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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Consistent with a crucial role of activin-like molecules in mesendoderm induction, activin response elements (AREs) have been directly investigated. ARF was first identified as a factor binding to an ARE in the *Mix.2* promoter in response to Vg1, TGFβ 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.

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 *Xnr* 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*, TGFβ

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-β (TGFβ) 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 has been directly investigated.

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 *goosecoid* (*gsc*; Watabe et al., 1995), *Mix.2* (Huang et al., 1995), *HNF1α* (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, TGFβ 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 Thise, 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 enhances 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 λDASH (gift from Eddy De Robertis; λD2 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 animal 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; Osaka 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 1x 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 Berthold 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’-ATTCAAGAGCTCTTGAAGCGGCCTGCAGAAATCTCCTGCTGACGACGACTACTTAACC-3’) contained HindIII, NotI, PsI, and EcoRI sites (underlined). Downstream primer: 5’-ATTCAAGAGCTCTTGAAGCGGCCTGCAGAAATCTCCTTCCA-3’ (HindIII site underlined). Sequenced to demonstrate that PCR amplification with Phu 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 PsI-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 11-ΔProx.

Reporter plasmids with the 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’-CTGTCTCATTTAAAGTTGTTCGTTATGGTTTCTG; mB, 5’-CCAAGCTGAAGTCTATAAAATATCGTGTTTTTG-3’; mC, 5’-CTATAAACTCCTAATCTAATTGTCGAGTGA-AC; mP 5’-CATGACTCATAATTCTGTATACAAATATG-AGAACC-3’. The oligonucleotides were used successively to eliminate multiple sites in mTA, mB, mC, mBC, and m(ALL). Primers used were: upstream, 5’-ACAAACACTGAAATAAGGCCT-3’; downstream, 5’-GGGAATTCAGTTGAGTTGGTTG-3’ (for ABC), 5’-CGGAATTCAGTTGAGTTGGTTG-3’ (for ΔABC), 5’-GGGAATTCAGTTGAGTTGGTTG-3’ (for ΔAC), and 5’-GGGAATTCAGTTGAGTTGGTTG-3’ (for ΔABC). PCR products were EcoRI-digested and inserted into Prox/Luc. Construction of activated (FV) and dominant-negative (Fe) forms of the Xnr1 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 into 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

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 32P end labeled with T4 polynucleotide kinase. PCR primers: for Xnr1-A and Xnr1-mA, 5’-TAAATAACCCAAACATCTC-3’ and 5’-TATAGATACAGAAGACTAAAACCA-3’; for Xnr1-B and Xnr1-mB, 5’-AACCTTTGTCTTTGCTATCT-3’ and 5’-TCATCTTGTGCACCTTTG-3’; for Xnr1-C and Xnr1-mC, 5’-TAAAGACAAATTACTGTG-3’ and 5’-TTGAGGGCTGTTGTTGTTAATT-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 Smal 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) F1 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’-TCCTAAGAATGACGTACGAGTGGTCTGAC; downstream, 5’-CCTTGCACCATGAGTAAAGGC.
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 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(AC)(CA)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 (GTCT or AGAC) are in bold. 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.

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![Diagram](image1.png)

**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 co-injected 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 un.injected 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).
Regulation of Xnr1 by FAST-1

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, which recognizes the Smad-interacting domain of xFAST, 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-ΔProx 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-ΔProx (data not shown). Single site mutation or deletion (mtA, mtB, mtC, and ΔC) 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 ΔABC), three (mtABC and ΔABC), 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 TGFβ-related factors

The specificity of the intron 1 ARE towards various TGFβ family members was examined (Fig. 5A). The Int 1-Prox/Luc or mt (ALL) luciferase constructs were coinjected with RNA
response to pathways. Int 1-Prox or mt (ALL) reporter activity was measured in or Smad 2/4. (B) Antagonism between activin and BMP4 signaling

mediators of TGFβ signaling. Smad1 and Smad2, which are essential nature of the intron 1 FAST sites for enhancer indicating 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 RNAs, 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.

Localisation 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 vegetal 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 vegetal 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 encoding various Xnrs, BVg1 (an active BMP-Vg1 chimera), or BMP4. Xnr1, Xnr2, Xnr4, and BVg1, which show dorsal mesendoderm 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 mesendoderm 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 mesendoderm inducers, and activated Int 1-Prox/Luc, while Smad1/4 did not. The failure of these dorsal mesendoderm 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 mesendoderm 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.

![Figure 5. Xnr1 intron 1 activity in animal caps is induced by 'dorsal'- and suppressed by 'ventral'-type mesendoderm 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.](image-url)
Regulation of Xnr1 by FAST-1 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 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).
Ectopic or no expression was also observed, presumably due to effects of the individual transgene integration sites. Compared to the wild-type intron 1 transgens, similar frequencies of transgenic embryos with asymmetric expression were seen for mtC and mtBC. However, mtC transgens 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 transgens, 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 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

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

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<th>Asymmetric</th>
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<td>wt</td>
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<td>2</td>
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<td>7</td>
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<td>mtC</td>
<td>4 (3) + to +++</td>
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<td>4 (4) +</td>
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<td>mtABC</td>
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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.
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; Sajioh 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, Sajioh 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 Int1-1-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 TGFβ-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; Osaka 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; Sajioh 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 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.

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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; Sajioh 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 F2 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 Xnr1 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; Sajioh et al., 2000). It is hard, however, to link the broad bilateral expression of Xnr1 FAST enhancer 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 (Sajioh 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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