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FAST-1 is a key maternal effector of mesoderm inducers in the early *Xenopus* embryo

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SUMMARY

We have examined the role of the maternally encoded transcription factor FAST-1 in the establishment of the mesodermal transcriptional program in *Xenopus* embryos. FAST-1 has been shown to associate with Smad2 and Smad4, transducers of TGFβ superfamily signals, in response to stimulation by several TGFβ superfamily ligands. The FAST-1/Smad2/Smad4 complex binds and activates a 50 bp activin responsive element identified in the promoter of the meso-endodermal marker *Mix.2*. We have now used three complementary approaches to demonstrate that FAST-1 is a central regulator of mesoderm induction by ectopic TGFβ superfamily ligands and during endogenous patterning: ectopic expression of mutationally activated FAST-1, ectopic expression of dominant inhibitory FAST-1, and injection of a blocking antibody specific for FAST-1.

Expression of constitutively transcriptionally active FAST-1 fusion protein (FAST-VP16^{A}) in prospective ectoderm can directly induce the same set of general and dorsal mesodermal genes, as well as some endodermal genes, as are induced by activin or Vg1. In intact embryos, this construct can induce secondary axes similar to those induced by activin or Vg1. Conversely, expression of a FAST-1-repressor fusion (FAST-En^{R}) in prospective ectoderm blocks induction of mesodermal genes by activin, while expression of FAST-En^{R} in intact embryos prevents general/dorsal mesodermal gene expression and axial development. Injection of a blocking antibody specific for FAST-1 prevents induction of mesodermal response genes by activin or Vg1, but not by FGF. In intact embryos, this antibody can prevent the expression of early mesodermal markers and inhibit axis formation, demonstrating that FAST-1 is a necessary component of the first steps in the specification of mesoderm.

Key words: FAST-1, Smad, Mesoderm induction, *Xenopus*, Signal transduction, Transcriptional regulation

INTRODUCTION

In the early amphibian embryo, the mesoderm is thought to be established in the equatorial region of the embryo by signals from the underlying endoderm. This induction, in combination with subsequent patterning signals from the Spemann organizer, defines the dorsoventral and anteroposterior pattern of the mesodermal components of the body axis (Conlon et al., 1996a; Cornell and Kimelman, 1994; Harland and Gerhart, 1997; Heasman, 1997). A number of extracellular factors have been identified as candidate signaling molecules in this process (see above reviews); only recently has the molecular nature of these signals begun to be understood. Several members of the TGFβ superfamily of factors, including activin, Vg1, and the nodal related proteins Xnr1 and Xnr2, can potently induce dorsal mesodermal markers and tissues when ectopically expressed or activated either in prospective ectoderm (animal caps) or prospective ventral mesoderm (ventral marginal zone) (Dale et al., 1993; Jones et al., 1995; Kessler and Melton, 1995; Lustig et al., 1996a; Smith et al., 1990; Thomsen and Melton, 1993; Thomsen et al., 1990). Generalized inhibition of TGFβ superfamily signaling in the early embryo by expression of a dominant negative type II activin receptor can prevent formation of mesodermal tissues and axes in the embryo, supporting a central role for TGFβ-like factors in mesoderm induction (Hemmati-Brivanlou and Melton, 1992).

Transcriptional regulation by TGFβ superfamily factors begins with activation of heteromeric complexes of serine-threonine kinase receptors on the plasma membrane, which in turn phosphorylate and activate a novel class of signal transducers known as Smads (Derynck and Feng, 1997; Heldin et al., 1997; Massagué, 1998; Whitman, 1998). In the case of activin, binding of a ligand-specific receptor (ActRIIB) leads to dimerization with and activation of a signaling receptor (ActRIB), which in turn phosphorylates the signal transducer Smad2. Phosphorylated Smad2 heterodimerizes with Smad4; this complex then translocates to the nucleus (Lagna et al., 1996; Macias-Silva et al., 1996; Zhang et al., 1996, 1997). For other TGFβ superfamily ligands, a similar signaling pathway is mediated by distinct receptors and receptor-phosphorylated Smads (Derynck and Feng, 1997; Heldin et al., 1997; Massagué, 1998).

How Smads regulate specific transcriptional responses is not completely understood (Whitman, 1998). Smad1, Smad2 and Smad4 contain intrinsic transcriptional activation domains (Liu et al., 1996), and may also interact with a variety of co-activator molecules (Feng et al., 1998; Janknecht et al., 1998; Pouponnot et al., 1998; Shioda et al., 1998). In addition, *Drosophila* MAD, Smad3 and Smad4 contain intrinsic, site-specific DNA-binding activity (Dennler et al., 1998; Kim et al., 1997; Yingling et al., 1997; Zawel et al., 1998). It is not clear, however, in which cases Smad DNA binding is either necessary or sufficient for TGFβ superfamily stimulated responses. In the case of the activin early response gene *Mix.2*, a 50 bp activin responsive element (ARE) has been identified in the promoter of the *Mix.2* gene that is necessary and sufficient to confer activin responsiveness on a reporter (Huang et al., 1995; Vize, 1996). The ARE is bound by a complex of Smad2, Smad4 and a maternally expressed forkhead domain DNA-binding protein, FAST-1 (Chen et al., 1996, 1997; Huang et al., 1995). Reconstitution of this complex in tissue culture cells by ectopic expression of FAST-1 is sufficient to confer activin/TGFβ induction of the ARE, indicating that the Smad-FAST complex is a positive regulator of the ARE (Hayashi et al., 1997; Liu et al., 1997). The DNA-binding activities of both FAST-1 and Smad4 recognize the ARE, but only DNA binding by FAST-1 is required for ARF binding or for transcriptional regulation by activin (Yeo et al., 1999). These observations suggest a fairly simple model for at least one component of activin/Smad regulation of the *Mix.2* gene: activated Smad2/Smad4 translocates to the nucleus and is directed to an early embryo-specific promoter response element through association with FAST-1 (Chen et al., 1997).

Over the past 10 years, a variety of early transcriptional responses to mesoderm inducers in the *Xenopus* embryo have been described. Many of these early response genes are transcription factors that when overexpressed can mimic or modulate aspects of mesoderm induction or patterning, indicating that they are functionally important determinants of the response to mesoderm inducers (Conlon et al., 1996a). Mesoderm-specific transcriptional responses can be induced as soon as zygotic gene transcription begins at the mid-blastula transition and therefore these responses must be regulated by maternally encoded transducers and transcription factors. The identification of FAST-1 as a maternally encoded transcription factor coupling Smad2 activation to regulation of an element in the *Mix.2* gene raises the question of whether it may be a more generalized temporal determinant of mesodermal transcriptional responses to TGFβ superfamily inducers in early embryos.

In the present study, we have used FAST-1 activator- and repressor-domain fusions and blocking antibodies specific for FAST-1 to examine the role of FAST-1 in the early specification of mesoderm. Expression of the activator-domain fusion induces the expression of not only *Mix.2*, but also a broad set of mesodermal genes that are induced by activin or Vg1. This constitutively active FAST-1 can, like activin or Vg1 (Dale et al., 1993; Thomsen et al., 1990), induce partial secondary axis formation in intact embryos. Conversely, expression of repressor-domain fusion blocks mesodermal gene expression in animal cap explants and blocks axis formation in embryos. Moreover, injection of anti-FAST-1-specific antibody blocks mesodermal gene expression induced by activin, but not gene expression induced by FGF. Anti-FAST-1 also blocks mesodermal gene expression and axis formation in embryos. We propose that FAST-1 is a critical maternal determinant of dorsal mesodermal specification by TGFβ superfamily factors.

MATERIALS AND METHODS

Embryos and animal cap assay

Eggs obtained from female frogs were artificially fertilized by using testis homogenates and dejellied with 3% cysteine (pH 7.8-8.0) 30 minutes after fertilization, followed by several 0.1× MMR (1× MMR: 100 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes (pH 7.4)) washes. Staging of embryos was according to Nieuwkoop and Faber (1994). Embryos were injected in 3% Ficoll/1× MMR with 5-10 nl RNAs per blastomere and the medium was diluted to 3- to 4-fold by adding water at 1 hour after injection. At stage 9, embryos were transferred to 0.1× MMR containing 50 μg/ml gentamicin (Gibco BRL) for long-term culture. For animal cap assay, animal cap cells were cut at stage 8.5-9 and transferred to 0.7× MMR containing 100 μg/ml BSA and 0.1% gelatin. Purified human activin protein (a gift of Dr Y. Eto, Ajinomoto Inc.) or recombinant human bFGF (Gibco BRL) was added to the medium at 200 pM or 100 ng/ml, respectively. Cycloheximide (Sigma, at final 5 ng/ml) was added to the medium to block protein synthesis throughout the experiments for the early markers or for 1 hour for the late marker described in Fig. 3. For the activation of FAST-VP16^{A}-GR protein, dexamethasone (Sigma) was added to the medium at 10 μM.

In vitro transcription, RNA extraction and RT-PCR

Capped RNAs were synthesized in vitro using SP6 polymerase (Promega; Krieg and Melton, 1987) or the Megascript RNA transcription system (Ambion). Total RNAs were extracted from animal cap explants or whole embryos by the proteinase K/phenol method (LaBonne and Whitman, 1994). Different numbers of amplification cycles were used for different primers/targets to maintain amplification in the linear range (not shown). The primer sets and cycles used for PCR amplification were:

Mix.2 (25 cycles, Vize, 1996)
U:5′-TGCCCCTAACAGCTCCTCATACCAA-3′,
D:5′-TGGCCGGCAAACAAACTCAT-3′.
Xlim-1 (25 cycles, Taira et al., 1992)
U:5′-GTTCGTCTGCAAAGAAGA-3′,
D:5′-GAGCGCTCAGCTGTTTCA-3′.
edd (23 cycles, Sasai et al., 1996),
U:5′-ATATAACGTTCCCCACCCCAAAGA-3′,
D:5′-GAATGTCCTGCTCCACCAAGAAAC-3′.
XFKH-1 (25 cycles, Dirksen and Jamrich, 1992: Knochel et al., 1992)
U:5′-ACCACCTTGTCCACTACTCTAAC-3′,
D:5′-CGCTGCCTGTCAAAAACAAT-3′.

Cer (25 cycles, Bouwmeester et al., 1996), globin (20 cycles, Graff et al., 1996), epidermal keratin (20 cycles, Wilson and Hemmati-Brivanlou, 1995), *Xombi* (23 cycles, Lustig et al., 1996b), *Chd* (25 cycles, Suzuki et al., 1997a), *Xvent-1* (23 cycles, Gawantka et al., 1995), *Xmsx.1* (23 cycles, Maeda et al., 1997), *Xsox17α* and *Xsox17β* (25 and 23 cycles, respectively, Hudson et al., 1997), *Xlhbox8* and IFABP (25 cycles, Henry et al., 1996), *Xbra*, *Gsc* and *Xwnt8* (25 cycles, Hemmati-Brivanlou et al., 1994), and m-actin and EF1α (20 cycles, Hemmati-Brivanlou and Melton, 1994). PCR conditions were described in LaBonne and Whitman (1994).

Immunoblotting, immunoprecipitation and gel shift assay

Proteins from RNA-injected embryos were separated in 10% acrylamide gel and transferred to nitrocellulose filter and incubated with 5% non-fat skim milk in 1× PBST (137 mM NaCl, 2.68 mM

KCl, 1.47 mM KH_2PO_4, 8.06 mM $Na_2HPO_4.7H_2O$, 0.05% Tween 20) to block nonspecific binding of antibodies. Anti-myc antibody (9E10) and affinity-purified anti-FAST antibody were used for immunoblotting and signals were detected by Amersham ECL kit. For the immunoprecipitation, embryonic proteins were labeled by injecting 20 nl of 800 Ci/mmol [^{35}S]methionine at the 2-cell stage. At blastula stage, embryos were homogenized with lysis buffer (10 μl/embryo, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10% glycerol, 1 mM DTT, 1 μM PMSF, 40 μg/ml leupeptin, 20 μg/ml aprotinin). The lysates was centrifuged twice and the supernatant was incubated with antibody for 1 hour at 4°C, followed by 1 hour incubation with Protein A Sepharose (PAS, Phamacia). After brief centrifugation, PAS-bound material was washed with: lysis buffer, lysis buffer containing 10% NP40, lysis buffer containing 1 M NaCl, and lysis buffer again, and boiled 5 minutes with SDS buffer (10% glycerol, 5% β-mercaptoethanol, 2.3% SDS, 62.5 mM Tris-HCl (pH 6.8)), and separated on a 10% acrylamide gel. Gel mobility shift assay was done according to Huang et al. (1995) using a ^{32}P-labeled ARE probe.

In situ hybridization

In situ hybridization was performed essentially as described (Harland, 1991) using albino embryos, except the removal of vitelline membrane, and RNase treatment were omitted and BM purple (Boehringer Mannheim) was used as a substrate. Antisense *Xbra* template (from D. Melton) was linearized with *Xho*I and transcribed with SP6 polymerase using digoxigenin-labeled UTP (Boehringer Mannheim).

Luciferase assay

A *Mix.2*-luciferase reporter construct was generated by subcloning the 2.5 kb promoter sequence from the *Mix.2* gene (Vize, 1996) into pGL3 basic vector (Promega), and a EF1α-luciferase was by 0.45 kb promoter/enhancer sequence from the pXeX vector (Johnson and Krieg, 1994) into pRL vector (Promega). Luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega).

β-gal staining

Embryos injected with RNA encoding nuclear β-galactosidase were fixed by MEMFA (0.1 M MOPS (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$ and 3.7% formaldehyde) for 15 minutes, washed by PBS containing 2 mM $MgCl_2$ and stained in PBS containing 0.5 mg/ml Magenta X-gal (ICN), 20 mM $K_3Fe(CN)_6$, 20 mM $K_4Fe(CN)_6.3H_2O$, 2 mM $MgCl_2$, 0.01% sodium deoxycholate and 0.02% NP-40 at room temperature.

Anti-FAST-1 antibody production

A C-terminal portion of the FAST-1 protein (366-518aa) was fused to glutathione S-transferase (GST) (Chen et al., 1997), affinity-purified from bacterial extracts and used for injection of rabbits (CoCalico). Anti-FAST antibody was purified from serum using Ultralink beads (Pierce) coupled with GST-FAST-1(366-518aa) according to the manufacture's protocol. Preimmune serum was carried through an identical purification protocol in parallel with non-immune serum. Anti-GST antibodies were co-purified in this procedure, and these antibodies were removed by a GST-column. The resultant anti-FAST antibody was used for western blotting (1:10,000 dilution), immunoprecipitation (undiluted, 1 μl/reaction), gel-shift assay (undiluted, 1 μl/reaction) and microinjection (undiluted, 20-40 nl/embryo).

Plasmid construction

FAST-VP16^A: the forkhead DNA-binding domain containing fragment (61-365aa) of FAST-1 was excised from pCSMT-FAST-1 (Chen et al., 1997) by *Hin*dIII-*Msc*I digestion, and subcloned into the pCSVP16 vector (provided by Adrian Salic) digested by *Hin*dIII-*Bst*EI-filled. FAST-En^R: the same fragment was subcloned into pCSEn vector (from Ralph AW Rupp) digested by *Hin*dIII-*Cla*I-filled. FAST-VP16^A-GR: FAST-VP16^A fragment was amplified from pCSMT-FAST-VP16^A by PCR using CS2 primer (5′-AAGCAATGCTCGTTTAGG-3′) and FAST-VP16^A primer (5′-TCTCTCGAGCCCACCGTACTCGTC-3′). The amplified product was digested with *Bam*HI-*Xho*I and subcloned into pCSGRZ vector (provided by David L. Turner) digested by the same enzymes. FAST-VP16^A(L169F) and FAST-En^R(L169F) were generated by PCR-based mutagenesis of their respective parent constructs. All these FAST-1 constructs were linearized by *Sac*II for in vitro transcription. ActivinβB, BMP4, Smad2 and Vg1 constructs used in this study were in pSP64T vector (provided by D. Melton), and Xnr1 construct was in pCS2 vector (provided by K. Lustig).

Histology

Embryos were fixed for 1 hour at room temperature in MEMFA and embedded in wax followed by sectioning and stained with hematoxylin/eosin.

RESULTS

Construction of transcriptional activator or repressor forms of FAST-1

FAST-1 was originally identified as a component of the transcriptional complex binding to a regulatory element (ARE) in the *Mix.2* promoter upon activin stimulation (Chen et al., 1996; Huang et al., 1995). To examine whether ectopic expression of FAST-1 was sufficient for transcriptional activation of *Mix.2*, we injected mRNA encoding wild-type FAST-1 into the animal pole region of embryos at the 2-cell stage and examined animal caps dissected from the resulting embryos for *Mix.2* expression. Overexpression of wild-type

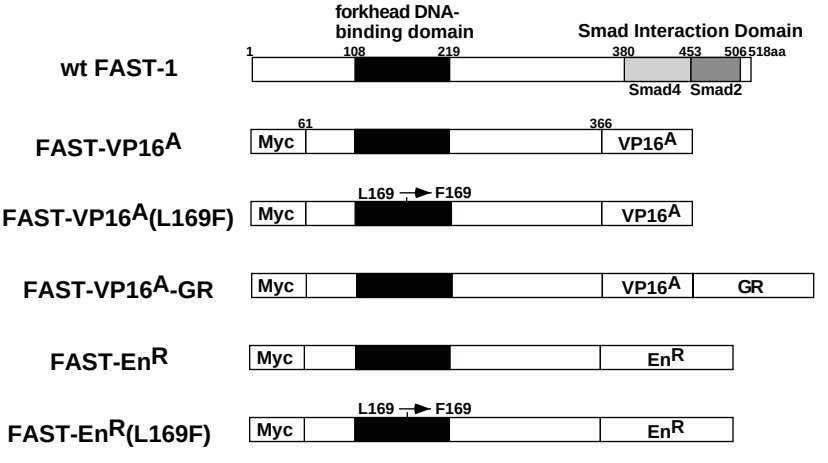
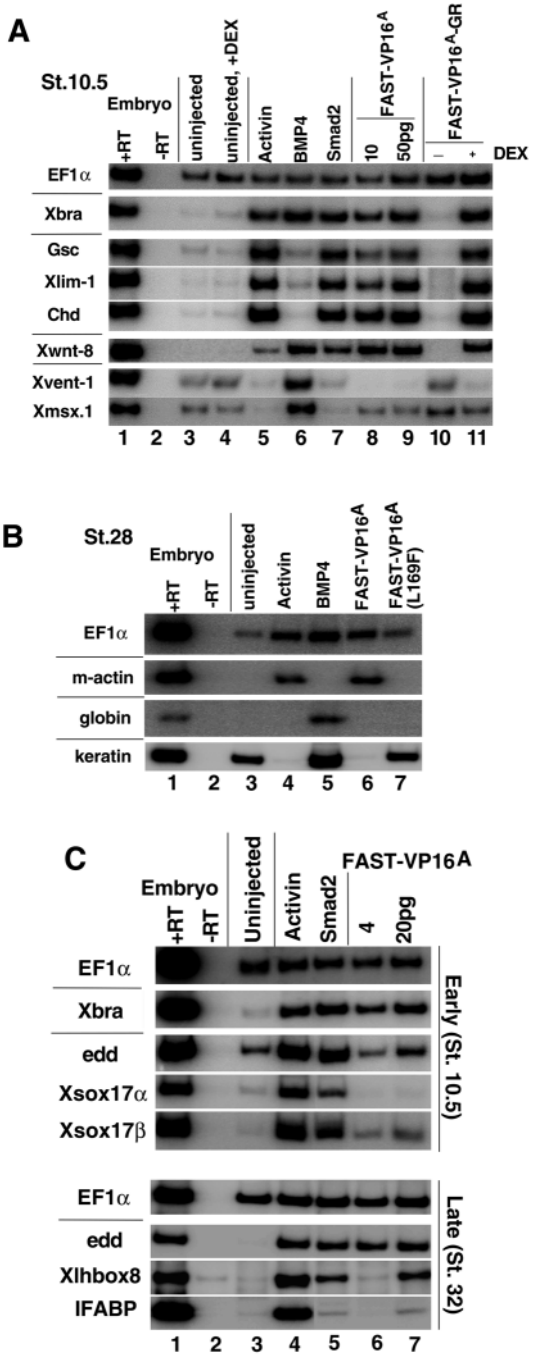


Fig. 1. Schematic representation of constructs used in this study. cDNA encoding a Myc epitope tagged FAST-1 containing the forkhead DNA-binding domain was fused to a VP16 activation domain, a VP16 activation domain-glucocorticoid receptor fusion, or an Engrailed repression domain (see methods for details). Dark shaded area indicates the forkhead DNA-binding domain of FAST-1, and light shaded areas indicates the Smad interaction domain (SID). The constructs L169F contain a Leu to Phe mutation at position 169 within the forkhead DNA-binding domain that eliminates DNA-binding activity.

FAST-1 did not induce the expression of either *Mix.2* or other marker genes tested (Fig. 5B lane 5), nor did it induce an ARE-luciferase reporter construct (data not shown). Ectopic FAST-1 does, however, cause toxicity at gastrulation at RNA doses greater than 100 pg/embryo. This toxicity appears to be a property of only the full-length wild-type protein, as it is not observed with the isolated SID, with FAST-En^{R} or FAST-VP16^{A} fusion proteins. The inability of ectopic FAST-1 to regulate *Mix.2* transcription suggested that regulated association FAST-1 with Smad2 and Smad4 and their intrinsic transcriptional activation domains might be required for FAST-1 to mediate transcriptional activation (Chen et al., 1997). To generate a form of FAST-1 that would be transcriptionally activated without Smad regulation, we fused the forkhead DNA-binding domain to the VP16 transcriptional activator domain from herpes simplex virus (FAST-VP16^{A}) (Sadowski et al., 1988; Triezenberg et al., 1988). To generate a form of FAST-1 that might repress rather than activate transcription, a fusion of the repressor domain from *Drosophila* Engrailed (FAST-En^{R}) (Jaynes and O'Farrell, 1991) was made (Fig. 1). Heterologous fusion proteins with these VP16^{A} and En^{R} domains have been shown to act as a constitutive activators or repressors, respectively, for transcription in a variety of systems including *Xenopus* (Conlon et al., 1996b; Fan and Sokol, 1997; Horb and Thomsen, 1997; Kessler, 1997). To confirm the importance of DNA-binding activity of the FAST-1 protein to the function of these fusion constructs, we also made control fusion constructs in which a point mutation was introduced into the DNA-binding domain (FAST-VP16^{A}(L169F) and FAST-En^{R}(L169F)) (Fig. 1). Leucine at position 169 is conserved among all forkhead DNA-binding domain proteins (Kaufmann and Knochel, 1996). A mutation at this position to phenylalanine was originally found in a *Drosophila* mutation in the forkhead-domain gene *crocodile* and was shown to abolish the DNA-binding activity of Crocodile protein (Hacker et al., 1995). By gel mobility shift assay, L169F fusion protein mutants did not bind to DNA, whereas fusion proteins with a wild-type DNA-binding domain bound to the ARE (data not shown). To permit conditional activation of the FAST-VP16^{A} construct, we also fused the FAST-VP16^{A} to the hormone-binding domain of glucocorticoid receptor (FAST-VP16^{A}-GR) (see below).



FAST-VP16^{A} induces the expression of mesodermal genes in animal cap explants

Prospective ectodermal cells of blastula stage embryos (animal cap cells) normally give rise to epidermal or neural tissue later in development, but they can be respecified as mesoderm either by the addition of extracellular inducing factors or by the injection of RNAs encoding transducers or regulators of inductive signals (Dawid, 1991). To test whether

Fig. 2. Overexpression of FAST-VP16^{A} induces mesodermal and endodermal marker gene expression in animal cap explants. (A) Early mesodermal gene induction by FAST-VP16^{A}. Capped, in vitro transcribed RNAs encoding activin (10 pg), BMP4 (2 ng), Smad2 (2 ng), FAST-VP16^{A} (10 or 50 pg), or FAST-VP16^{A}-GR (2 pg) RNA were injected into the animal pole region of embryos at the 2-cell stage. Animal cap cells were cut at stage 9 and expression of early mesodermal marker genes induced by RNA injection was analyzed by RT-PCR at stage 10.5. Animal cap explants injected with FAST-VP16^{A}-GR RNA were cultured with or without 10 μM dexamethasone (DEX). EF1α was used as a loading control. +RT represents the RT-PCR products from whole embryonic RNA, which served as a positive control, –RT represents PCR amplification from whole embryo RNA samples in which reverse transcriptase was omitted as a negative control. (B) Late mesodermal and epidermal marker gene induction by FAST-VP16^{A}. Activin (4 pg), BMP4 (2 ng), FAST-VP16^{A} (10 pg) or FAST-VP16^{A}(L169F) (10 pg) RNA-injected animal cap explants were studied for late mesodermal and epidermal gene expression by RT-PCR at stage 28. (C) Endodermal gene induction by FAST-VP16^{A}. The same amount of RNAs as in A or indicated amount of RNA was injected and both early (stage 10.5) and late (stage 32) endodermal marker genes expression in animal cap explants was studied by RT-PCR.

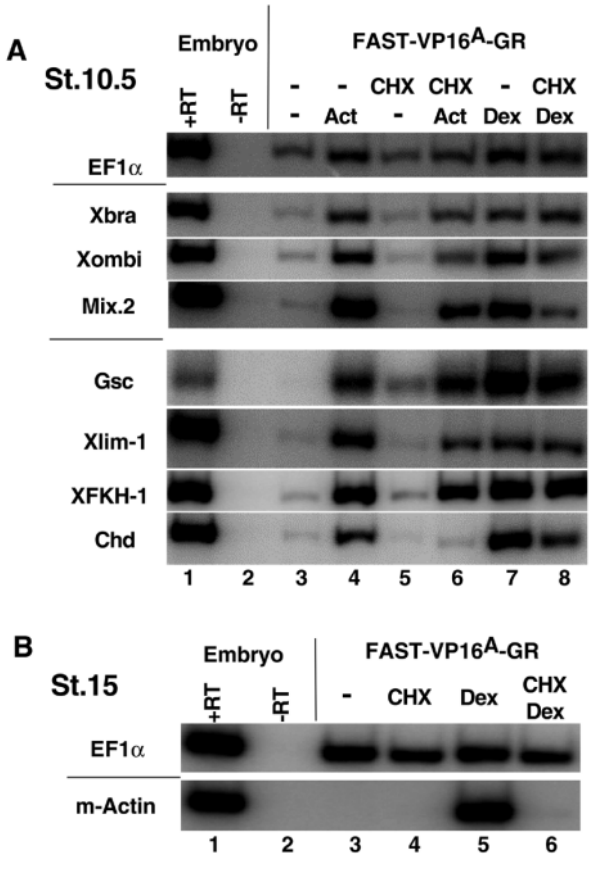


Fig. 3. FAST-VP16^A induces early mesodermal genes directly. (A) FAST-VP16^A-GR RNA (1 pg)-injected animal cap explants (stage 9) were cultured with cycloheximide (CHX) for 30 minutes to block protein synthesis, and then DEX or human recombinant activin protein was added in the medium to activate the FAST-VP16^A-GR protein or to induce mesodermal gene expression, respectively. The explants were maintained in the presence or absence of CHX and/or DEX or activin protein until stage 10.5 and harvested for RT-PCR analysis. Mesodermal gene expression by FAST-VP16^A-GR alone (lane 3), with DEX (lane 7), or with DEX and CHX (lane 8) was shown. Activin induction of these genes in the presence or absence of CHX (lanes 4 and 6) was also compared. (B) FAST-VP16^A-GR-injected explants were treated as in A, except CHX treatment was done for 1 hour. At stage 10.5, the explants were transferred into normal medium and harvested at stage 15 for RT-PCR analysis.

overexpression of FAST-VP16^A in prospective ectodermal cells modifies their developmental fate, FAST-VP16^A RNA or RNAs encoding activin, BMP4, or Smad2 were injected into the animal hemisphere of 2-cell stage embryos, and animal caps were cut at late blastula (stage 9) and harvested for analysis of early mesodermal gene expression at gastrulation (stage 10.5). FAST-VP16^A RNA injection, like activin or Smad2 RNA injection, induced the expression of a variety of mesodermal genes: the pan-mesodermal marker gene *Xbra* (Smith et al., 1991), the dorsal mesodermal genes *Gsc* (Blumberg et al., 1991; Cho et al., 1991), *Xlim-1* (Taira et al., 1992) and *Chd* (Sasai et al., 1994) and the lateral mesodermal gene, *Xwnt8* (Christian et al., 1991) (Fig. 2A lanes 5-9). In contrast, early ventral mesodermal genes, *Xvent-1* (Gawantka et al., 1995) and *Xmsx.1* (Maeda et al., 1997; Suzuki et al., 1997b) were not induced either by FAST-VP16^A RNA injection or by activin, while BMP4 RNA injection induced these genes in sibling explants (Fig. 2A lanes 5-9). When animal cap explants injected with FAST-VP16^A RNA were harvested at tail bud stage (stage 28) and analyzed for expression of markers of differentiated dorsal and ventral mesoderm, the dorsal mesodermal marker m-actin was induced, while the ventral mesodermal marker globin was not induced (Fig. 2B lanes 4-6). In the absence of added factors, animal cap explants isolated from blastula stage embryos express epidermal marker genes; endogenous BMP signaling appears to be necessary for this epidermal specification (Wilson and Hemmati-Brivanlou, 1995). Epidermal keratin is expressed in uninjected or BMP4 RNA-injected animal caps, while activin or FAST-VP16^A overexpression suppressed the expression of keratin (Fig. 2B lanes 3-6). This suggests that FAST-VP16^A, like activin addition, suppresses the epidermal gene program while stimulating the mesodermal program. Injection of RNA encoding wild-type FAST-1 protein did not induce the expression of any mesodermal genes (Fig. 5B lane 5, and data not shown), nor did FAST-VP16^A(L169F) (Fig. 2B lane 7 and data not shown). These results strongly suggest that FAST-VP16^A mimics the activity of dorsal mesoderm inducers such as activin and Vg1, and that the DNA-binding activity is essential for FAST-VP16^A function.

To establish a means for conditional control of FAST-VP16^A, we also characterized a construct in which FAST-VP16^A was fused to a glucocorticoid receptor hormone-binding domain (FAST-VP16^A-GR). The glucocorticoid domain causes sequestration of the fusion protein in the heat-shock apparatus; this sequestration is relieved and the protein is activated by addition of dexamethasone (DEX) (Kolm and Sive, 1995; Mattioni et al., 1994). As shown in Fig. 2A, FAST-VP16^A-GR induced the same subsets of mesodermal genes as FAST-VP16^A did, but only in the presence of DEX (Fig. 2A lanes 10-11); DEX treatment in the absence of FAST-VP16^A-GR did not induce these genes in the animal cap cells (Fig. 2A lane 4). This result demonstrates that FAST-VP16^A-GR induces the same genes as FAST-VP16^A does and that the activity of FAST-VP16^A-GR protein is strictly regulated by DEX.

FAST-VP16^A induces the expression of some endodermal genes in animal cap explants

Activin is able to induce not only mesodermal genes, but also endodermal genes in animal cap explants, and involvement of TGFβ superfamily signaling in endodermal patterning has been proposed (Gamer and Wright, 1995; Henry et al., 1996; Hudson et al., 1997). Since FAST-1 was identified as a downstream signal transducer for activin, and overexpression of FAST-VP16^A could, like activin, induce a range of mesodermal genes, we also tested whether FAST-VP16^A could induce endodermal genes. Overexpression of FAST-VP16^A induced the expression of early endodermal markers *edd* (Sasai et al., 1996) and *Xsox17β* (Hudson et al., 1997) but did not induce another early endodermal marker, *Xsox17α* (Hudson et al., 1997) (Fig. 2C lanes 6-7). At stage 32, FAST-VP16^A induces the late endodermal markers *edd*, *Xlhbox8* (Wright et al., 1988) and IFABP (Shi and Heyas, 1994). For comparison, induction of these endodermal genes by overexpression of

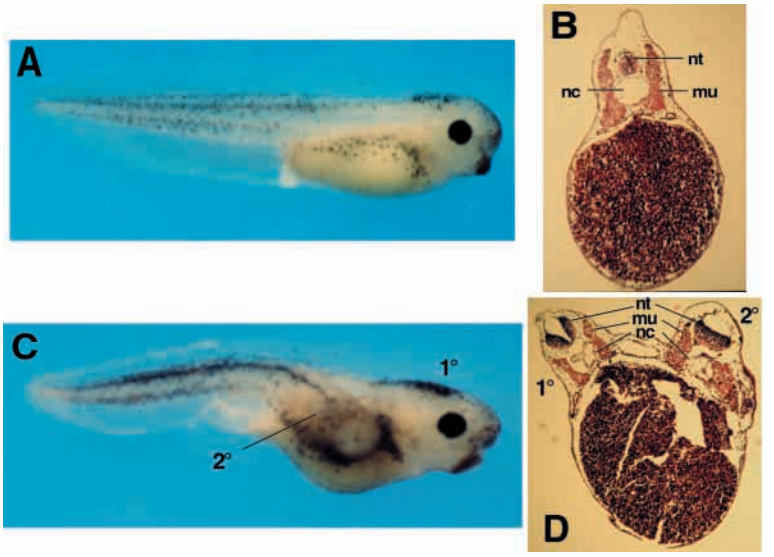


Fig. 4. FAST-VP16^{A} induces a secondary dorsal axis. (A,B) Uninjected control embryo and cross-section at stage 40. (C,D) Embryo injected with FAST-VP16^{A} RNA (5 pg) into two ventrovegetal blastomeres at the16-cell stage and fixed at the same stage as uninjected control. Abbreviations: 1°, primary axis; 2°, secondary axis; nt, neural tube; nc, notochord; mu, muscle.

activin or Smad2 in animal cap explants is shown (Fig. 2C lanes 4-5). The observation that Smad2 potently induces endodermal markers indicates that it may mediate the action of activin on these genes. The ability of FAST-VP16^{A} to mimic some, but not all, of these inductive effects suggests that it is one component of regulation of endodermal markers by activin/Smad2 signaling.

FAST-VP16^{A} directly induces early mesodermal genes in animal cap explants

Induction of mesodermal markers by expression of FAST-VP16^{A} does not distinguish direct effects of FAST-VP16^{A} on target promoters from indirect effects mediated by induction of genes that themselves regulate mesodermal gene expression. To address this question, we took advantage of the hormone-inducible activation of FAST-VP16^{A}-GR. Animal cap explants expressing FAST-VP16^{A}-GR were cultured with cycloheximide (CHX) for 30 minutes to block protein synthesis (Cascio and Gurdon, 1987), and then DEX was added in the medium to activate the FAST-VP16^{A}-GR protein. The

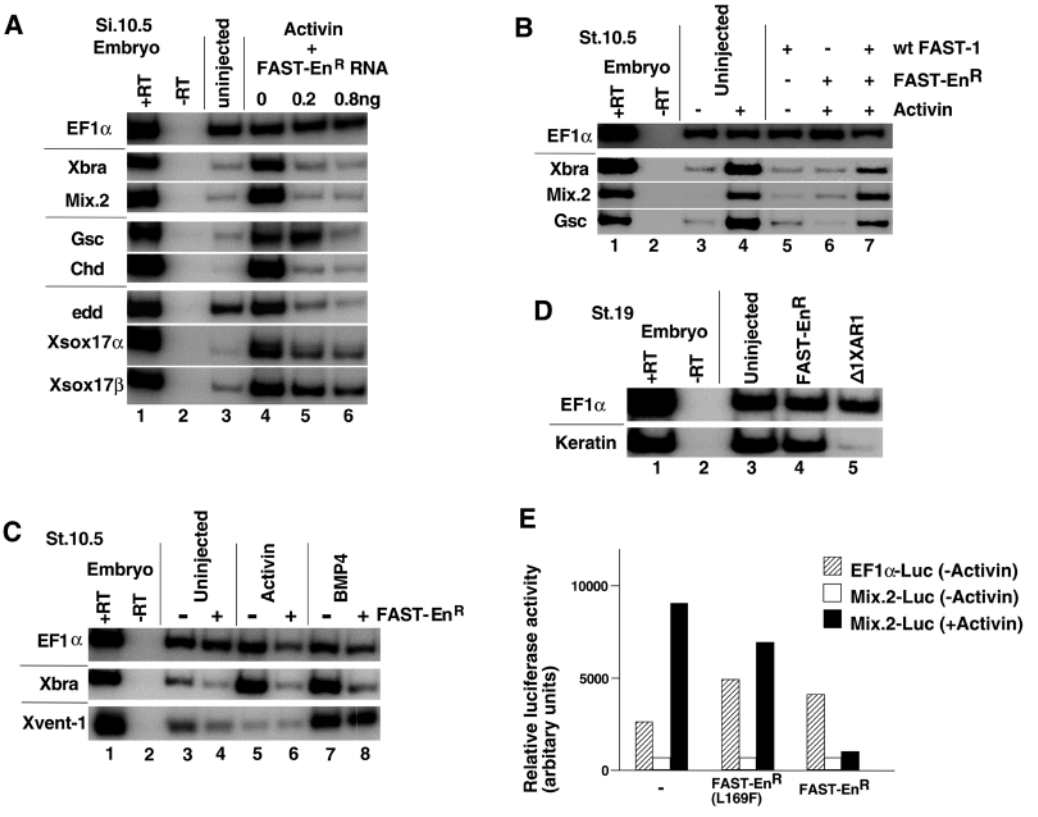


Fig. 5. Overexpression of FAST-En^{R} blocks mesodermal/endodermal gene expression in animal cap explants. (A) FAST-En^{R} blocks mesodermal/endodermal gene induction by activin. Animal cap explants injected with FAST-En^{R} RNA (stage 9) were treated with activin protein and expression of mesodermal/endodermal marker genes was studied by RT-PCR at stage 10.5. (B) Overexpression of wild-type (wt) FAST-1 protein restores activin signaling blocked by FAST-En^{R} in animal cap explants. Animal cap explants injected with wt FAST-1 (100 pg) and/or FAST-En^{R} (200 pg) RNA were cultured with or without activin protein, and mesodermal gene expression was studied at stage 10.5 by RT-PCR. (C) FAST-En^{R} does not block ventral mesodermal gene expression. Activin (4 pg), or BMP4 (2 ng) RNA was injected into the embryos with or without co-injection of FAST-En^{R} (400 pg) RNA, and marker gene expression in animal cap explants was studied as in (A). (D) FAST-En^{R} does not block epidermal gene expression in animal cap explants. Animal cap explants injected with FAST-En^{R} (400 pg) or dominant negative activin type II receptor Δ1XAR1 (1 ng) RNA were examined for the expression of epidermal keratin gene expression by RT-PCR at stage 19. (E) FAST-En^{R} blocks activin-induced *Mix.2*-reporter gene expression in animal cap explants. *Mix.2* and EF1α luciferase reporter construct DNAs (25 pg/embryo) were injected animally with or without FAST-En^{R} (400 pg) or FAST-En^{R} (L169F) (400 pg) RNA at the 2-cell stage. Animal caps were cut at stage 9 and treated with activin protein and luciferase activity was measured at stage 10.5.

explants were maintained in the presence or absence of CHX and/or DEX until stage 10.5 and harvested for RT-PCR analysis. Without DEX, FAST-VP16^{A}-GR protein did not induce mesodermal genes (Fig. 3A lane 3). Addition of DEX, however, induced expression of *Xbra*, *Mix.2* (Vize, 1996), *Xombi* (Lustig et al., 1996b) (also called *Brat/Apod/VegT*) (Horb and Thomsen, 1997; Stennard and Gurdon, 1996; Zhang and King, 1996), *Gsc*, *Xlim-1*, *XFKH-1* (Dirksen and Jamrich, 1992; Knochel et al., 1992) and *Chd* (Fig. 3A lane 7), even in the presence of CHX (Fig. 3A lane 8). This result clearly demonstrates that FAST-VP16^{A}-GR protein directly activates the transcription of these genes. These mesodermal genes could also be induced by activin directly, except *Chd* (Fig. 3A lanes 4-6). Induction of *Chd* by activin has previously been reported to require new protein synthesis (Sasai et al., 1994); inhibition of *Chd* induction by activin provides a positive control that CHX treatment in this experiment effectively blocked new protein synthesis. These observations also suggest that *Chd* induction by activin requires other, protein-synthesis-dependent, factors in addition to FAST-1. That FAST-1 is necessary for *Chd* induction was independently confirmed by injection of blocking antibodies to FAST-1 (see below). Although activation of FAST-VP16^{A}-GR protein is sufficient to induce early mesoderm marker expression in animal cap explants without new protein synthesis, induction of the late mesoderm marker, m-actin, was completely blocked by CHX (Fig. 3B).

These results indicate that FAST-VP16^{A} protein directly binds to regulatory sites for target genes to activate their transcription and that FAST-1 protein is a common downstream signal transducer for early mesodermal gene induction by activin or activin-like TGFβ super family members.

FAST-VP16^{A} induces a secondary axis in embryos

Since FAST-VP16^{A} RNA injection induced a variety of general/dorsal mesodermal genes in animal cap explants, we

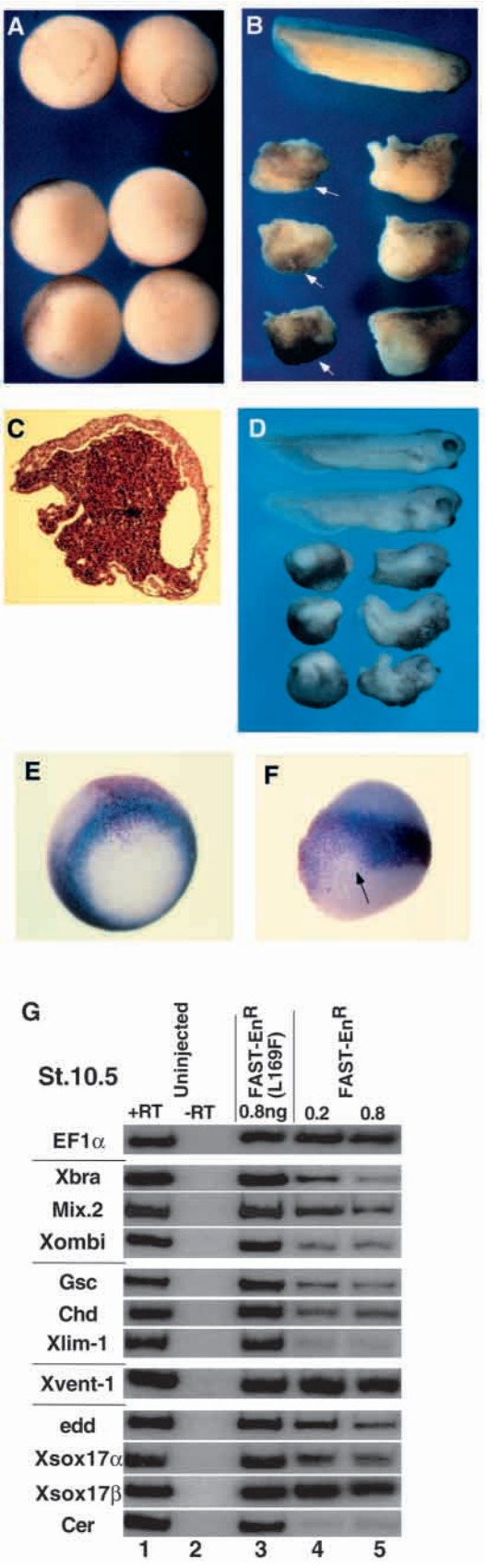


Fig. 6. FAST-En^{R} blocks endogenous mesoderm/axis-inducing signal(s). (A) Formation of the blastopore at gastrulation (stage 11.5) in FAST-En^{R} (800 pg RNA)-injected embryos (bottom), or uninjected sibling embryos at the same stage (top). (B) Effects of FAST-En^{R} on axial patterning at stage 34. 200 pg (right) or 800 pg (left) RNA was injected. (Top) Uninjected sibling embryo at the same stage. (C) Cross-section of a FAST-En^{R}-injected embryo (stage 34). (D) Overexpression of FAST-VP16^{A} protein restores axial structures of FAST-En^{R}-injected embryos. (Top) Uninjected sibling embryo at stage 37/38. (Second from the top) Embryos injected with FAST-VP16^{A} RNA (20 pg) into two dorsovegetal blastomeres at the 8-cell stage. (Bottom) Embryos injected with FAST-En^{R} RNA (400 pg) into both blastomeres at the 2-cell stage, with (right) or without (left) injection of FAST-VP16^{A} RNA (20 pg) into two dorsovegetal blastomeres at the 8-cell stage. (E,F) FAST-En^{R} blocks *Xbra* expression in the embryo cell autonomously. RNA encoding nuclear β-galactosidase (200 pg) was injected into a single blastomere at the 4-cell stage marginally without (E, vegetal view) or with (F, lateral view) FAST-En^{R} RNA (200 pg), and injected embryos were harvested at stage 10.5. The expression of β-galactosidase was detected by magenta X-gal staining (red) and the expression of *Xbra* was studied by in situ hybridization using a BM purple stain (blue). Arrow indicates site of inhibition of endogenous *Xbra* expression. (G) Early mesoderm and endoderm marker gene expression in FAST-En^{R}-injected embryos. Embryos were injected with RNA encoding FAST-En^{R} or FAST-En^{R}(L169F) into all cells marginally at the 4-cell stage, and expression of endogenous marker genes was studied by RT-PCR at stage 10.5.

tested the effect of FAST-VP16^{A} in whole embryos. RNAs were injected into two ventrovegetal or dorsovegetal blastomeres of the 16-cell-stage embryos and the effects of injection were observed at tail bud stage. Fig. 4 shows that the injection of FAST-VP16^{A} RNA caused secondary axis formation in embryos if injected ventrally. Uninjected embryos, embryos injected with FAST-VP16^{A} (L169F) (5 pg RNA/embryo, *N*=35), wild-type FAST-1 (50 pg, *N*=32) or embryos injected dorsally with FAST-VP16^{A} RNA (5 pg, *N*=39), never showed secondary axis formation (data not shown). Dorsal injection of FAST-VP16^{A} enhanced anterior/head structures of the embryos (see Fig. 6D; second from the top). Roughly two thirds of embryos (65%, *N*=60) injected ventrally with FAST-VP16^{A} (5 pg RNA) showed at least partial secondary axis formation (Fig. 4C) and 7% of them showed a secondary cement gland and/or eye formation (data not shown). The phenotype caused by FAST-VP16^{A} overexpression (5 pg RNA) was partially rescued by co-injection of wild-type FAST-1 RNA (50 pg, secondary axis formation was 19%, *N*=31), suggesting that the effect of FAST-VP16^{A} is specific to the FAST-1 signaling pathway. Frequency or degree of secondary axis formation was not changed by higher concentrations of FAST-VP16^{A} RNA injection (50 pg, 70%, *N*=31). Sectioning of FAST-VP16^{A}-injected embryos revealed that the secondary axis contained well-organized internal structures, such as notochord, neural tube and muscle (Fig. 4D). At later stages, a secondary beating heart was formed in some injected embryos (data not shown). Complete secondary axes, as can be observed in embryos injected with *Xwnt* RNA (Sokol et al., 1991), were not observed. FAST-VP16^{A}-GR RNA injection also induced secondary axis formation, again only in the presence of DEX (data not shown). Ectopic expression of activin and Vg1 have each been shown to induce anteriorly defective secondary dorsal axes when expressed on the ventral side (Dale et al., 1993; Thomsen et al., 1990). Expression of FAST-VP16^{A} is sufficient to mimic the effects of these inducing factors on early axial patterning.

FAST-En^{R} blocks mesodermal and endodermal gene expression in animal cap explants

If FAST-1 is indeed involved, at least in part, in mesoderm-inducing signaling in vivo, inhibition of FAST-1 activity should result in the inhibition of mesodermal formation in embryos. We therefore made a transcriptional repressor form of FAST-1, a fusion protein of FAST-1 DNA-binding domain with the transcriptional repressor domain of the *Drosophila* Engrailed protein (Jaynes and O'Farrell, 1991). Fusion proteins with this domain have been used to inhibit the function of putative transcription factors in many systems (Conlon et al., 1996b; Fan and Sokol, 1997; Horb and Thomsen, 1997). We first studied the effects of FAST-En^{R} overexpression in animal cap explants. Explants expressing FAST-En^{R} were cultured in the presence of recombinant human activin protein until stage 10.5 and harvested for RT-PCR. Injection of FAST-En^{R} RNA alone did not induce mesodermal gene expression in explants (Fig. 5C lane 4). Activin induced the expression of both mesodermal and endodermal genes in animal caps and this induction could be inhibited overexpression of FAST-En^{R} (Fig. 5A lanes 4-6). Expression of FAST-En^{R} also blocked induction of mesoderm by additional TGFβ superfamily dorsal mesoderm inducers Vg1 and Xnr1 (Jones et al., 1995; Lustig et al., 1996a; Thomsen and Melton, 1993) (data not shown). Activin induction of mesodermal genes can be restored, at least partially, by co-expression of wild-type FAST-1 with FAST-En^{R} (Fig. 5B). The rescue of FAST-En^{R} by wild-type FAST-1 is incomplete because high levels of FAST-En^{R} are required for full inhibition of signaling; the amount of wild-type FAST-1 RNA required to effectively compete with FAST-En^{R} is toxic to embryos.

In contrast to its effects on dorsal and pan-mesodermal gene induction by activin, FAST-En^{R} did not block induction of *Xvent-1*, a ventral mesodermal gene induced by BMP4 (Fig. 5C lanes 7-8), indicating that the effects of FAST-En^{R} are specific to target genes for activin-like inducers. FAST-En^{R} also did not block the expression of epidermal gene (epidermal keratin) expression in animal cap cells at later stages (stage 19), whereas an inhibitor of both activin and BMP-like signaling, dominant negative type II activin receptor (Δ1XAR1), blocked the expression of the epidermal keratin gene (Fig. 5D). Epidermal keratin is induced by endogenous BMP signaling present in animal cap cells (Wilson and Hemmati-Brivanlou, 1995), again suggesting that FAST-En^{R} does not block BMP signaling. FAST-En^{R} did, however, inhibit induction of *Xbra* by both activin-like and BMP ligands (Fig. 5C). FAST-En^{R}, but not FAST-En^{R} (L169F), also inhibits activin induction of a *Mix.2* luciferase reporter construct, but not the constitutive expression of the EF1α promoter (Fig. 5E). Similar results were observed with the activin responsive elements of the *Xlim-1* (M. Watanabe, M. Rebbert, I. Dawid, and M. Whitman, unpublished data) gene. Inhibition of induction by FAST-En^{R} may reflect the presence of FAST-1-binding sites in a promoter rather than utilization of these sites by any particular signal. For this reason, we have used blocking antibodies for FAST-1 as an additional approach that permits elucidation of the relative necessity of FAST-1 in different signaling pathways that target the same promoter (see below).

FAST-En^{R} blocks axial development in embryos

Since FAST-En^{R} protein blocked the expression of mesodermal genes in animal cap explants, we expected that overexpression of FAST-En^{R} would cause loss of mesoderm-derived tissues in embryos. To test this possibility, RNAs were injected in both blastomeres marginally at the 2-cell stage and the phenotype of injected embryos was observed at later stages. The first morphological abnormality of injected embryos was the failure of blastopore formation and subsequent gastrulation movements (Fig. 6A bottom). This phenotype could be observed in almost all embryos injected with a high concentration of FAST-En^{R} RNA (800 pg/embryo, *N*=88). Vital dye staining showed that there was no internalization of vegetal pole cells at later stages (data not shown). When uninjected sibling embryos had reached the neurula stage, FAST-En^{R}-injected embryos retained a large blastocoel, which subsequently contracted leaving a wrinkled, darkly pigmented ectodermal layer covering the vestigial blastocoel (arrows in Fig. 6B). By tailbud stage, these embryos showed losses of axial structure dependent on the dose of FAST-En^{R} mRNA injected (Fig. 6B). When a low concentration of RNA (200 pg/embryo, Fig. 6B right) was injected, truncated axes were formed that retained some anterior structures, such as cement gland (cement gland formation was 44% of the cases, *N*=121). At a higher dose of injected mRNA (800 pg/embryo, Fig. 6B

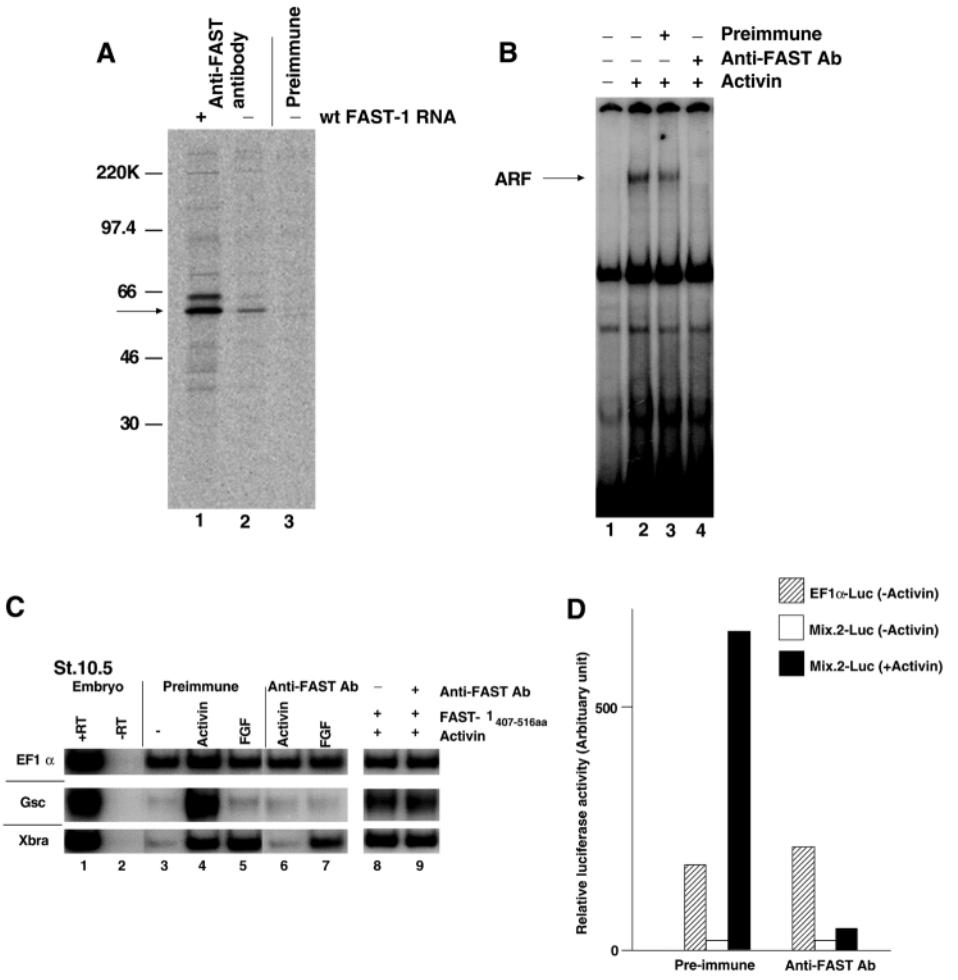


Fig. 7. Anti-FAST antibody blocks ARF formation and activin-induced mesodermal gene expression. (A) Anti-FAST antibody specifically recognizes FAST-1 protein. [^{35}S]methionine was injected into embryos at the 2-cell stage to label proteins with or without co-injection of wt FAST-1 RNA (1 ng). Proteins from embryos were immunoprecipitated with anti-FAST or preimmune antibody, and run on a 10% SDS-PAGE gel and visualized by autoradiography. Arrow indicates the position of FAST-1 protein. (B) Anti-FAST antibody blocks ARF formation. Embryonic lysates stimulated by activin were incubated with ^{32}P-labeled ARE probe or preincubated antibodies before adding ARE probe and ARF formation was analyzed by gel mobility shift assay. Arrow indicates the position of ARF. (C) Anti-FAST antibody blocks mesodermal genes expression by activin, but not FGF. Preimmune or anti-FAST antibody was injected into animal pole of embryos at the 2-cell stage with or without co-injection of an RNA (1 ng) encoding a partial FAST-1 protein (407-516aa) used for antibody production, and caps were cut at stage 9 and treated with activin or FGF protein. *Gsc* and *Xbra* expression induced by activin or FGF was studied at stage 10.5 by RT-PCR. (D) Anti-FAST antibody blocks activin-induced *Mix.2*-reporter gene expression in animal cap explants. *Mix.2* and EF1α luciferase reporter DNAs (25 pg/embryo) were injected animally with preimmune or anti-FAST antibody at the 2-cell stage. Animal caps were cut at stage 9 and treated with activin protein and luciferase activity was measured at stage 10.5.

left), all embryos (N=88) showed a complete loss of axial structure. Sectioning of these embryos revealed that there were no organized internal structures, such as notochord, muscle or neural tube (Fig. 6C). These phenotypes are reminiscent of those observed with dominant negative type II activin receptor (Δ1XAR1)-injected embryos (Hemmati-Brivanlou and Melton, 1992), although in the case of FAST-En^R injection ectopic neuralization is not observed, suggesting that BMP signaling is not inhibited (Wilson and Hemmati-Brivanlou, 1995).

FAST-VP16^A was tested for its ability to rescue the effects of FAST-En^R protein in embryos. 400 pg of FAST-En^R RNA was marginally injected at the 2-cell stage embryo, and then 20 pg of FAST-VP16^A RNA was injected into two dorsovegetal blastomeres at the 8-cell stage in the same embryos and phenotypes were observed at tailbud stage. As shown in Fig. 6D, while overexpression of FAST-En^R almost completely blocked formation of axial structures (80%, N=122), coinjection of FAST-VP16^A restored at least partial body axis and anterior structures of these embryos (cement gland formation was observed in 65% of the cases, N=80). Higher concentrations of FAST-VP16^A do not improve frequency of rescue. The frequency and degree of rescue are probably limited by the incomplete effectiveness of FAST-VP16^A at inducing a secondary axis, and may be due to the effective induction by FAST-VP16^A of transcriptional responses (e.g. *Mix.2*) that may be inhibitory for head formation. Co-expression of wild-type FAST-1with FAST-En^R does not effectively restore normal embryonic phenotype. This may be because levels of wild-type FAST-1 RNA that exceed the levels of FAST-En^R RNA necessary to block axial patterning are themselves weakly disruptive to gastrulation, possibly because excess levels of the FAST-1 Smad interaction domain (SID) can sequester endogenous Smads from other downstream targets important in cell movement or patterning (Chen et al., 1997). Co-injection of RNA encoding a β-galactosidase tracer with FAST-En^R into a single blastomere at the 4-cell stage results in the exclusion of *Xbra* expression in a region limited to the region expressing β-galactosidase, indicating that the effects of FAST-En^R are cell autonomous (Fig. 6E,F).

FAST-En^R blocks expression of endogenous mesoderm/endoderm markers in embryos

The effects of FAST-En^R RNA injection on embryos indicated a block to endogenous induction of mesodermal/endodermal gene expression. To further characterize this phenomenon,

FAST-En^R RNA was injected into all blastomeres marginally at the 4-cell stage and the embryos were harvested at stage 10.5 for RT-PCR. As shown in Fig. 6G, injection of FAST-En^R RNA into embryos resulted in the inhibition of mesodermal and endodermal marker gene expression, as in activin-treated animal cap explants. Expression of the ventral mesodermal marker *Xvent-1* was unaffected by FAST-En^R (Fig. 6G). Incomplete inhibition of *Mix.2* and endodermal genes may be due to the uneven distribution of injected RNA, because injection was done marginally and these genes are expressed vegetally (Hudson et al., 1997; Sasai et al., 1996; Vize, 1996). The DNA-binding deficient form of FAST-En^R, FAST-En^R(L169F), did not block the expression of these genes (Fig. 6G lane 3). These results, together with the inhibition of mesodermal gene induction by activin, Vg1 or Xnr1, strongly suggest that FAST-En^R blocks endogenous mesodermal/axial-inducing signal in embryos.

Anti-FAST antibody blocks mesoderm induction in animal caps and in embryos

Although overexpression of FAST-En^R provides a useful probe for inhibiting expression of genes regulated by endogenous FAST-1, it does not provide a true representation of loss of function of FAST-1. As in the case of FAST-En^R inhibition of *Xbra* induction by BMP4 noted above, inhibition of transcriptional responses by FAST-En^R provides an indication that FAST-1-binding sites are present in the regulatory regions of mesoderm response genes, but does not demonstrate that any given signaling pathway acts through FAST-1. To more specifically characterize the effects of loss of function of FAST-1 on activin-like signaling, we selectively targeted endogenous FAST-1 protein by injection of an anti-FAST-1 antibody. Rabbit anti-FAST-1 antibody was raised against a GST fusion with the FAST-1 Smad interaction domain (SID, FAST-1$_{366\text{-}518aa}$, see Fig. 1) and affinity purified against this antigen. In immunoprecipitations from [^{35}S]methionine-labeled embryos, this antibody recognizes a single predominant band that co-migrates with overexpressed wild-type FAST-1(Fig. 7A). Similar results were obtained in western blot analysis (data not shown). These observations indicate that this antibody is monospecific for FAST-1 in early embryos. Upon injection into embryos, this antibody is stable through the end of gastrulation (data not shown).

We tested this highly specific affinity-purified antibody for its ability to block known FAST-1 functions. Upon activin stimulation, FAST-1 protein forms a complex with Smad2 and Smad4 referred to as activin responsive factor (ARF) (Fig. 7B lanes 1-2) (Chen et al., 1996, 1997). When activin-stimulated embryonic lysate was preincubated with anti-FAST antibody before incubation with ^{32}P-labeled ARE probe, ARF formation was completely blocked, while preincubation with preimmune IgG purified in parallel with the immune serum had no effect (Fig. 7B lanes 3-4). This indicated that the antibody effectively inhibits the interaction of Smads and FAST-1 to form the ARF complex. When injected into embryos, the antibody inhibited activin induction of *Xbra* or *Gsc* expression in animal cap explants, while preimmune IgG had no effect (Fig. 7C lanes 4 and 6). The induction of other mesodermal markers, including *Mix.2*, *Xlim-1*, *Xombi* and *Chd*, induced by activin were also blocked by this antibody (data not shown). Induction of *Xbra* by Vg1 was also inhibited by antibody injection (data not shown). To test the specificity of this inhibition, RNA encoding a portion of the region of FAST-1 used as antigen in the preparation of the anti-FAST serum was tested for its ability to rescue the effects of anti-FAST antibody injection. Because overexpression of FAST-1$_{366\text{-}518aa}$ (corresponding to the complete SID) is itself inhibitory for activin signaling (Chen et al., 1997), a partial SID that does not block activin signaling (M. Watanabe and M. Whitman, unpublished) was used for this rescue experiment. When this partial SID was overexpressed, activin-induced expression of *Gsc* and *Xbra* was restored in anti-FAST-injected cap explants (Fig. 7C lanes 8-9), indicating that the inhibitory effects of the antibody are indeed due to its recognition of the SID of FAST-1. To demonstrate the specificity of the effects of anti-FAST antibody for the activin/Smad2 signaling pathway, we examined its effects on FGF induction of *Xbra*. FGF induces *Xbra* through a MAP kinase-dependent pathway that does not involve Smads or FAST-1 (Huang et al., 1995; LaBonne et al., 1995; LaBonne and Whitman, 1997). When antibody-injected animal cap explants were treated with human recombinant FGF and FGF-induced *Xbra* expression was examined by RT-PCR, no inhibition of *Xbra* induction by anti-FAST antibody was observed (Fig. 7C, lanes 5 and 7). Injection of anti-FAST-1 antibody, but not preimmune serum, also blocks activin induction of a *Mix.2* promoter-luciferase reporter plasmid, but not of a constitutive EF1α promoter (Fig. 7D).

When injected into whole embryos, anti-FAST antibody, but not preimmune serum, inhibited expression of *Xbra*, *Mix.2*, *Gsc*, *Xlim-1* and *Chd* at gastrulation. The ventral mesodermal marker *Xvent-1* expression in embryos was not affected (Fig. 8A). The expression of mesodermal genes in anti-FAST antibody-injected embryos was restored by co-injection of RNA encoding a portion of the antigen against which the antibody was made, the FAST-1 SID (data not shown). In anti-FAST antibody-injected embryos, gastrulation movements were severely disrupted. At high doses of anti-FAST antibody, the blastopore lip does not form, but a localized indentation does appear in the dorsovegetal epithelium at stage 10.5-11. At lower doses of antibody, anti-FAST antibody-injected embryos form a dorsal blastopore lip with the same timing as control embryos, but this lip fails to extend circumferentially and close normally, and subsequent axial development is disrupted (Fig. 8E). Roughly half of anti-FAST antibody-injected embryos show, in addition to severe axial patterning defects, substantial dissociation of cells in the vegetal hemisphere. At all doses tested, the effects of anti-FAST antibody on gastrulation, axial patterning, and vegetal cell adhesion could be rescued by injection of RNA encoding a partial FAST-1 SID (Fig. 8F, Table 1), indicating that the effects of antibody injection are due to antibody recognition of endogenous FAST-1 rather than to non-specific toxic effects. The specific effects of the anti-FAST antibody on ectopic induction and endogenous expression of mesodermal markers corroborate the data obtained from expression of FAST-En^R, indicating that FAST-1 plays a central and essential role in the specification of mesoderm.

DISCUSSION

By expression of transcriptional activator or repressor FAST-1

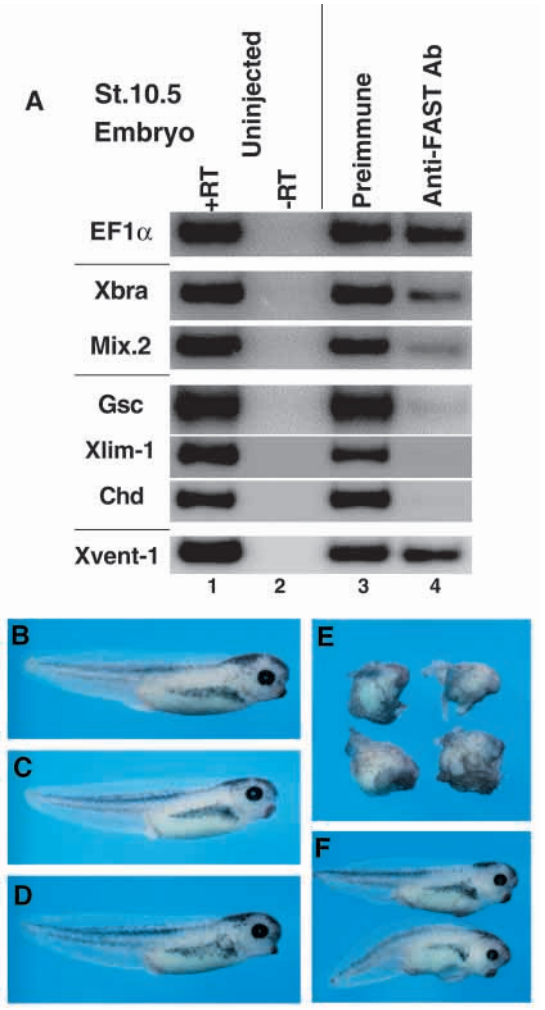


Fig. 8. Anti-FAST antibody blocks mesodermal gene expression and axis formation in embryos. (A) Anti-FAST antibody blocks mesodermal gene expression in embryos. Antibodies were injected into all blastomeres marginally at the 4-cell stage and expression of endogenous mesodermal marker genes was studied by RT-PCR at stage 10.5. (B-F) Effects of anti-FAST antibody on axial patterning on embryos. (B) Uninjected sibling embryo at stage 37. (C) Preimmune antibody-injected embryo. (D) Embryo injected with RNA (200 pg) encoding a partial FAST-1 protein (407-516aa) into both blastomeres at the 2-cell stage marginally. (E) Anti-FAST-injected embryos. Antibodies were injected as in A with two-fold dilution. (F) Embryos injected with both partial FAST-1 protein encoding RNA and anti-FAST antibody. Injection was done as in D and E.

fusions and injection of anti-FAST-1 antibody, we have shown here that FAST-1 directly regulates mesodermal gene expression and is a necessary component of activin/Vg1/Xnr1-mediated mesodermal induction in the *Xenopus* embryo. While a large number of genes that are transcribed in response to mesoderm inducers have been defined, and many of these response genes shown to be important components of mesodermal specification, this is the first characterization of a maternal transcription factor that mediates the regulation of these early transcriptional responses by TGFβ superfamily inducers. That FAST-1 transcripts are present ubiquitously in the early embryo (Chen et al., 1996) and overexpression of wild-type FAST-1 has relatively little effect on early patterning, is consistent with a patterning scheme in which localized expression/activation of inducing ligands, rather than localization of signal transducers, is a primary determinant of spatial patterning. These properties of FAST-1 also suggest that maternal transcription factors mediating early responses may not be easily isolated by the functional expression screens that have been so powerful in identifying gastrula-stage determinants of mesodermal and neural patterning (Lemaire et al., 1995; Lustig et al., 1996b; Smith and Harland, 1992).

In addition to the ARE from the *Mix.2* gene, activin-responsive promoter elements have been identified in genomic sequence from several other mesoderm early response genes: *Gsc*, *XFKH-1*, *Xbra*, *HNF1α*, *Xlim-1* and *Xombi* (Huang et al., 1995; Kaufmann et al., 1996; Latinkic et al., 1997; Rebbert and Dawid, 1997; Watabe et al., 1995; Weber et al., 1996; G. Naco and M. Whitman, unpublished). These elements do not share extensive sequence similarity to the *Mix.2* ARE or to one another. It was therefore initially surprising that all of the activin response genes that we examined, including *Gsc*, *Xbra*, *XFKH-1*, *Xlim-1* and *Xombi*, can be activated by FAST-VP16^{A}-GR in a cycloheximide-insensitive manner. This observation indicates that FAST-VP16^{A}-GR can directly bind to regulatory sites controlling expression of these genes. The recent definition of a FAST-1 consensus binding site (Zhou et al., 1998), however, does permit the identification by inspection of potential FAST-1 target sites in these promoters, consistent with their direct regulation by FAST-1. We have experimentally confirmed FAST-1-binding sites and/or FAST-1-dependent regulation of enhancer sequences from the *Xombi*, *Xbra*, *Gsc* and *Xlim-1* promoters (C. Y. Yeo, G. Naco, M. Watanabe, M. Rebbert, I. Dawid and M. Whitman, unpublished), and Labbe et al. (1998) have demonstrated FAST-1/FAST2 binding and regulation of the mouse *goosecoid* promoter, supporting the direct regulation of these genes by FAST-1 in vivo.

Since the original identification of FAST-1 as a transcription factor that targets activated Smad2 to an activin responsive

Table 1. Effects of anti-FAST antibody on development

	Vegetal cell loss, no body axis (%)	Intact, no body axis (%)	Partial body axis (%)	Normal body axis (%)	Other defects (%)	Total (*n*)
Preimmune serume*	0	0	0	90	10	50
Anti-FAST antibody*	43	52	6	0	0	51
FAST01$_{407\text{-}516aa}$‡	0	0	10	77	13	66
Anti-FAST antibody*,‡ + FAST-1$_{407\text{-}516aa}$	0	5	50	40	5	47

*Total 20 nl equivalent of affinity purified antibody was injected marginally all blastomeres at the 4-cell-stage embryos.
‡Total 200pg of RNA encoding FAST-1$_{407\text{-}516aa}$ was injected marginally into both blastomeres at the 2-cell-stage embryos.
Death ratio after gestrulation was less than 5% in each injection and dead embryos were excluded from total number.

promoter element (Chen et al., 1996), both *Drosophila* and vertebrate Smads have been shown to have intrinsic DNA-binding activities (Dennler et al., 1998; Kim et al., 1997; Yingling et al., 1997; Zawel et al., 1998). These observations raise the issue of to what extent Smads have intrinsic affinity for regulatory element in mesoderm early response genes, and to what extent they require additional factors, such as FAST-1, to regulate these responses. Cell types that lack FAST-1 but express Smad2 and Smad4 cannot activate an ARE-luciferase reporter construct unless FAST-1 is ectopically expressed (Hayashi et al., 1997; Liu et al., 1997; Weisberg et al., 1998), indicating that Smad activation in the absence of FAST-1 is not sufficient for regulation of the ARE. We have identified both FAST-1 and Smad4 (but not Smad2)-binding sites in the ARE; elimination of the FAST-1-binding site eliminates activin responsiveness, while elimination of the Smad4-binding site reduces, but does not eliminate, activin responsiveness (Yeo et al., 1999). This result also indicates that FAST-1 is the major determinant of targeting of the ARE by the FAST-1/Smad2/Smad4 complex.

The identification of FAST-1 as a mediator of activin/Vg1 induction of mesoderm response genes raises the question of how many different transcription factors are involved in mediating these responses. The complex, and quite different, spatial and temporal patterns of expression of, for example, *Gsc*, *Xombi* and *Mix.2* are unlikely to be regulated solely by a single extracellular signal or a single transcriptional mediator. In the case of the *gsc* promoter, detailed analysis has revealed distinct sites and transcription factors mediating responses signaling through the activin/Vg1 and Wnt/β catenin pathways (Watabe et al., 1995). Furthermore, experiments with the activin-regulated enhancer from the *Xenopus Gsc* promoter suggest that there may be FAST-independent (Candia et al., 1997), as well as potential FAST-dependent (Labbe et al., 1998), sites targeted by Smad2/Smad4-containing complexes, indicating that the Smad2 signaling pathway may use multiple transcription factors for regulation of the same gene. It seems likely that it will turn out to be the general case that developmental gene regulation involves integration of control of a complex set of transcription factors at target promoter/enhancers (Arnone and Davidson, 1997).

Dorsal mesodermal inducers such as activin and Vg1 can also induce both early and late endodermal markers, as well as genes (e.g. *Mix.2*) that are expressed in both endoderm and mesoderm (Gamer and Wright, 1995; Henry et al., 1996; Hudson et al., 1997; Vize, 1996). To what extent the differential specification of mesoderm and endoderm may involve distinct signals or different doses of the same signal is not clear. Our observations indicate that both Smad2 and FAST-1 may have roles as mediators of endodermal as well as mesodermal induction by activin/Vg1-like signals, in that both early response genes expressed in mesoderm and endoderm (*Mix.2*), and genes expressed exclusively in the endoderm (*edd*) can be induced by either Smad2 or FAST-VP16^{A} and are inhibited by FAST-En^{R}. On the contrary, expression of another endodermal marker, *Xsox17α*, is only minimally affected by manipulation of FAST-1 but potently activated by expression of Smad2, indicating that Smad2 is likely to regulate at least some endodermal genes through mechanisms not involving FAST-1. These observations suggest that there may be diversity not only in the extracellular ligands specifying endoderm and mesoderm, but also in the intracellular signal transducers and transcription factors mediating these specifications.

Although manipulation of FAST-1 function is sufficient to control mesodermal gene expression, the signals regulating FAST-1 in vivo are not clear. Ectopic activin, Vg1, Xnr1 and Xnr2 are all capable of activating ARF formation (Huang et al., 1995; X. Chen and M. Whitman, unpublished), and have been postulated to act through either Smad2 or Smad3. Smad2 and Smad3 are capable both of participating in ARF with FAST-1 and of activating transcription through FAST-1 (Chen et al., 1997; Weisberg et al., 1998; Yeo et al., 1999), but to date Smad2 is the only member of the Smad family demonstrated to be downstream of activin-like signals and present in the early *Xenopus* embryo (Baker and Harland, 1996; Graff et al., 1996). If Smad2 is the only transducer of this subclass of the TGFβ superfamily in early embryos, the only important functional distinction between ligands may be the spatial and temporal patterns of their expression (and that of their respective receptors). In this case, FAST-1-mediated gene expression should be regulated similarly by any member of this set of ligands. Alternatively, there may be still unidentified Smads or other signal transducers that act differentially downstream of activin, Vg1, Xnr1 and Xnr2, creating the possibility of distinct sets of transcriptional responses to each ligand. Early transcriptional responses that distinguish among these ligands have not been reported, consistent with the idea that they share a common signal transduction mechanism.

TGFβ superfamily ligands evoke a wide range of biological effects that depend in large part on the developmental history of the target cell (Moses and Serra, 1996). Early embryonic blastomeres express mesodermal genes as a response to activin/Vg1 signals, but the capacity for this response disappears by the end of gastrulation (Jones and Woodland, 1987). Our observations indicate that FAST-1 is a maternal determinant of the competence of the pregastrula embryonic response to an activin/Vg1-like signal. Levels of mRNA encoding FAST-1 drop sharply at gastrulation (Chen et al., 1996); whether this is a major mechanism by which FAST-1-mediated responses are restricted to early development, or whether there are additional levels of regulation at which FAST-1 function is restricted, is not known. Expression of mRNA encoding a mouse homologue of FAST-1 is similarly restricted to early embryogenesis (Labbe et al., 1998; Weisberg et al., 1998). The similarities in expression pattern and transcriptional specificity of *Xenopus* and mouse FASTs suggest that the function of these factors in specification of early pattern in response to TGFβ-like signals may be conserved in vertebrate embryogenesis.

Endogenous patterning of mesodermal genes undoubtedly involves the integration multiple signals through a complex set of transcription factors. The definition of one complete pathway, however, from ligands (activin, Vg1, Xnr1 and Xnr2) through receptors (ALK4) (Armes and Smith, 1997) and intracellular signal transducers (Smad2 and Smad4) (Baker and Harland, 1996; Graff et al., 1996) to an enhancer-specific transcription factor (FAST-1) provides the beginning of a framework in which to understand the full complexity of the signals patterning the early mesoderm.

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