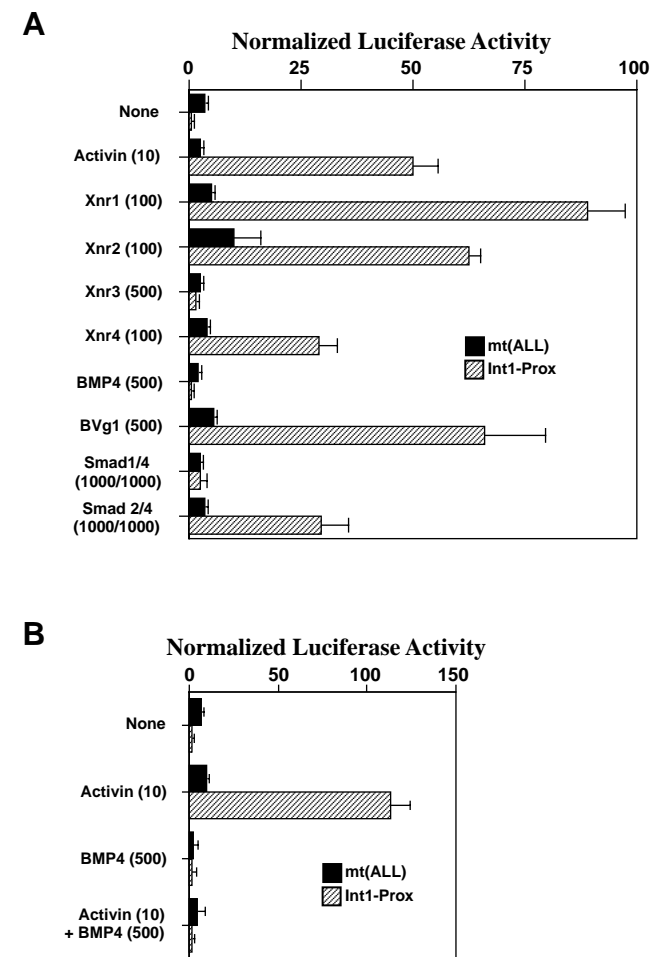
site C more profoundly reduced activin responsiveness than removing sites A or B, which may be related to the finding that the strongest gel shift band occurred with site C (Fig. 3A). Suppression was progressively augmented in mutants in which two (mtAB, mtAC, mtBC, and  $\Delta$ BC), three (mtABC and  $\Delta$ ABC), or all sites (mt(ALL)), were mutated or deleted. Thus, the number of FAST sites is a critical regulator of the enhancer activity of *Xnr1* intron 1.

### Specificity of *Xnr1* intron enhancer towards TGF $\beta$ -related factors

The specificity of the intron 1 ARE towards various TGF $\beta$  family members was examined (Fig. 5A). The Int 1-Prox/Luc or mt(ALL) luciferase constructs were coinjected with RNA



**Fig. 4.** FAST sites in intron 1 are essential for ARE activity. Black boxes indicate FAST sites (A, B, C or P) that are mutated. Fold induction was calculated as the ratio of the normalized luciferase activity of activin-injected embryos to uninjected ones. ARE activity decreased depending on the number of mutated FAST sites.



**Fig. 5.** *Xnr1* intron 1 activity in animal caps is induced by ‘dorsal’- and suppressed by ‘ventral’-type mesoderm inducers. (A) Int 1-Prox or mt (ALL) construct (see Fig. 4) were injected with indicated amounts (pg) of RNA(s) encoding various TGFβ ligands, Smad 1/2, or Smad 2/4. (B) Antagonism between activin and BMP4 signaling pathways. Int 1-Prox or mt (ALL) reporter activity was measured in response to *activin* and *BMP4* RNAs.

encoding various Xnrs, BVg1 (an active BMP-Vg1 chimera), or BMP4. Xnr1, Xnr2, Xnr4, and BVg1, which show dorsal mesoderm inducing activities in ectodermal explants, strongly activated Int 1-Prox/Luc, while BMP4 did not. Xnr3, which lacks mesoderm inducing activity and can act as a neural inducer (Hansen et al., 1997), did not activate Int 1-Prox/Luc. We also examined the effects of Smads, major mediators of TGFβ signaling. Smad1 and Smad2, which are implicated in ventral and dorsal mesoderm induction during *Xenopus* embryogenesis, respectively (Graff et al., 1996), make protein complexes with a common mediator, Smad4, and their nuclear accumulation activates transcription of the downstream targets of TGFβ signaling (Whitman, 1998). As expected, Smad2/4 coexpression mimicked the dorsal mesoderm inducers, and activated Int 1-Prox/Luc, while Smad1/4 did not. The failure of these dorsal mesoderm inducing molecules to activate mt (ALL) indicates the essential nature of the intron 1 FAST sites for enhancer activity. These results suggest that *Xnr1* intron 1 responds to

dorsal mesoderm inducing signals, including potential autoregulatory Xnr signals, but not ventral inducers.

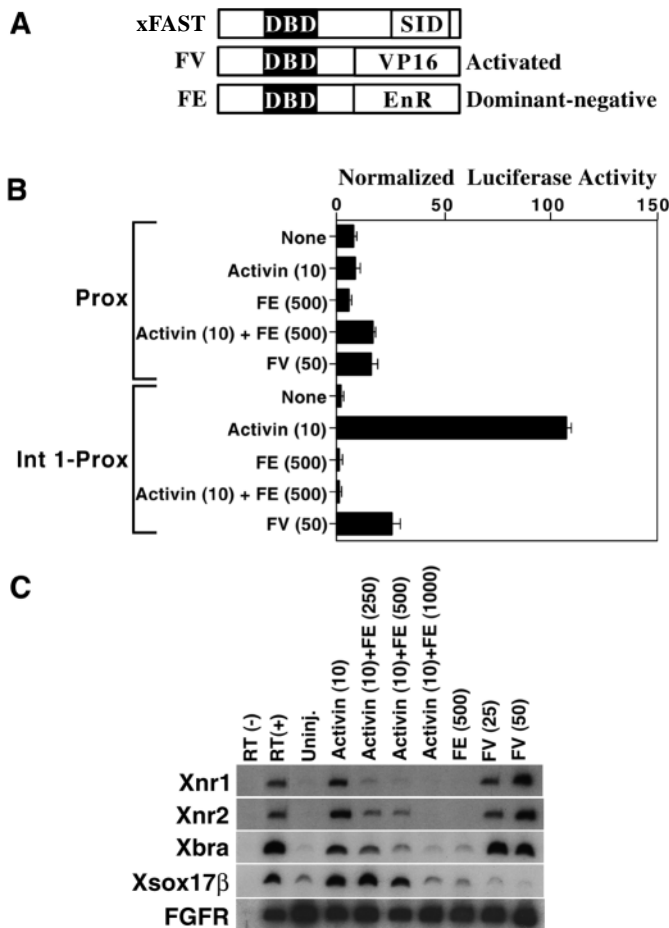
During gastrulation, *BMP* transcripts become excluded from the organizer region by antagonists secreted from this region (Harland and Gerhart, 1997). Thus, cells within the presumptive organizer region are likely to receive and interpret both dorsal and ventral signals, and modulate gene expression appropriately to adopt different dorsal fates. Antagonism between activin/Vg1 (dorsal) and BMP2/4 (ventral) signaling pathways has been reported on the *XFD-1*’ (Kaufmann et al., 1996) and *gsc* (Candia et al., 1997) promoters. We found that activation of Int 1-Prox/Luc expression by *activin* RNA was almost completely blocked by coexpression of BMP4 (Fig. 5B), indicating that the intronic enhancer in *Xnr1* can register an antagonistic relationship between activin/Vg1 and BMP signals.

We next tested more directly the involvement of xFAST in the activation of *Xnr1* intron 1 induction by activin, using dominant-negative (FE) or activated (FV) forms of xFAST, in which the DNA binding domain is fused to the engrailed repressor or VP16 activation domain, respectively (Fig. 6A; Watanabe and Whitman, 1999). Fig. 6B shows that FE alone did not activate Int 1-Prox/Luc and, when coexpressed with activin, could completely suppress activin-induced reporter expression. In contrast, FV alone mimicked the activation of the reporter construct by activin. Since FV did not activate Prox-Luc, we conclude that the functional FAST sites in intron 1 are necessary for the FV-mediated activation. In these experiments, the level of activation by FV was usually lower than that induced by *activin* RNA, perhaps reflecting the lack of a Smad interaction domain (SID) in FV. A previous report showed that FE suppressed mesendoderm induction by activin (Watanabe and Whitman, 1999). Consistent with this, FE dose-dependently suppressed the induction of the endogenous *Xnr1* and *Xnr2* genes by activin, while FV induced their expression (Fig. 6C). These results suggest that xFAST mediates activin-like signaling to Xnr and the potential autoregulation by Xnr signaling in mesendoderm induction.

### Localization of endogenous *Xnr1* activators

We attempted to map the location of endogenous *Xnr1* activators in the *Xenopus* embryo. Since *Xnr1* and *Xnr2* are initially expressed throughout the vegetal region at the blastula stage (Jones et al., 1995), we first tested whether delivering Int 1-Prox/Luc to the animal or vegetal region at the 1-cell stage resulted in differences in reporter activity at early gastrula (stage 10.5). Vegetal injections gave rise to higher activation than animal injections (approx. 5–24 fold depending on the batch of embryos and dose of Int 1-Prox/Luc reporter; data not shown). We next injected Int 1-Prox/Luc into blastomeres in each of the A–D tiers in 32-cell embryos, on the dorsal or ventral side, to locate endogenous *Xnr1* activators more precisely. Highest activity at stage 10.5 was reproducibly observed in tier C, with lower activity in tier D (Fig. 7B). In addition, we detected significant differences between dorsal and ventral injections at stage 10.5: activation in C1 blastomeres was up to sixfold that in C4 blastomeres (Fig. 7C). In contrast, mt (ALL) was not activated in tier C, but still significantly activated in tier D, indicating that tier C activation is primarily dependent upon functional xFAST sites. These results are consistent with the endogenous *Xnr1* expression



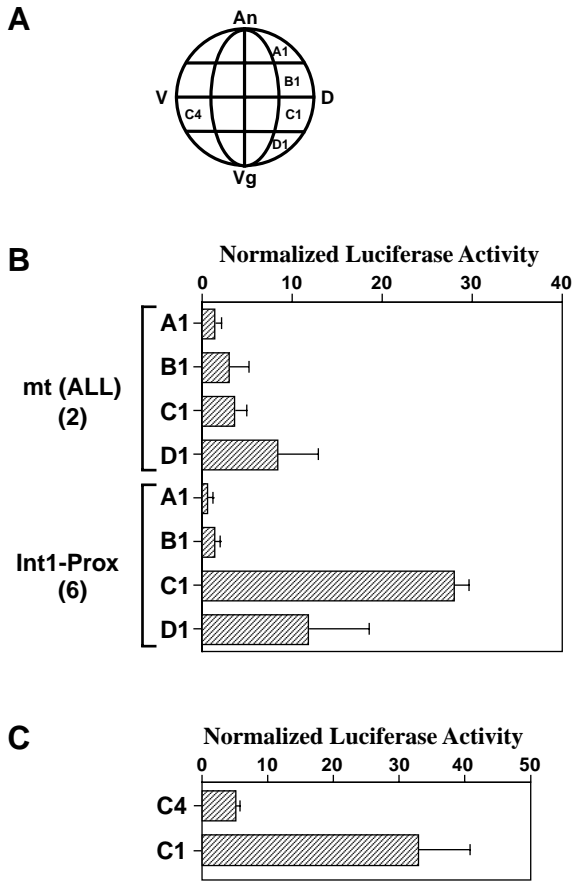


**Fig. 6.** A dominant-negative xFAST chimera abolishes activin responsiveness of *Xnr1* intron 1. (A) Activated (FV) and dominant-negative (FE) forms of xFAST represent fusions of VP16 activation or engrailed repressor domains (EnR) to the xFAST DNA-binding domain (DBD). SID, Smad-interacting domain. (B) FE abolishes activin responsiveness in *Xnr1* intron 1 (parentheses, pg RNA injected). (C) FE abolishes *Xnr1* and *Xnr2* induction by activin. Indicated amounts of RNA (pg) were injected animally into 1-cell embryos, and gene expression measured by RT-PCR in animal caps at stage 10.5. *Xbra*, a pan-mesodermal marker; *Xsox17β*, a pan-endodermal marker. RT (-) and RT (+) indicate whole embryo RNA transcribed without or with reverse transcriptase (RT).

pattern during embryogenesis: initial vegetal expression around late blastula, followed by expression in the equatorial region at gastrula with more prominent dorsal expression (Jones et al., 1995).

***Xnr1* intron 1-directed transgenes mimic mouse *nodal* expression patterns**

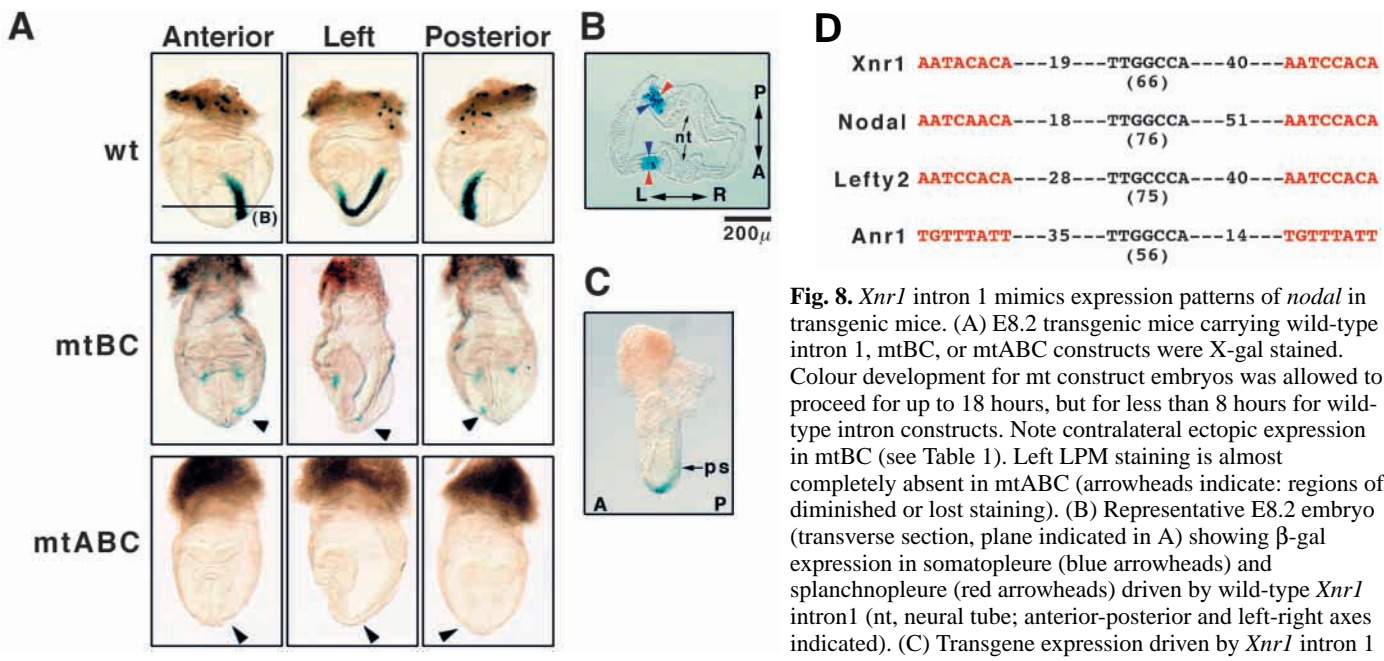
*Xnr1* is implicated in left-right asymmetric morphogenesis (Lowe et al., 1996; Sampath et al., 1997). Recently, *cis*-acting regions responsible for left side-specific expression in mouse *nodal* (Adachi et al., 1999; Norris and Robertson, 1999) and *lefty2* (Saijoh et al., 1999) were identified, and mFAST sites in these regions are essential for their asymmetric expression (Saijoh et al., 2000). We first tested whether *Xnr1* intron 1 could drive asymmetric reporter gene expression in frog embryos by non-transgenic transient expression assay. We



**Fig. 7.** Localization of endogenous *Xnr1* intron 1 activators. (A) Diagram of a *Xenopus* 32-cell stage embryo (An, Vg: animal, vegetal poles; D, V: dorsal, ventral). Subsequent luciferase assays were carried out at stage 10.5. (B) Two dorsal blastomeres of each tier received 100 pg (total) of mt(ALL) or Int 1-Prox/Luc plasmid (number of independent experiments showing this result indicated in parentheses). (C) C1 or C4 blastomeres were injected as above. Three independent experiments showed similar results.

injected 4-cell embryos with Int 1-Prox/Luc into locations fated to become the prospective left or right LPM, and assayed reporter activity at stage 19-20, when *Xnr1* is endogenously expressed in left LPM. Although we often detected up to approx. twofold higher activity in left side-injected embryos, considerable activity was also observed in right side injections.

Presumably, the earlier activation of intron 1 during mesendoderm induction and perdurance of luciferase protein may corrupt the study of L-R enhancer activity as described above. Moreover, chromosomal integration of the reporter construct (e.g. by using transgenic frogs in the future) may allow tighter control of enhancer activity. To test the cross-species conservation of enhancer function, we generated transgenic mice in which 'wild-type' or FAST-mutant *Xnr1* intron 1 enhancers were used to drive a minimal *hsp68* promoter and *lacZ* reporter. Expression domains were visualized by X-gal staining of 8.2 dpc embryos, as summarized in Table 1. In transgenics showing *lacZ* expression ( $n=3/7$  transgenic embryos), wild-type *Xnr1* intron 1 reproducibly directed robust left-sided LPM expression (Fig. 8A), which was confirmed by transverse sections (Fig. 8B).



(overnight colour reaction). A, anterior; P, posterior; PS, primitive streak. (D) Evolutionary conservation of a ‘Paired FAST’ module in intron 1 of vertebrates and ascidian *nodal*-related and *lefty* genes (FAST sites in red, nucleotide spacing indicated). Sites are reversed in *Anr1*; both fit the FAST binding consensus AAT(A/C)(A/C)ACA. Another conserved sequence, TTG(G/C)CCA, lies between the two FAST sites. *Xnr1*, frog; *nodal* and *lefty2*, mouse; *Anr1*, ascidian (*Molgula oculata*).

Ectopic or no expression was also observed, presumably due to effects of the individual transgene integration sites. Compared to the wild-type intron 1 transgenics, similar frequencies of transgenic embryos with asymmetric expression were seen for mtC and mtBC. However, mtC transgenics showed highly variable *lacZ* expression levels and increased ectopic expression, implying a requirement for the integrity of this site for proper spatiotemporal function of the enhancer. The intensity of asymmetric expression was greatly reduced in mtBC construct transgenics, which also tended to display ectopic expression and, finally, was almost completely eliminated in mtABC (note that colour development times for mtBC/mtABC embryos were longer than for the WT intron). Thus, an expression trend emerges based upon the number of FAST sites in the enhancer: site A may be partially functional in the mtBC construct, but sites B and C are essential for the normal asymmetric expression properties of the *Xnr1* intronic enhancer.

The mouse *nodal* asymmetric enhancer (ASE) also drives reporter expression to epiblast and visceral endoderm of gastrulating embryos (Adachi et al., 1999; Norris and Robertson, 1999). Endogenous *nodal* expression in the epiblast and visceral endoderm at 6.0 dpc becomes confined to the posterior side of the embryo at early streak stages (6.5 dpc), and then to a domain including the primitive streak at 7.5 dpc (Conlon et al., 1994; Varlet et al., 1997). We examined whether *Xnr1* intron 1 could recapitulate this pattern. No evidence was found of *Xnr1* intron 1-driven expression in the epiblast and visceral endoderm at 6.5 dpc (0/8 transgenic embryos). At 7.5 dpc, expression was seen in the primitive streak (Fig. 8C, 6/18 embryos), albeit weakly compared to the *nodal* ASE (Adachi et al., 1999; Norris and Robertson, 1999). Transverse sections showed that the staining was distributed around the posterior

**Table 1. Expression patterns of wild type or mutated *Xnr1* intron 1-driven transgene in mice**

	Asymmetric	Ectopic	No Exp	Total
wt	3 (1) +++	2	2	7
mtC	4 (3) + to +++	2	1	7
mtBC	4 (4) +	5	4	13
mtABC	2 (0) +/-	4	3	9

Asymmetric are transgenic embryos with left LPM expression; ectopic are embryos with expression in yolk sac or ectoplacental cone, but no expression in left LPM. Transgenic embryos with no X-gal staining are classified as ‘No Exp’. Numbers in bracket indicate embryos showing asymmetric expression in left LPM plus ectopic expression. Relative intensity of X-gal staining in the left LPM is indicated by the number of + symbols (+/-, very low expression in few cells). Note that the frequency of embryos with asymmetric expression decreased according to the number of mutated FAST sites.

midline, corresponding to primitive streak cells (data not shown). Primitive streak expression then declined before asymmetric expression began during early somitogenesis. The enhancer activity in the mouse primitive streak is consistent with its role in driving *Xnr1* expression during frog gastrulation. Together, these data suggest that several expression properties of the mouse *nodal* ASE are mimicked by the *Xnr1* intronic enhancer.

Conservation of a DNA module consisting of two similarly spaced FAST sites between vertebrate *nodal*-related genes (*nodal* and *Xnr1*) and *lefty2* (Fig. 8D) prompted us to examine whether this module exists in vertebrate ancestors. We chose the ascidian *Molgula oculata*, a chordate, from which we recently isolated a *nodal*-related gene, *Anr1*, displaying bilateral expression during gastrulation and transient asymmetric expression during tadpole stages (B. Swalla, personal communication). Based upon the conserved intron 1



location in frog, mouse, and zebrafish *nodal* genes (not shown), we PCR-amplified a 520 bp intron sequence from a genomic DNA library. Two sequences in the 3' region of the intron match the FAST consensus perfectly (Fig. 8D), and are spaced similar to those in *Xnr1* intron 1. Comparisons of the intronic enhancers between vertebrate and ascidian *nodal* genes, and mouse *lefty2* (Fig. 8D; Saijoh et al., 2000) suggest that the core cassette controlling gastrulation and left-side activity consists of paired FAST sites with conserved spacing. Thus, direct or indirect interactions between FAST monomers may be critical for enhancer function. In this regard, we speculate that another conserved sequence located between the FAST sites (TTG(G/C)CCA) may act as an additional platform for recruiting 'bridging factors' or cooperatively acting transcriptional modulators.

## DISCUSSION

In studies concurrent with those presented here, Saijoh et al. (2000) used yeast one-hybrid screening to identify FAST as an essential mediator of the asymmetric activation of enhancer elements shared between mouse *nodal* and *lefty2*, and showed evidence for FAST-dependent *nodal* autoregulation. Our studies on frog *Xnr1* confirm and extend these observations. Comparisons of the required sequences between species allows the definition of a 'paired FAST site' motif as an essential evolutionarily conserved component of an enhancer acting as a fundamental regulator of *nodal*-related expression in two crucial embryonic patterning events: mesendoderm induction and L-R axis specification. Moreover, the finding of a FAST regulatory module in an ascidian *nodal*-related gene suggests an ancestral linkage of this cassette to both patterning processes. Furthermore, we show that the intronic *Xnr1* enhancer can also register BMP repression, and we have functionally linked the enhancer activity to the spatiotemporally restricted pattern of *Xnr1* expression during gastrulation stages in *Xenopus* embryogenesis. Thus, together with the extensive analyses of mouse *nodal* (Adachi et al., 1999; Norris and Robertson, 1999), we are now gaining insights into the mechanisms regulating what seem to be emerging as vital signaling factors in all vertebrates.

### Positive and negative regulation of *Xnr1*

*Xnr1* expression during early *Xenopus* embryogenesis is dynamic. Expression is initiated broadly across the vegetal region of the blastula (Jones et al., 1995), with some dorsal enhancement. At early gastrulation, vegetal expression decreases and *Xnr1* expression primarily occurs equatorially with a dorsal bias (Jones et al., 1995). In both of these phases, *Xnr1* expression overlaps widely with that of *Xnr2*, which has similar inducing properties (Jones et al., 1995). After a period of post-gastrulation inactivity, transient *Xnr1* expression occurs in the left LPM at late neurula/tailbud stages (Lowe et al., 1996).

Our mapping of the spatial activation profile of the *Xnr1* intronic enhancer at gastrulation (Fig. 7), and the presence of VegT and FAST sites in Int1-Prox-luciferase construct is broadly consistent with the idea that *Xnr1* expression is initiated by VegT and subsequently maintained/upregulated in equatorial regions via intercellular TGF $\beta$ -related signaling

(Agius et al., 2000; Clements et al., 1999; Hyde and Old, 2000; Kimelman and Griffin, 1998; Kofron et al., 1999; Yasuo and Lemaire, 1999; Zorn et al., 1999). While wild-type and mt(ALL) enhancer constructs were both activated in tier D derivatives, mt(ALL) does not respond to BVg1, *Xnr* or activin. Thus, a substantial fraction of this vegetal activity represents non-FAST-dependent activation, probably involving VegT (Hyde and Old, 2000; Kofron et al., 1999), although additional factors may also be involved. Our finding that the highest FAST site-dependent intron 1 enhancer activity occurred in tier C derivatives is consistent with the proposal that FAST-dependent mechanisms are a major regulator of the endogenous equatorial *Xnr1* expression at gastrulation stages.

The strong ARE in *Xnr1* helps to explain the induction of *Xnr* expression by activin-like signals reported previously (Jones et al., 1995; Osada and Wright, 1999). Here, we extended this observation by showing that the induction of both *Xnr1*-driven reporter genes and endogenous *Xnr* genes was blocked by specific interference with xFAST activity (Fig. 6). The similar response of the intronic ARE to activin, BVg1 and *Xnr* (Fig. 5A) – all dose-dependent dorsal-type mesoderm inducers – could mean that FAST-dependent *Xnr1* transcription is activated by several ligands in vivo, including activin or Vg1. As described in the Introduction, there are difficulties establishing a requirement for activin in frog mesendoderm induction. On the other hand, one explanation for the defects caused by dominant negative Vg1 ligands in *Xenopus* embryos (Joseph and Melton, 1998) could be that Vg1 signaling is involved in activating *Xnr* expression. We propose that the evidence for a *nodal* autoregulatory loop (Meno et al., 1999; Saijoh et al., 2000; and this work), and a conserved requirement for Nodal signaling in early vertebrate embryogenesis, strongly suggests that the FAST-dependent ARE defined here actually functions as a Nodal response element, or 'NRE'. It will be important to determine if the mechanisms regulating *Xnr1* are analogous in *Xnr2*, which has similar inducing activities and early expression to *Xnr1*, but is not asymmetrically expressed at later stages.

In many developmental processes, refinement of cell fate specification is achieved by a balance between positive and negative regulatory signals, and some form of antagonism may be critical to prevent excessive *Xnr/nodal* signaling by breaking the autoregulatory loop. BMPs are good candidates for negative regulatory signals. We showed that BMP signaling negatively modulates intron 1 activity (Fig. 5B). Endogenous BMPs, which are themselves antagonized by the dorsally secreted factors chordin and noggin, and Wnt-signaling (Baker et al., 1999), are therefore likely to influence the overall level of *Xnr1* transcription in the embryo and, consequently, the instruction of mesendodermal cell fates. Since Smad4 is a component of ARF, the activin/BMP4 antagonism on *Xnr1* intron 1 could occur via titration of the Smad4 co-activator between 'dorsal' and 'ventral' signaling pathways, as proposed for the *gsc* promoter (Candia et al., 1997). However, BMPs could induce a variety of as yet unknown cross-regulators that antagonize the intron enhancer activity. Further experimentation, including additional regulatory element mapping within the enhancer will help to address the mechanism of BMP antagonism. In addition, the *lefty*/antivin factors, whose expression is induced by *Xnr/nodal* signaling, have been also proposed to act as critical negative feedback

inhibitors of nodal/Xnr signaling (Cheng et al., 2000; Meno et al., 1999). Further issues to be addressed include how these negative regulatory signals regulate 'NRE' activity.

Based upon our findings, we hypothesize that the intron 1 enhancer represents a simple integrating cassette through which multiple signals converge to affect *Xnr1* expression, allowing precise and rapid adjustments of *Xnr1* expression. This fine tuning of *Xnr1* expression could affect the level of *Xnr* signaling, and the induction of different mesendodermal fates: low levels activating, for example, pan-mesodermal genes like *Xbra*, while increasing levels progressively induce more dorsal mesodermal fates (e.g. muscle-specific *actin* and *gsc*) and, at the highest levels, endodermal fates (Henry et al., 1996; Osada and Wright, 1999).

### L-R Specification and Intron 1 enhancer function

The activity of *Xnr1* intron 1 as a FAST-dependent asymmetric enhancer during mouse somitogenesis (Fig. 8A) mimics the intronic asymmetric enhancer (ASE) of mouse *nodal* (Adachi et al., 1999; Norris and Robertson, 1999; Saijoh et al., 2000). The mouse *nodal* ASE also drives expression during gastrulation (Adachi et al., 1999; Norris and Robertson, 1999). We find that the *Xnr1* intron drives similar expression in the primitive streak of transgenic mice (Fig. 8B) but, unlike the nodal ASE, may not direct expression in the epiblast and visceral endoderm at 5.5–6.5 dpc. More work is required to determine if this is a species-specific difference, or if analysis of transgenic lines, rather than F<sub>0</sub> embryos, will reveal greater similarities with the mouse enhancer. Nevertheless, we conclude that the FAST-dependent enhancer plays a conserved role in gastrula stage expression and later asymmetric expression of *nodal* genes in vertebrates. The presence of a similar cassette in an ascidian *nodal* gene supports and extends this idea. The bilateral expression of *Anr1* during gastrulation and later transient left-sided expression (B. Swalla, personal communication) is strikingly reminiscent of vertebrate *nodal* gene expression. Thus, studies in chordates and more primitive organisms may provide insight into the core regulatory mechanisms regulating *nodal* expression during both phases. It will also be interesting to determine whether the xFAST intronic enhancer functioned first in non-asymmetric mesendoderm induction, being co-opted for L-R determination early in chordate/vertebrate evolution, or if both processes have been intimately linked and co-evolved over a longer period.

### Asymmetric *nodal* activation by BMP derepression

FAST-mediated *Xnr/nodal* autoregulation could contribute to the spreading of *nodal* expression through the left LPM during early somitogenesis, perhaps initiated by Nodal produced at the node, at least in species for which there is evidence of asymmetric expression in this structure (Collignon et al., 1996; Levin et al., 1995; Saijoh et al., 2000). It is hard, however, to link the broad bilateral expression of xFAST/mFAST during these stages of embryogenesis (Chen et al., 1996; Weisberg et al., 1998) to the left-sided activation of *nodal* expression. Recent studies in chick embryos suggest that Caronte, a left-side expressed Cerberus/Dan-related secreted factor, may activate *nodal* by antagonizing bilaterally expressed BMPs (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999; Zhu et al., 1999). Our observation that activin/BMP antagonism can be efficiently registered via the *Xnr1* intron 1 FAST-regulated

enhancer provides, in principle, a simple underpinning for this derepression. Thus, it is possible that progressive release from BMP-mediated repression, via a FAST/Smad-dependent intronic enhancer, is a common mechanism of activating/upregulating *nodal* expression during both mesendoderm induction and left-right axis specification.

### Differential action of FAST sites on *Xnr1* intron 1 enhancer activity

The three FAST sites in intron 1 have different contributions to reporter gene expression in vivo (Figs 3, 4). The finding that the 3'-most FAST site (site C) is most effective in Xnr/activin induction assays is consistent with transgenic data (Saijoh et al., 2000) showing that mutation of the corresponding site in *nodal* and *lefty2* greatly reduces asymmetric enhancer activity. Our assays of asymmetric *Xnr1* intron 1 enhancer activity in transgenic mice (Table 1) mostly agree with those on the *nodal* ASE. Generally, *Xnr1* left-sided enhancer activity depends on the number of FAST sites present. The relative imprecision in quantitating *lacZ* expression levels in whole mounts, plus the variability in transgene position effect and copy number between founder embryos, make it difficult to conclude precisely the relative role of individual FAST sites between mouse and frog enhancers. Nevertheless, the finding that mutating the paired FAST sites B and C severely debilitates the *Xnr1* enhancer underscores the conserved role of this regulatory module. Future issues to be addressed include the basis for the functional difference between the various FAST sites: whether the proximity, quality, and relative orientations of Smad motifs are contributing factors (Johnson et al., 1999; Labbe et al., 1998; Yeo et al., 1999), and if the FAST sequence itself or the surrounding context regulates ARF affinity and the assembly of transcriptional activation complexes.

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