

The *Xenopus Eomesodermin* promoter and its concentration-dependent response to activin

Kenneth Ryan^{a,b,1}, Nigel Garrett^{a,b}, Pierre-Yves Bourillot^{a,b}, Fiona Stennard^{a,b}, J.B. Gurdon^{a,b,*}

^aWellcome CRC Institute, Tennis Court Road, Cambridge CB2 1QR, UK

^bDepartment of Zoology, University of Cambridge, Cambridge, UK

Received 25 November 1999; received in revised form 24 February 2000; accepted 24 February 2000

Abstract

Eomesodermin is an essential early gene in *Xenopus* mesoderm formation and shows a morphogen-like response to activin. Here we define the regions of the *Eomesodermin* promoter required for mesodermal expression and for concentration-dependent response to activin. We find an activin response element (ARE) located between -5.6 and -5.0 kb which contains two critical FAST2 binding sites. The ARE alone is necessary and sufficient for concentration-dependent response to activin. A 5.6 kb promoter recapitulates *Eomes* expression in normal mesoderm cells. A repressor element extinguishes *Eomes* expression in the endoderm. We relate our results to mesoderm patterning in early *Xenopus* development and to a mechanism of morphogen gradient response. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: *Xenopus*; *Eomesodermin*; Promoter; Mesoderm; Induction; Morphogen; Gradient; ARE; FAST2; Endoderm; Repressor; Germ; Layer

1. Introduction

One mechanism by which tissue-specific differentiation is achieved in metazoans is through regional activation of gene transcriptions in early embryos. Owing to its rapid rate of development, easy accessibility and experimental manipulability, *Xenopus* is a useful organism in which to study how germ layer-specific gene expression occurs. As mesoderm is the earliest induced embryonic tissue in *Xenopus*, for which there is also an abundance of early gene markers (Stennard et al., 1997), it is a natural choice for further study of mechanisms of developmental gene regulation.

Recent progress has been made in identifying some of the molecular components of early signalling pathways important in germ layer specification. Formation of the full range of early mesodermal cell types requires several maternally encoded, vegetally localized signalling pathways. This

includes the early-acting dorsalization pathway (for a review see Moon and Kimelman, 1998), and a second, mesoderm inducing pathway, which appears to act slightly later and entails cell–cell communication. This inducing pathway in *Xenopus* is thought to work through secreted factor members of both the FGF family (Slack et al., 1989; Smith et al., 1989; Amaya et al., 1991) and the TGF- β superfamily (Smith et al., 1988; Thomsen et al., 1990; Hemmati-Brivanlou and Melton, 1992; Dyson and Gurdon, 1997). Both of these gene families are also important in endoderm differentiation (Cornell et al., 1995; Henry et al., 1996; Zorn et al., 1999).

As the TGF- β factor activin is capable of inducing a full range of early mesodermal genes and cell types, it has frequently been used in studying gene promoter activation. Activin and other TGF- β factors signal by direct phosphorylation of Smad proteins (Derynck et al., 1996; Graff et al., 1996) and association with cellular transcription factors (Wotton et al., 1999; for a review see Zhang and Derynck, 1999). The receptor-activated Smads (Smads 2 and 3) are phosphorylated by activated activin and TGF- β receptor type I kinases, respectively, enabling them to form heterooligomers with the DNA binding ‘common’ Smad (Smad 4; Lagna et al., 1996; Wu et al., 1997). These Smad complexes then travel to the nucleus, form protein-DNA complexes with other transcription factors like FAST proteins (in the case of activin and TGF- β s; Chen et al., 1996, 1997; Labbé

* Corresponding author. Wellcome CRC Institute, Tennis Court Road, Cambridge CB2 1QR, UK. Tel.: +44-1223-334-090; fax: +44-1223-334-185.

E-mail addresses: ryank@email.chop.edu (K. Ryan), j.gurdon@welc.cam.ac.uk (J.B. Gurdon).

¹ Present address: Children’s Hospital of Philadelphia and the University of Pennsylvania School of Medicine, Department of Cardiology, 34th Street and Civic Center Boulevard, Abramson Research Building #704F, Philadelphia, PA 19104, USA.

et al., 1998) and with p300/CBP (Feng et al., 1998; Janknecht et al., 1998; Poupponnot et al., 1998), where they activate target genes.

A number of early *Xenopus* gene promoters have been analyzed to date, among which the ones most relevant to this study are those expressed in the mesoderm and endoderm (see below). The activin response gene *Mix.2* is expressed throughout the endoderm and mesoderm (Huang et al., 1995; Vize, 1996). Its promoter contains an activin responsive element (ARE; Chen et al., 1996) that mediates signaling through the Smad/FAST pathway (Chen et al., 1997). Another mesendodermal gene, *Bix.1*, appears to be regulated by *Xbra* and *VegT/Apod* (Tada et al., 1998). *Xbra* is pan-mesodermal, and its promoter, like the endogenous gene, responds to FGF and to low doses of activin, but is inhibited by high activin concentrations (Latinkic et al., 1997) or by *gsc* (Artinger et al., 1997). Consistent with this modulated *Xbra* response, there appear to be at least several co-operative cis-acting regulatory regions within the *Xbra* promoter (Latinkic et al., 1997). A general theme in *Xenopus* promoter analysis to date is a lack of extensive sequence similarity among the various AREs (and activin responsive regions) that have been identified.

Eomes is a crucial early gene in *Xenopus* mesoderm differentiation (Ryan et al., 1996), and its recently cloned mouse homologue (Wattler et al., 1998) is required for the epithelial-mesenchymal transition which characterizes primitive streak and node formation (Russ et al., 2000). *Eomes* is also expressed later in development in the forebrain (Ryan et al., 1998; Ciruna and Rossant, 1999; Hancock et al., 1999; Kimura et al., 1999), where it is important in neuronal differentiation (Ryan and Gurdon, in preparation). *Eomes* encodes a transcription factor of the T-box family, of which *brachyury* (Smith et al., 1991) is the founding member. It is among the few earliest mesoderm-specific genes, and can ectopically activate most early mesodermal genes, suggesting a role at the head of a mesoderm gene cascade. Its requirement for mesoderm development is indicated by the effect of a dominant negative *Eomes-engrailed* fusion construct (Ryan et al., 1996).

A particularly interesting feature of early *Eomes* induction is its concentration-dependent responsiveness to activin. The early expression of *Eomes* is in a dorsal to ventral gradient across the future mesoderm. Animal cap cells show the same graded response when treated with increasing concentrations of activin, but this gene does not respond to eFGF or early Wnt signalling (Ryan et al., 1996). This situation is an exceptionally simple system in which to analyze the mechanism by which cells make a concentration-related response to a known signalling molecule or morphogen. Previous work from this laboratory has shown that cells respond to three-fold increases in activin concentration by only a three-fold difference in receptor occupancy (Dyson and Gurdon, 1998) and by a three-fold difference in nuclear Smad concentration (Shimizu and Gurdon, 1999). The desirable next step in this analysis is to identify the

region of an early mesoderm gene promoter that shows a concentration-related response to activin.

In order to achieve this, we have made a detailed investigation of the promoter of *Xenopus Eomesodermin*. We have defined a particular region of its promoter that gives correct developmental expression and that also shows a normal pattern of response to activin concentration.

2. Results

2.1. A genomic clone represents an expressed *Eomes* gene

We used a probe generated from the first 200 bp of the *Eomes* cDNA (Ryan et al., 1996) within the 5' untranslated region (UTR) to screen a *Xenopus* muscle genomic library cloned in Lambda FIX[®]II (Stratagene). Screening 2 million clones from this library yielded four positive clones (numbered 1, 3, 5 and 8). Sequencing of clones 3 and 5 upstream of the *Eomes* 5' UTR revealed a nearby TATA box (Fig. 1A, underlined). We therefore used PCR to generate RNase protection clones spanning the region of the putative start site. Using these probes in an RNase protection assay, we find that each of the four genomic clones (1, 3, 5 and 8) represents a gene that is expressed in *Xenopus* development (Fig. 1B, stage 11), and that the protected fragment lengths were as expected. Furthermore, these expressions display the same temporal kinetics of accumulation as the original *Eomes* mRNA (Ryan et al., 1996, 1998) (Fig. 1B). Further sequencing data revealed a 99% identity within the coding region near the transcription start site of clone 3 compared to the original *Eomes* cDNA, which we therefore chose for further analysis.

To more precisely define the start site of *Eomes* transcription, we used primer extension analysis (Fig. 1C, lane PE) employing three antisense primers (EP59, 60, 61) each 10 nucleotides apart within the *Eomes* 5' UTR. In agreement with the RNase protection assay results, each primer gave the expected length extension product, locating the start of transcription to an adenosine residue (Fig. 1A, +1 under thick arrow). Depending on the primer used, a major stop was alternately found at T (Fig. 1A, thick arrow alone). These data indicate that the TATA box is located at -34. In conclusion, *Eomes* genomic clone 3 is expressed with the same kinetics as the original *Eomes* mRNA, and therefore represents a good candidate for an *Eomes* gene.

2.2. Structure of an *Eomes* genomic clone

We sequenced nearly the entire 14 kb *Eomes* genomic clone 3, including 8.5 kb of promoter (GenBank accession number AF179418), revealing a typical RNA polymerase II (pol II) promoter structure near the start site of transcription; the *Eomes* gene contains, in addition to the TATA box, two CCAAT boxes at -178 and -213 (Fig. 1A, underlined). Mapping studies (data not shown) revealed a number of restriction enzyme sites present within the 8.5 kb promoter

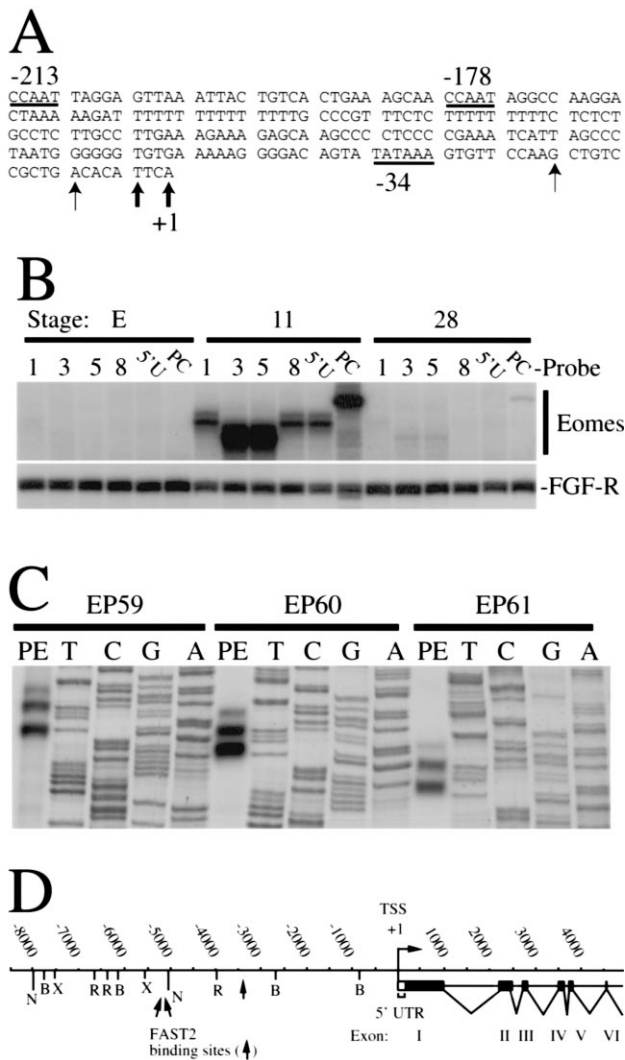


Fig. 1. Several *Eomes* genomic clones represent genes expressed in *Xenopus* development. (A) *Eomes* genomic sequence (sense strand) flanking the transcription start site reveals a TATA box (−34) and two CCAAT boxes (−178, −213). The transcription start site is marked by +1 and thick arrows; thin arrows mark weak reverse transcriptase stops. (Note that the sequence data shown on the gel in (C) is antisense, while that shown in (A) is sense strand.) (B) RNase protection analysis of several independent *Eomes* genomic clone isolates using probes spanning the transcription start site. Clones are numbered 1, 3, 5 and 8 (above lanes). Control probes 5′U (*Eomes* 5′ UTR), and PC (*Eomes* 3′ UTR) are indicated above lanes. RNA was isolated from eggs (E), mid-gastrula (11) or tailbud (28) embryos (stages shown above lanes). (C) Primer extension confirms results in (B) and defines the start site of transcription. Antisense primers EP59, 60 and 61 are each approximately 10 nucleotides apart. Sequencing reactions (lanes T, C, G, A) using these same primers are shown to the right of primer extension (lane PE) products. (D) Structure of *Eomes* genomic clone 3. The *Eomes* gene contains 8.5 kb of promoter sequence upstream of the +1 transcription start site (TSS; horizontal arrow). The 5′ UTR (open box) is 222 bp long. Clone 3 contains 4.9 kb of the *Eomes* coding region containing exons I through VI (filled boxes), introns I through V (bracketed by inverted carets) and part of the sixth intron. Exons I through VI encode the N-terminal two-thirds of the *Eomes* protein. *EcoRI* (R), *BamHI* (B), *NcoI* (N) and *XbaI* (X) sites are shown. Vertical arrows indicate FAST2 binding sites.

region. The 5.0 kb of *Eomes* coding region present within clone 3 contains a 222 bp 5′ UTR and the first six exons of the *Eomes* gene encoding the N-terminal two-thirds of the *Eomes* protein (Fig. 1D). The *Eomes* intron/exon structure was deduced by comparing its sequence to that of the *Eomes* cDNA. In each case canonical GT/AG splice donor/acceptor sites were found flanking each intron. We found that exons I through VI (spanning 1537 bp) were 99% identical overall to the original *Eomes* cDNA. Based on temporal expression kinetics (discussed above; Fig. 1B) and coding sequence, we conclude that genomic clone 3 represents an expressed *Xenopus Eomes* gene.

2.3. The *Eomes* promoter can be used to drive a heterologous globin reporter gene

To test whether the *Eomes* 5′ flanking genomic sequence could be used to drive the expression of a heterologous reporter gene, we fused the 8.5 kb *Eomes* promoter 5′ to the coding region of the human β -globin gene, which starts at the globin initiator methionine and includes all three β -globin exons (Fig. 2A, top diagram) (Mohun et al., 1989; Howell and Hill, 1997). We injected *Eomes-globin* plasmids into the animal pole of *Xenopus* embryos at the one- to two-cell stage and allowed these embryos to develop until a mid-blastula stage (Fig. 2B). Animal pole cells (animal caps) were dissected, and either treated with activin protein or left untreated until a mid-gastrula stage, when they were assayed for gene transcripts. As a control for levels of injected plasmid gene expression, we used the constitutively active EF1- α promoter fused to human globin from which the last 50 bp of β -globin exon II have been removed (Mohun et al., 1989) (Fig. 2A, bottom diagram). These injected plasmids drive expression of human globin RNA in activin-treated animal caps, yielding five different globin RNA fragments in an RNase protection assay (Fig. 2C). For plasmid DNA and unspliced RNA, *Eomes-globin* yields a 434 nucleotide (nt) protected fragment (Fig. 2A, see numbered segments above and below globin), while *Ref-globin* fragments are 50 bp smaller at 384 nt. For correctly spliced RNA, the missing introns are degraded in the probe. This leaves two protected fragments per reporter, one corresponding to each exon. Globin exon I is common to both constructs, and yields a 94 nt protected probe fragment. *Eomes-globin* exon II produces a 208 nt fragment, and *Ref-globin* 158 nt. For simplicity we henceforth show only the 208 and 158 nt fragments.

2.4. The *Eomes* promoter contains an activin response element

In order to locate any element(s) acting in cis in the *Eomes* response to activin, we generated an initial 5′ deletion series in *Eomes-globin* by digestion with appropriate restriction enzymes, yielding 6.5, 4.0, 1.8 kb and 253 bp promoter constructs. We tested these constructs in animal caps that were either treated with 32 U/ml (Green et al.,

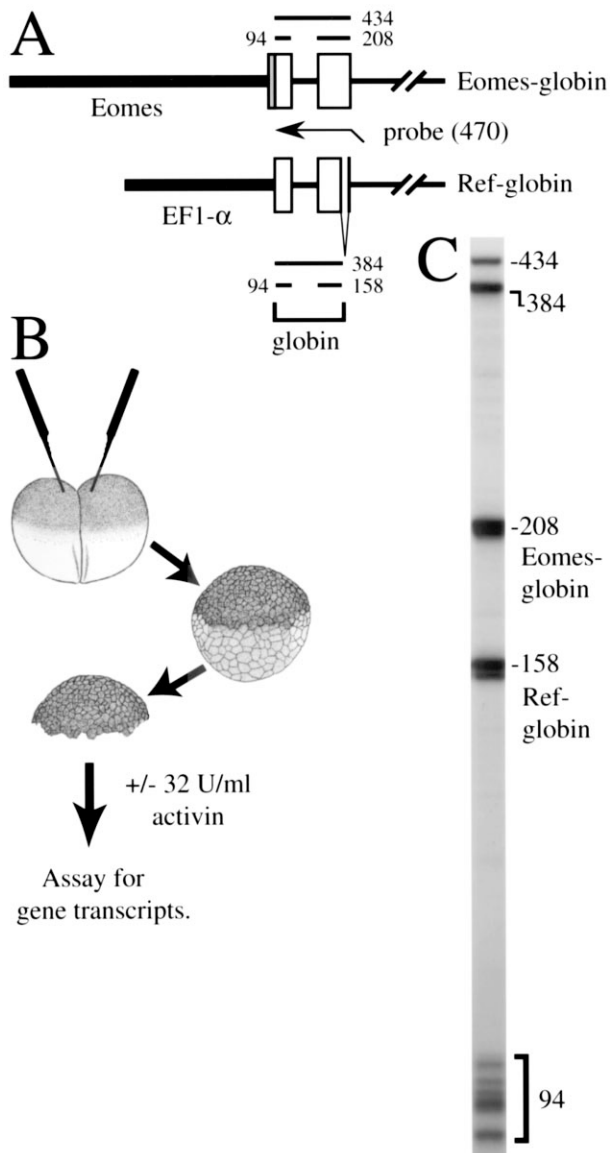


Fig. 2. *Eomes* promoter-reporter construction. (A) The 8.5 kb *Eomes* promoter was fused to the human β -globin gene (*Eomes-globin*) at *Asp*I (+107). The shaded box demarcates 107 bp of the *Eomes* 5' UTR. The constitutive, ubiquitous EF1- α promoter was fused to a deleted globin gene that is missing 50 bp from the 3' end of globin exon II (*Ref-globin*). The antisense RNase protection probe used contains 36 nucleotides of vector (5' end), and includes all of globin exons II through I. Expected RNase digestion fragments (see text) are shown as numbered bars above and below globin. (B) Supercoiled plasmids were injected at the one- to two-cell stage. Animal pole explants (caps) were made at mid-blastula and either treated with 32 U/ml activin or left untreated. (C) Injected caps assayed for globin transcripts yield 434 and 384 base fragments from DNA and unspliced RNA, and a 94 base fragment from exon I (mRNA). *Eomes-globin* mRNA yields a 208, while *Ref-globin* a 158, base exon II fragment.

1992; Howell and Hill, 1997) activin protein or left untreated. To do this, we injected as DNA a mixture of the 8.5 kb (or each deleted) *Eomes-globin* construct plus *Ref-globin* as described above. RNA was prepared as previously described and used in an RNase protection assay (Ryan et al., 1996) for either human globin or

Eomes, *Xbra* and FGF-receptor (*FGF-R*) transcripts. The results indicate that sequences between -6.5 and -4.0 kb fully confer activin responsiveness to the *Eomes* promoter; the 6.5 and 8.5 kb promoters are equivalently active within error, while the 4.0 kb promoter is unresponsive to activin (Fig. 3A; quantitation and diagram of deletions shown in

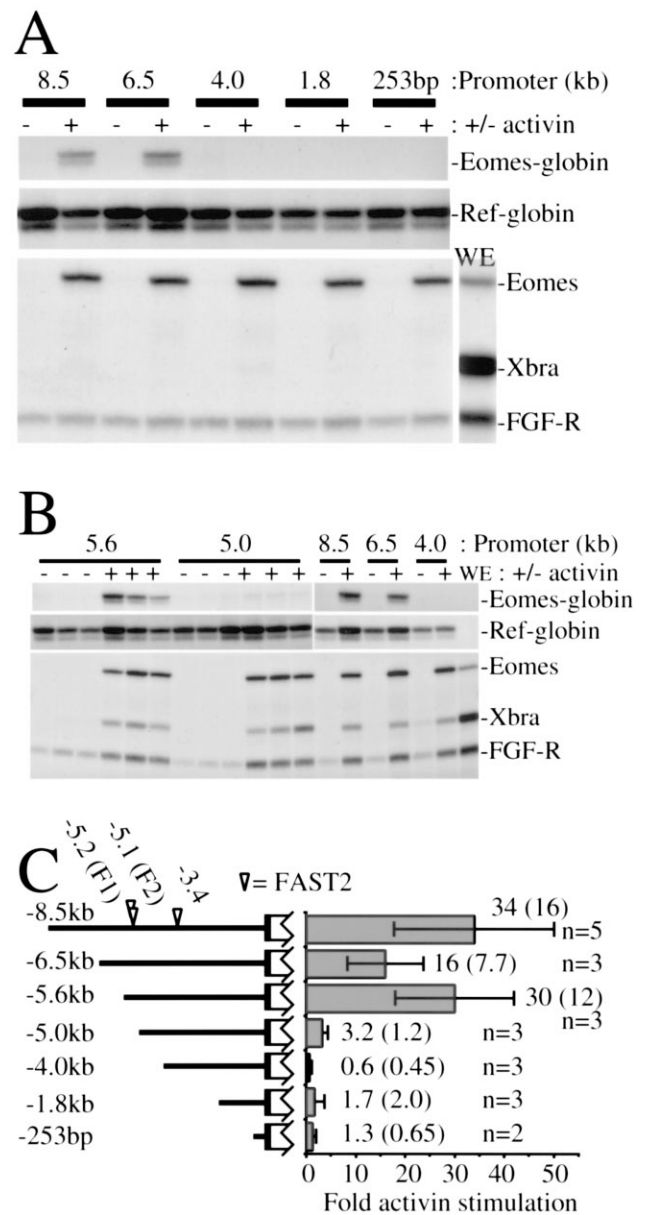


Fig. 3. Activin responsiveness is conferred by DNA element(s) located between -5.6 and -5.0 kb within the *Eomes* promoter. (A) An initial deletion series reveals an activin response element (ARE) between -6.5 and -4.0 kb. (B) The *Eomes* ARE is located between -5.6 and -5.0 kb. Endogenous gene transcripts (A,B) confirm activin responsiveness of caps. Promoter constructs and treatment \pm activin (32 U/ml) are shown above the lanes. Unlike *Eomes*, high doses of activin yield variable *Xbra* transcription in intact caps; hence, *Xbra* is mostly not seen in (A). (C) Quantitation confirms the results in (A,B). Promoter lengths are shown to the left, with levels of *Eomes-globin* activation (\pm 1 SD in parentheses) to the right. The number of independent measurements (*n*) for each construct is indicated. One error bar equals one standard deviation.

Fig. 3C). Similar to the 4.0 kb promoter, further deletions to the 5' end (Fig. 3A, lanes 1.8 and 253 bp) also failed to respond to activin treatment. Endogenous gene activations confirm activin treatment (shown beneath globin bands, Fig. 3A,B).

Our sequence data revealed three FAST2 binding sites (Labbé et al., 1998) within the *Eomes* promoter, at -5.2 , -5.1 and -3.4 kb, respectively (Fig. 3C, arrowheads). Two of these sites are removed by deletion to -4.0 kb, which as mentioned above also abolishes responsiveness to activin, implicating these sites in *Eomes* activation. We therefore made two further 5' end deletions, at *Xba*I (-5.6 kb) and *Nco*I (-5.0 kb), closely bracketing the two FAST2 sites. On testing these two constructs in animal caps we found that sequences between -5.6 and -5.0 kb within the *Eomes* promoter confer full activin responsiveness to the *Eomes* promoter (Fig. 3B; compare lanes 5.6 with 5.0). As a measure of reproducibility, all promoter constructs (except 253 bp) were assayed at least in triplicate, which is shown for comparison for the 5.6 and 5.0 kb *Eomes* promoters in Fig. 3B. To reduce batch variability we find it necessary to directly compare test and control constructs in eggs from the same female. Uninjected control *Xenopus* embryos contain no human β -globin transcripts, as expected (Fig. 3B, lane WE).

Quantitation of the results from several assays including the ones shown in Fig. 3A,B revealed a ten-fold mean reduction in *Eomes-globin* response to activin on deletion of sequences between -5.6 and -5.0 kb within the *Eomes* promoter (Fig. 3C). We found that the 8.5, 6.5 and 5.6 kb promoters were all activated by activin to a similar degree, and that 5' end deletions to 5.0 kb and further were all equally inactive within error.

In conclusion, we find that a 571 bp region within the distal *Eomes* promoter containing two FAST2 transcription factor binding sites is necessary for *Eomes* response to activin signalling, while a more proximal region containing a FAST2 site found at -3.4 kb has no effect on its own in this assay.

2.5. DNA elements important in *Eomes* activin responsiveness

To specifically assess the role of these distal FAST2 sites (at -5.1 and -5.2 kb, called F1 and F2, respectively) in mediating the transcriptional activation of the *Eomes* promoter in response to activin, we used PCR to generate a double point mutation in each FAST2 site individually (Fig. 4A). This double point mutation has been shown by Labbé et al. (1998) to specifically prevent FAST2 binding to the mouse *gsc* promoter and block *gsc* activation in response to TGF- β . As shown in Fig. 4B, similarly mutating either FAST2 site individually (lanes F1 μ and F2 μ , plus FAST2) results in a promoter to which in vitro translated mouse FAST2 (or *Xenopus* FAST1; Bourillot and Gurdon, in preparation) protein still binds in an electrophoretic mobi-

lity shift assay, compared to the wild type sequence (lane WT, plus FAST2). In contrast to the singly mutated constructs, when both FAST2 sites are mutated, binding is

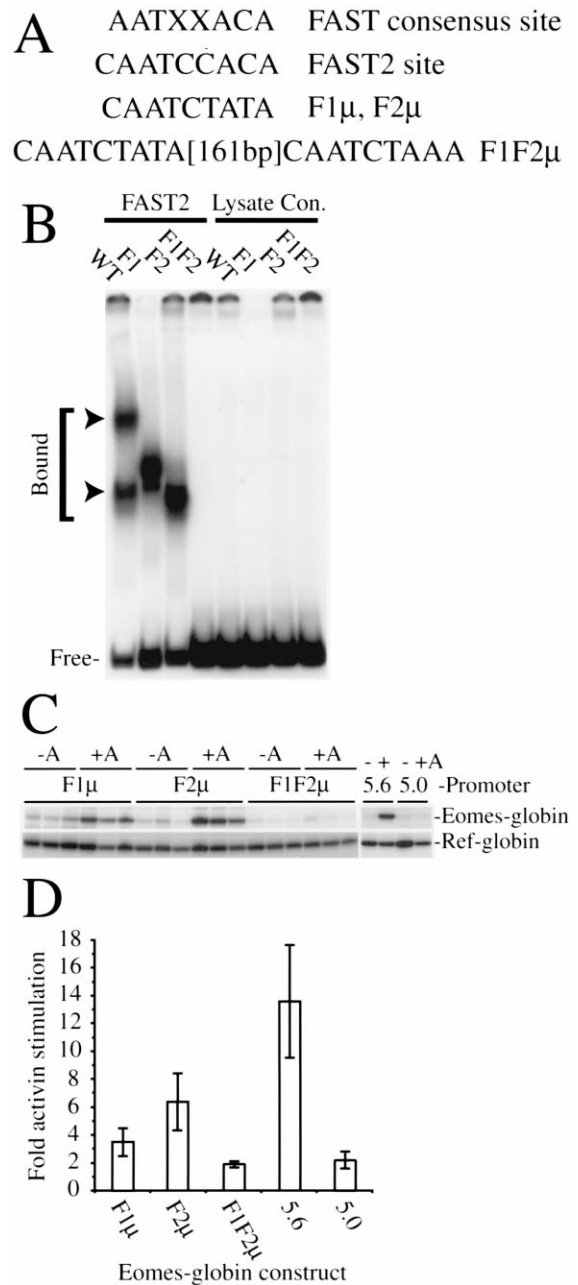


Fig. 4. FAST2 binding sites are important in the *Eomes* response to activin stimulation. (A) Double point mutations were made (sequence shown) in either distal FAST2 site alone (F1 μ or F2 μ), or in both sites (F1F2 μ). (B) Mutations in either FAST2 site alone (lanes FAST2: F1 μ , F2 μ , compared to wild type, lane WT) still bind mouse FAST2 in vitro, while mutations in both sites prevent binding (lanes F1F2 μ , FAST2 versus Lysate con.). Bound and free probe indicated to left of lanes. (C) Mutating either FAST2 site alone results in a partial response to activin, while mutating both sites eliminates response. Promoter constructs and activin treatment (\pm A) are shown above the lanes. Gene transcripts are indicated to the right of lanes. (D) Quantitation confirms the results shown in (C). Mutated constructs were verified by sequencing to contain the desired mutations.

completely abolished (Fig. 4B, lane F1F2 μ plus FAST2 versus lane Lysate con.). We note that while the WT probe yields two distinct, well-resolved complexes with FAST2 (arrowheads), the F1 μ and F2 μ probes each yield

a single shift species, perhaps reflecting their only having one intact FAST2 site. Also, the F1 μ complex migrates more slowly, and the F2 μ complex slightly more quickly than the faster of the two WT complexes (see below), perhaps due to a slightly different protein-DNA conformation in each case. Taken together, these data confirm that our mutations abolish the binding of FAST2 to either one or both FAST2 sites within the *Eomes* promoter.

On testing these mutated constructs in animal caps with or without activin, promoters containing individually mutated sites still respond to activin (Fig. 4C, lanes F1 μ and F2 μ , \pm activin [A]), but at a reduced level (normalized to *Ref-globin*) compared to that seen for the wild type promoter (lane 5.6). On average, the F1 μ construct is less active in this assay than the F2 μ construct, which correlates with their respective binding properties mentioned above. However, we feel that the error in measuring *Eomes-globin* promoter activations in caps precludes a definitive correlation between transcriptional activity and binding for these two constructs (see below, Fig. 4D).

In contrast with individual mutations, when both FAST2 sites are destroyed we obtain an activin non-responsive *Eomes* promoter indistinguishable from the control 5.0 kb construct missing these sites (Fig. 4C, compare lanes F1F2 μ to 5.0). Quantitation of the results shown in Fig. 4C confirms these results (Fig. 4D). (As before, activin treatment of animal caps was confirmed by RNase protection analysis for endogenous gene transcripts; data not shown.) We conclude that each FAST2 site at -5.1 or at -5.2 kb can at least partially compensate for the loss of the other site, in conferring activin responsiveness to the *Eomes* promoter, and that at least one FAST2 site in this position is absolutely required for *Eomes* activation in response to activin.

We noticed that each activin responsive FAST2 site (Fig. 5A, F1 and F2; Fig. 5B, thick underlines) is embedded within a 43 bp imperfect direct repeat (Fig. 5B, arrows). These repeated sequences are separated by unrelated sequence in three blocks of 43 bp each. In an attempt to more sharply define the minimal *Eomes* activin response element (ARE), we truncated the *Eomes* promoter at -5.3 kb, at the beginning of the first 43 bp repeat (Fig. 5A,

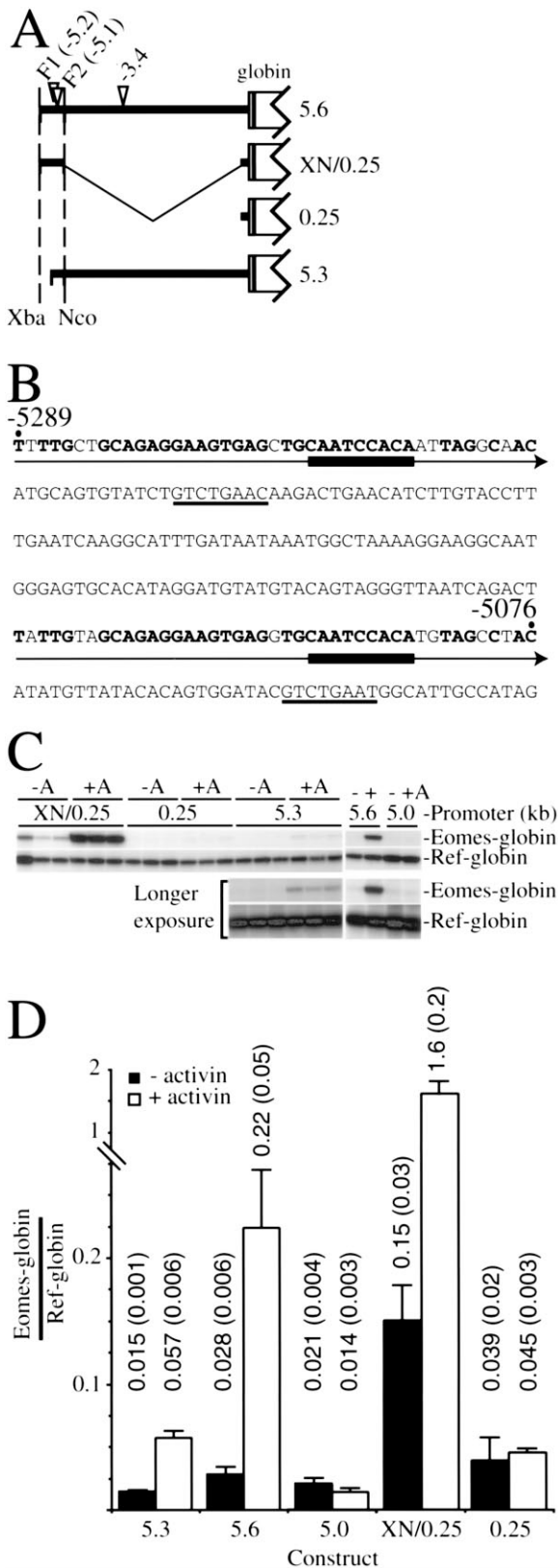


Fig. 5. The *Eomes* ARE can confer activin responsiveness to a minimal promoter; a repressor element is located between -0.25 and -5.0 kb; the upstream half of the ARE is important for its activity. (A) Diagram of deletion constructs. 5.6, wild type; XN/0.25, internal deletion; 0.25, minimal promoter; 5.3, 5' end deletion. Dashed vertical lines demarcate the *Eomes* ARE. The inverted caret marks the internally deleted segment. X, *Xba*I; N, *Nco*I. Arrowheads indicate FAST2 sites. (B) The *Eomes* ARE contains two direct repeats (arrow underlines) encompassing FAST2 sites (thick underlines). Potential Smad binding elements are underlined. Nucleotides in bold indicate identities between the repeats. (C) *Eomes-globin* constructs shown in (A) assayed in caps \pm activin (shown above lanes). 5.6 and 5.0 controls are shown in the last 4 lanes. Gene transcripts are shown to the right of lanes. (D) Quantitation of (C). Levels of *Eomes-globin* normalized to *Ref-globin* are shown above the columns; 1 SD (parentheses).

construct 5.3, truncated at nucleotide -5289 (Fig. 5B)). This retains both 43 bp repeats. We find that the 5.3 kb *Eomes-globin* construct yields a low absolute level of activin stimulated response in animal caps compared to the 5.6 kb promoter (Fig. 5C, lanes 5.3 versus 5.6, +activin; longer exposure shown to verify 5.3 response). Despite this, the 5.3 kb promoter (containing both FAST2 sites and direct repeats) is still about half as activin-responsive as the parental 5.6 kb one (Fig. 5D, 5.3 versus 5.6, compare plus/minus activin ratio). Quantitation further indicates a two-fold reduced baseline activity of the 5.3 kb promoter (Fig. 5D, 5.3 versus 5.6, both minus activin). These results suggest that the missing sequences between -5.6 and -5.3 kb are more important for increasing the pol II initiation rate than for activin response per se. A more precise definition of the minimal *Eomes* ARE is currently underway.

A visual inspection of the *Eomes* promoter sequence flanking the ARE reveals two potential Smad binding elements (SBEs) as identified by Zawel et al. (1998). The first SBE is located 24 bp $3'$ of the F1 FAST2 site, while the second is 33 bp downstream of F2 (Fig. 5B, underlined). These sites consist of a Smad box (GTCT; Shi et al., 1998) followed by GAAC (F1) or GAAT (F2), respectively (see Stroschein et al., 1999). This is consistent with a requirement for FAST and supports the view that *Eomes* is activated via the Smad/FAST pathway (see Section 3).

In sum, at least one FAST2 site is required for *Eomes* responsiveness to activin, and an important stimulatory element is located upstream of the FAST2 sites between -5.6 and -5.3 kb. We now test whether the intervening sequences between the FAST2 sites and the proximal promoter region at -0.25 kb (which represents a minimal, activin non-responsive promoter) affect activin responsiveness. Specifically, we wished to know if the *Eomes* ARE could confer activin responsiveness to a minimal *Eomes* promoter.

2.6. The *Eomes* ARE confers activin responsiveness to a minimal promoter

In order to test whether the *Eomes* ARE itself is sufficient for activin responsiveness, we placed the ARE *XbaI-NcoI* fragment (-5.0 to -5.6 kb) immediately upstream of -0.25 kb and tested this construct (called XN/0.25; Fig. 5A) in our assay. As shown in Fig. 5C, the *Eomes* ARE when used to drive a minimal promoter is activin hyper-responsive (XN/0.25 versus 5.6, lane +A), while the control construct with only 0.25 kb of promoter is unresponsive to activin (lane 0.25 \pm A). However, the baseline level of transcription occurring on XN/0.25 is elevated significantly (lane $-$ A). This result is consistent with the loss of a repressor element, and was confirmed by quantitation (Fig. 5D). Interestingly, the level of baseline transcription driven by the internally deleted XN/0.25 construct (Fig. 5C, lane $-$ A) is nearly as high as the activated level seen for the intact 5.6 kb *Eomes* promoter plus activin (lane +A). However, the

activin responsiveness of XN/0.25 is left unchanged relative to 5.6 kb at about ten-fold (Fig. 5D, compare plus/minus activin ratio). We conclude that our data are consistent with the idea of a repressor element or elements located between -5.0 and -0.25 kb within the *Eomes* promoter.

However, the elevated baseline transcription seen in untreated caps for the XN/0.25 construct raises the possibility that we have simply created an artifactually 'leaky' promoter instead of a bona-fide de-repressed one. To distinguish between these two possibilities, we turned to a different kind of assay which would allow us to assess the XN/0.25 construct independently of the animal cap assay, in a more physiologically relevant context. Such an assay would be provided in normal, untreated embryonic cells, left to develop undisturbed in situ in their natural context within the embryo. In the case of a generally leaky promoter, we might expect to see a baseline transcriptional level that is similarly elevated to the level seen in untreated caps. This elevated baseline might be expected to occur in (untreated) cells located in different regions within intact embryos irrespective of their location. In contrast, in the case of a missing repressor, one might not expect all cells to be equal in respect of this construct. To determine whether this approach is feasible, we first need to examine whether a fully active *Eomes-globin* construct is active in embryonic cells when they are left undisturbed and allowed to develop normally.

2.7. 5.6 kb of the *Eomes* promoter confers regionally restricted *Eomes-globin* expression

Having determined that 5.6 kb of upstream *Eomes* promoter sequence is sufficient for full activin responsiveness, we wished to know whether this promoter segment was also sufficient in mimicking the normal expression pattern seen for *Eomes*. To do this, we injected 5.6 kb *Eomes-globin* mixed with *Ref-globin* into cells located in different tiers along the animal vegetal axis of 32-cell embryos (tiers A through D, Fig. 6A) (Watabe et al., 1995). Tier A cells are mostly fated to become neuroectoderm and epidermis which do not express *Eomes* during gastrulation (in the absence of added activin), and therefore should not express injected *Eomes-globin*. Likewise, tier D cells predominantly represent presumptive endoderm, and left undisturbed in situ in injected embryos also should not activate *Eomes-globin* expression. In contrast, the equatorial tier C cells are mostly fated to become mesoderm, with the exception of the dorsal most blastomere, which is fated to form dorsal endoderm (Slack, 1991). Both mesodermal and some dorsal endodermal cells express *Eomes* at gastrulation (Ryan et al., 1996; Ryan and Gurdon, in preparation); hence, tier C cells would be expected to strongly activate *Eomes-globin* constructs should these contain all the necessary information for mesoderm-specific expression.

On injection of the 5.6 kb *Eomes-globin* construct into four representative blastomeres of tiers A, C and D, and

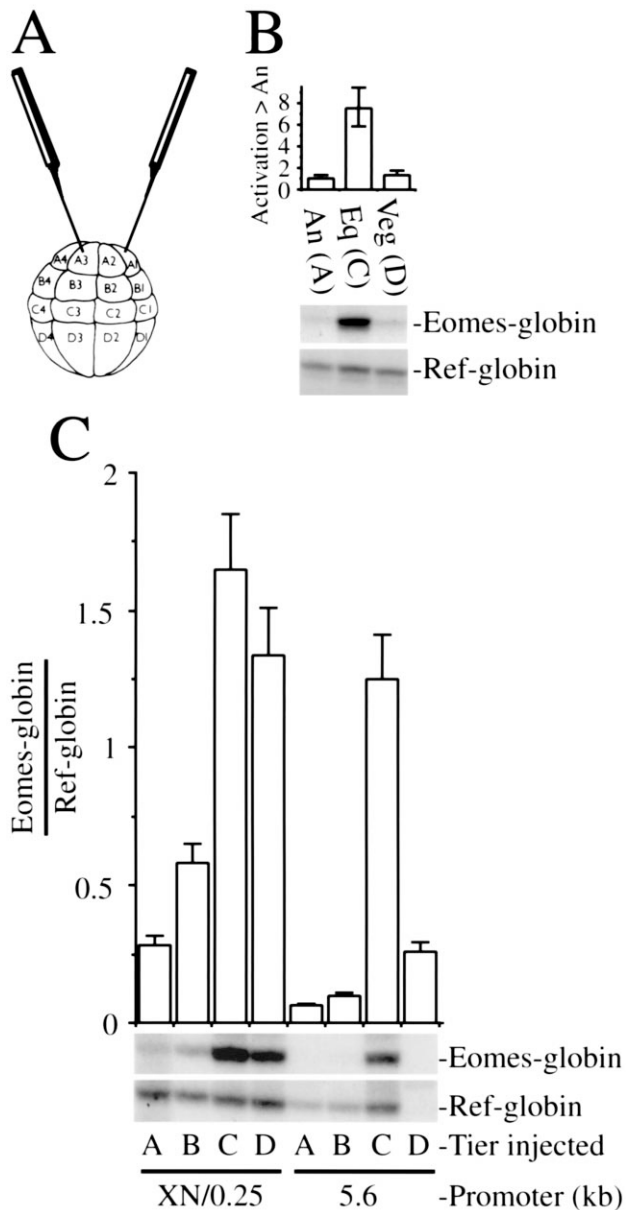


Fig. 6. Deletion of the *Eomes* repressor allows *Eomes-globin* expression in the endoderm. (A) A mixture of 5.6 kb *Eomes-globin* plus *Ref-globin* was injected into four representative blastomeres of tiers A, C and D at the 32-cell stage. (B) Injected embryos assayed at mid-gastrula stage for globin transcripts; quantitation shown above lanes. *Eomes-globin* expression is restricted to the mesoderm in injected embryos. (C) Constructs shown beneath, gene transcripts and tiers indicated to the right of lanes. Quantitation shown above lanes. The low level of globin transcripts seen in lane 5.6, D tier, gave a similar result, on quantitation, to Fig. 5B.

allowing the injected embryos to develop until a mid-gastrula stage (11), we find that *Eomes-globin* is strongly expressed in the tier C-injected embryos, but not in tiers A and D (Fig. 6B), approximating the normal mesodermal expression of the endogenous *Eomes* gene (Ryan et al., 1996). We conclude that the 5.6 kb *Eomes* promoter contains all the necessary information for mesodermal expression in this assay. To assess the significance of the

elevated baseline transcriptional levels seen for XN/0.25 in caps, we now ask whether this construct yields globally high baseline levels in whole embryos, or if its expression is instead regulated.

2.8. A repressor element extinguishes *Eomes* expression in endoderm

On injection of a mixture of XN/0.25 plus *Ref-globin* into different tiers of 32-cell embryos (as described above), we obtain the result that this construct is not expressed at equal levels in all cells. Instead, it is expressed at high levels after injection into the C and D tiers, relative to A and B tier-injected cells (Fig. 6C). In addition, the elevated levels seen for XN/0.25 in the C and D tier-injected whole embryos do not represent a hyper-activation, unlike the levels seen in injected, activin-treated caps (Fig. 5C,D). This result indicates that the high baseline level of XN/0.25 in untreated caps is unlikely to be attributable to an artifactually leaky promoter. It is much more likely that the high baseline in caps reflects the loss of a repressor element that would normally be required to block inappropriate *Eomes* expression in endoderm. This interpretation gains further weight given a wealth of evidence to show that TGF- β signalling is highly active throughout the vegetal hemisphere in early *Xenopus* embryos (for a particularly relevant example see Watabe et al., 1995). We conclude that we have identified a region within the *Eomes* promoter that behaves as an endoderm-specific element, and suggest that it normally works to block inappropriate *Eomes* transcription in these cells. Determination of the minimal repressor DNA element and its cognate target protein(s) is currently in progress. More importantly for the main thrust of this study, we further conclude that the *Eomes* ARE is necessary and sufficient for a high-dose responsiveness of the *Eomes* promoter to activin.

2.9. The *Eomes* promoter responds directly to activin signalling

One essential feature expected of a gene that directly transduces morphogen gradient signals is that it takes place directly, i.e. without intermediary gene activations. *Eomes* falls into this class of genes, as it is an immediate-early response to activin mesoderm induction, occurring in the absence of protein synthesis (Ryan et al., 1996). Hence, it is essential that the *Eomes* promoter under study should reflect this behaviour. To examine this, we tested whether *Eomes-globin* transcription can occur in response to activin in the presence of cycloheximide (Cascio and Gurdon, 1987). Fig. 7A shows that *Eomes-globin* makes an immediate-early response to a mesoderm inducing treatment with activin, even in the presence of cycloheximide (lane CA, compare *Eomes-globin* to *Eomes*). *Chordin* (*chd*) transcription is dependent on protein synthesis (Sasai et al., 1994) and is shown as a control for cycloheximide effectiveness. Early *Xbra* transcription is known to occur in the absence of



Fig. 7. *Eomes-globin* responds to activin in an immediate-early and dose-dependent way. (A) Injected caps were either left untreated (lane -) or treated with: 5 μ g/ml cycloheximide (CHX; lane C), activin (13 U/ml; lane A), or CHX plus activin (lane CA). For this experiment, 13 U/ml activin gave a 22-fold increase in *Eomes-globin* and a 43-fold increase in *Eomes* (lane CA). Endogenous gene transcripts confirm activin and CHX treatments. (B) Injected caps were dissected and treated at stage 8 with different concentrations (shown above lanes) of activin protein, or left untreated. Reporter and endogenous gene transcripts are shown to the right of the lanes. (C) Quantitation of the gel in (A) shows fold stimulation above nil for *Eomes-globin* (boxes), and endogenous *Eomes* gene response above nil in these same caps (crosses). Input dose of activin (abscissa) and *Eomes* level in uninjected whole embryos (WE; *Eomes* normalized to *FGF-R*, horizontal line) are shown. Arrows in (B,C) signify that this horizontal line derives from the indicated single band, normalized to *FGF-R*, on gel.

protein synthesis (Smith et al., 1991) and is shown as a positive control, while *Ref-globin* and the maternal level of *FGF-R* are unaffected by these treatments and are shown as loading controls. We conclude that *Eomes-globin*

faithfully reproduces the direct response of *Eomes* to activin treatment.

2.10. The *Eomes* ARE is sufficient to confer activin concentration responsiveness

A mixture of the 8.5 kb *Eomes-globin* construct plus *Ref-globin* injected and analyzed as above shows that *Eomes-globin* responds to mesoderm induction in a dose-dependent way, and that the level of *Eomes-globin* correlates well with the dose of activin used (Fig. 7B,C, compare boxes and circles). Furthermore, *Eomes-globin* normalized to *Ref-globin* (Fig. 7C, boxes) very closely approximates *Eomes* normalized to *FGF-R* (Fig. 7C, crosses) for each dose of activin used. We conclude that 8.5 kb *Eomes-globin* contains all of the cis-acting elements required to faithfully reproduce the same activin-response kinetics seen for the endogenous *Eomes* gene.

To determine whether the *Eomes* ARE alone is sufficient to confer activin responsiveness over a wide range of activin doses, we repeated the cap assay using the XN/0.25 construct, and compared the results with those using the complete 8.5 kb promoter. To further assess reproducibility, we also used a different batch of activin protein (see Section 4) for each test. This entailed measuring the actual dose, in each case, of activin received by treated caps, by both morphological (Green et al., 1992) and molecular (as described above) criteria. The results indicate that the *Eomes* ARE can confer activin dose responsiveness over the full range of doses tried (Fig. 8A), with the exception of the highest dose (see below). The 8.5 kb *Eomes-globin* control indicates that although there are quantitative differences among different batches of injected eggs in the relative levels of *Eomes* and *Eomes-globin* seen in response to activin treatment (compare Figs. 8B and 7C), *Eomes-globin* dose responsiveness still very closely parallels that of *Eomes*. We note that at the highest dose of activin treatment (200 U/ml) using XN/0.25-injected cells, *Eomes-globin* responds at a significantly higher level than might be expected for *Eomes*. This is likely to be due to the induction of endoderm at the expense of mesoderm in these caps, because of the very high dose of activin used in this instance. In this case *Eomes-globin* is free to respond fully to activin since it lacks the endodermal repressor element, while *Eomes*, with its intact promoter, responds at a lower level due to repression in the induced endodermal cell environment. We conclude that the *Eomes* ARE is sufficient to provide the full range of activin concentration responsiveness to activin shown by this gene.

3. Discussion

We have isolated an *Eomes* genomic clone with overall 99% identity within the coding region to the original *Eomes* cDNA and mapped the 5' end of the *Eomes* mRNA to a position 34 bp downstream of the TATA box. Fusion of

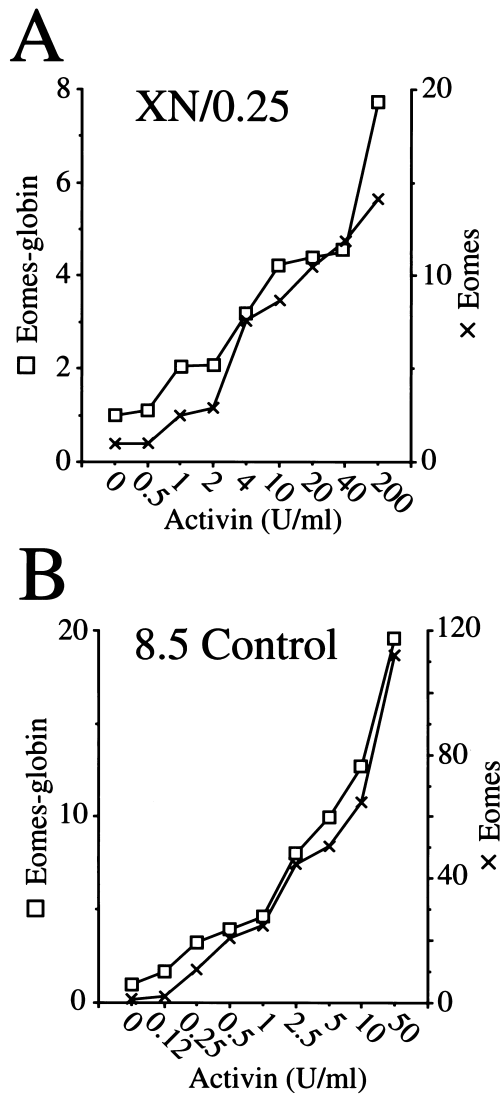


Fig. 8. The *Eomes* ARE is sufficient to confer activin dose responsiveness to a minimal promoter. (A) XN/0.25 *Eomes-globin* (boxes) and *Eomes* (crosses) stimulation in response to different doses of activin (abscissa). (B) 8.5 kb *Eomes-globin* control caps presented as in (A).

the *Eomes* promoter to the human β -globin reporter gene provides an in vivo assay for *Eomes* promoter function (Mohun et al., 1989; Howell and Hill, 1997). Deletion of upstream promoter sequences reveals an activin response element (ARE) within the *Eomes* promoter, located between -5.6 and -5.0 kb. This 571 bp DNA fragment contains two binding sites for mouse FAST2, and PCR site-directed mutagenesis reveals that at least one of either of these sites is required for response to activin. Internal deletion of sequences between -5.0 and -0.25 kb within the *Eomes* promoter reveals (1) that an endodermal repressor element, which resides in the promoter region between -0.25 and -5.0 kb, confines *Eomes* expression to the mesoderm and (2) that the *Eomes* ARE is necessary and sufficient for the activin concentration responsiveness of this gene.

Eomes is unique among *Xenopus* gene promoters

analyzed to date in its having a strong activator (the ARE) separated from a strong repressor, where the function is to control germ layer-specific gene expression. Other repressors that have been identified in *Xenopus* promoters are found in genes that are restricted to the dorsal mesoderm (organizer) such as *Xlim-1* (Rebbert and Dawid, 1997) and *XFD-1'* (Friedle et al., 1998). These genes differ from *Eomes* in that they repress expression in ventral mesoderm. In *Xlim-1* an intronic repressor is thought to restrict the otherwise constitutive and activin non-responsive activity of *Xlim-1* to the organizer, while for *XFD-1'* a BMP inhibitory element performs this same function through Xvent-1 binding.

The concentration dependence of *Eomes* expression is closely paralleled by *Eomes-globin*, and requires only the *Eomes* ARE as a mediator of activin signalling, a finding which has yet to be reported for other genes. The *Eomes* ARE has a close juxtapositioning of two FAST protein binding sites, and is the first such ARE in which two closely spaced FAST sites are of demonstrable importance in activin signalling. This close spacing of FAST sites may be a critical determinant in encoding a graded gene response at the promoter level to TGF- β like signalling, and hence would have important implications in explaining how morphogen gradients work at the molecular level. We are thus in a strong position to pursue a molecular analysis of the factors that mediate this tight regulation in response to an experimental morphogen gradient such as can be achieved using activin protein.

Based on our findings reported here, we propose a model

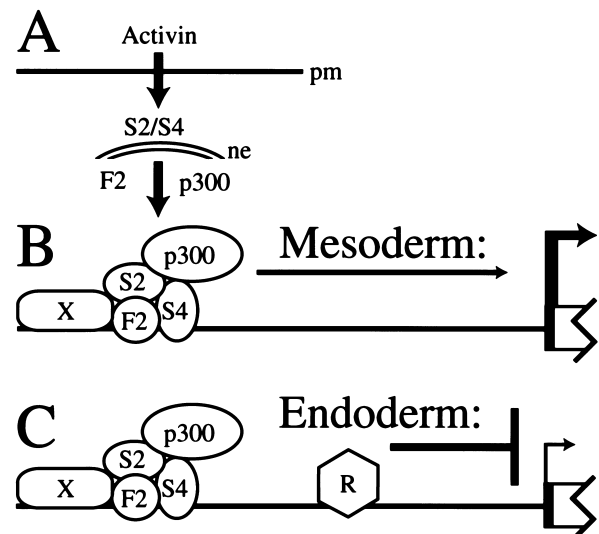


Fig. 9. Model for *Eomes* regulation in *Xenopus* gastrulae. (A) Activin signalling leads to phosphorylation of Smad2 (S2), Smad2/4 heterodimerization, and (B) association with FAST2 (F2) and p300 proteins at the *Eomes* ARE in the cell nucleus. In the mesoderm, this multiprotein complex, possibly in combination with factors interacting with the 5' half of the ARE (indicated as 'X'), activates the *Eomes* gene for transcription. (C) An endoderm-specific repressor protein(s), R, extinguishes *Eomes* expression in this tissue. pm, plasma membrane; ne, nuclear envelope.

for *Eomes* transcriptional regulation in early *Xenopus* embryos (Fig. 9). Activin (or activin-like) signalling via the Smad/FAST pathway (Fig. 9A) (Chen et al., 1996, 1997; Graff et al., 1996; Labbé et al., 1998) would be required to activate *Eomes* in the early embryonic mesoderm, and this signal would lead to the formation of a multi-protein complex at the FAST2 sites within the *Eomes* ARE. This complex would include Smads 2 and 4, FAST2, p300, and other as yet unidentified proteins (represented as X in Fig. 9) that function through interaction with the 5' half of the *Eomes* ARE (Fig. 5C,D, 5.3 construct). This enhancer complex is both necessary and sufficient for graded responsiveness to activin (Figs. 3B and 5C), and we propose that it is also important in mesoderm-specific *Eomes* activation in embryos (Fig. 9B), since *Eomes* is expressed in an equatorial dorsoventral gradient in normal embryos. In the endoderm, we propose that *Eomes* transcription is suppressed by an endoderm-specific repressor. This repressor might work as a decoy for binding to either the basal transcription machinery or to the *Eomes* ARE complex, as it interacts with a DNA element that does not physically overlap with the ARE (Fig. 9C). Thus, activin/TGF- β signalling that would otherwise inappropriately activate *Eomes* in the endoderm is blocked.

4. Materials and methods

4.1. Isolation of an *Eomes* genomic clone

Approximately 2×10^6 plaques of a *Xenopus* genomic library in Lambda FIX[®] II (Stratagene) were screened with a random primed cDNA probe corresponding to nucleotides 1–200 of the *Eomes* mRNA (Ryan et al., 1996), as described previously (Feinberg and Vogelstein, 1983). Four positive clones were identified and plaque-purified. Limited sequencing was initially performed for clones 3 and 5 within the 5' UTR and coding region. Further sequencing of clone 3 revealed a 99% identity to *Eomes* within the coding region. An apparent 7 bp deletion in genomic clone 3 (at a position corresponding to nucleotide 164) relative to the original *Eomes* cDNA sequence suggests that this clone would encode a message with a different initiator methionine, corresponding to M27 of the original decoded *Eomes* protein. Clone 3 was further analyzed by restriction mapping (data not shown) to generate the map shown in Fig. 1D. The entire 14 kb clone was sequenced with the exception of about 500 bp at the 3' end.

4.2. *Eomes-globin* reporter constructs

To place the entire 8.5 kb *Eomes* promoter upstream of β -globin (creating a plasmid called 5-656), a partially digested restriction fragment was isolated from the 14 kb *Eomes* genomic clone 3 (in pBluescript (Stratagene)) between *Asp*I at nucleotide 107 within the *Eomes* 5' UTR and a *Not*I site in lambda phage at the 5' end of the *Eomes* promo-

ter. This fragment was blunted using Klenow and cloned in the *Sma*I site of a vector called PRO6, in which we replaced the multicloning site (MCS) of pBluescript between *Not*I and *Xho*I with a new MCS (see below). The entire 8.5 kb *Eomes* promoter was then excised and cloned 5' *Pme*I to 3' *Asc*I in a vector plasmid called 4-627. To make 4-627 we cloned the PRO6 MCS blunt in –102SN-globin (Howell and Hill, 1997) that had been previously digested with *Sac*I and *Xba*I, and blunted using T4 DNA polymerase. The order of sites in the 4-627 MCS is: *Not*I, *Bss*HIII, N₆, *Pme*I, *Sma*I, *Asc*I/*Bss*HIII, N₆, *Nhe*I, *Not*I, globin; N₆ signifies a 6 bp spacer. 5' deletions were made to the *Eomes* promoter as follows. The 6.5 and 4.0 kb *Eomes-globin* deletion constructs were truncated at the corresponding *Eco*RI sites within the *Eomes* promoter by digesting 5-656 with *Pme*I, and then partially with *Eco*RI. Appropriate length partial digestion products were isolated and plasmids were re-closed using a *Pme*I-*Not*I-*Eco*RI blunt/sticky double stranded DNA adaptor. The 5.6 and 5.0 kb *Eomes-globin* constructs were made similarly, digesting with *Pme*I plus *Xba*I, or plus *Nco*I, and using the appropriate adaptor. The 1.8 kb *Eomes-globin* construct was made by excising the 1.8 kb *Asp*I fragment from a 6.5 kb *Eco*RI-partial-*Bam*HI promoter fragment (called 1-259, cloned in Bluescript), blunting this *Asp*I fragment, and re-cloning it in –102SN-globin that had been cut with *Sac*I and *Xba*I, and blunted with T4 polymerase. The 253 bp construct was similarly cloned blunt into this same vector by PCR. For the F1 μ , F2 μ , and F1F2 μ constructs, the *Xba*I-*Nco*I fragment containing the two distal FAST2 sites was PCR amplified (Expand[™] High Fidelity PCR System, Boehringer catalogue #1 732 641) using the appropriate mutant oligos, and then cloned into the *Xba*I-*Nco*I site of the 5.6 kb construct, replacing the natural *Eomes* sequence. (In this case a two-step PCR was performed, first generating upstream and downstream segments of the 571 bp *Xba*I-*Nco*I fragment, purifying these, and subsequently 'splicing' them together in a second PCR.) The XN/0.25 kb construct was made by placing the natural *Eomes Xba*I-*Nco*I fragment blunt into a PCR-engineered *Pme*I site (upstream of and adjacent to –0.25 kb) in a clone called 1-535, which was further digested using *Not*I and partially using *Bam*HI, then Klenow-blunted and re-closed. The 0.25 kb *Eomes-globin* construct was generated from 1-535 by digestion with *Pme*I, partial digestion with *Bam*HI, Klenow blunting and re-closing, and it contains nucleotides –253 to +107 of the *Eomes* gene promoter. The 5.3 kb construct was generated by PCR in which a 5'-truncated *Xba*I-*Nco*I fragment was used to replace the wild type sequence in the 5.6 kb construct. Mutated constructs were sequenced to verify that the desired mutations had been made. Ends of all other constructs were sequenced, except for the 1.8 kb one, which was restriction mapped, to verify the procedure. For each *Eomes-globin* construct, 50 pg per embryo of supercoiled plasmid DNA was mixed with 80 pg per embryo of *Ref-globin* and injected into the animal pole at the one- to two-cell stage. To ensure

obtaining supercoiled plasmids, these were prepared by standard alkaline lysis (followed by PEG precipitation; Sambrook et al., 1989), but mixing very gently during the lysis and neutralization steps. Plasmids were further purified using glass milk (Bio 101, Inc., GeneClean II Kit, catalogue #1001-400) prior to injection into *Xenopus* eggs.

4.3. Culturing embryos and producing activin protein in oocytes

Embryos and animal pole explants (caps) were cultured as described (Ryan et al., 1996); 1× MBG (0.45 μm-filtered) signifies 1× MBS containing 0.1% BSA and 25 μg/ml Gentamycin. For promoter assays, caps (15 at a time) were dissected in 1× MBG containing 0.1 mM Ca²⁺ and 0.1 mM Mg²⁺, then transferred to 1× MBG containing normal (0.4 mM) amounts of these divalent cations. Caps were either treated with 32 U/ml activin (Green et al., 1992) or left untreated, except in the case of Fig. 3C where 13 U/ml activin protein was used instead, and then cultured until the equivalent of stage 11 before freezing them in 1× MBG in liquid nitrogen for later assay. Activin protein was produced by micro-injection of manually de-folliculated *Xenopus* oocytes with 50 ng *activin βb* (Thomsen et al., 1990) synthetic mRNA per oocyte. Injected oocytes were cultured in 1× MBG, in 1% agarose/1× MBS-coated 96-well plates (12–14 oocytes in 200 μl 1× MBG per well), for 3 days (removing dead and dying oocytes daily). Each well also contained 10 μl (4.1 MBq) [³⁵S]methionine (43.5 TBq/mmol) per well. After 3 days of culture, the supernatant containing secreted activin protein was collected and centrifuged for 5 s at 12 000 × g, yielding 1.4 ml supernatant for seven wells. To increase its stability at –70°C, this radiolabeled activin protein was then partially purified by G50 gel filtration chromatography (10 ml column) in 1× MBG at room temperature (24°C; Dyson and Gurdon, 1998). Column fractions containing most (80% of the first-eluting counts) of the activin protein (the first-eluting radioactive peak, assayed by scintillation counting) were pooled, frozen in 20 μl aliquots on dry ice and stored at –70°C. Four different activin protein preparations were used in the experiments described in Figs. 7 and 8; two were prepared as described above (Figs. 7A,B and 8B) and two were used as unlabeled oocyte conditioned media (Figs. 7C and 8A). Cycloheximide treatment of caps was performed as described (Casacio and Gurdon, 1987; Smith et al., 1991; Ryan et al., 1996).

4.4. RNase protection and primer extension analysis

RNase protection was performed as previously described (Ryan et al., 1996). To reduce variability due to mosaic expression of injected DNA in *Xenopus* cap assays (Howell and Hill, 1997), 15 reporter plasmid-injected caps were pooled per plasmid DNA construct and frozen in liquid nitrogen. RNA was prepared for each sample as described (Ryan et al., 1996), and one-tenth (1.5 caps' worth) was

removed and assayed separately for endogenous gene transcripts (*Eomes*, *Xbra*, and *FGF-R*), while the remaining nine-tenths was assayed for human *β-globin* transcripts. To reduce batch variation, injected constructs and controls were assayed in parallel in eggs from the same female. Quantitation was performed for all RNase protection gels using a Fujifilm BAS-2500 PhosphorImager and MacBAS V2.5 software. Primer extension analysis was performed using standard methodology (Sambrook et al., 1989) using 6 μg per primer of poly A+ RNA selected from gastrula stage embryos, except for the following modifications. After cDNA synthesis using reverse transcriptase, samples were digested in 100 μl with RNase A (5 μg/ml, 15 min at 37°C), and then with Proteinase K (0.5 mg/ml, 0.5% SDS, 15 min at 37°C). Digested samples were phenol extracted and ethanol precipitated with 10 μg carrier tRNA before re-suspension in 8 μl formamide dyes (20 mM EDTA, 0.03% xylene cyanol, 0.03% Bromophenol Blue in formamide) and electrophoresis on 6% polyacrylamide/urea gels. The sequence of extension primers used is: EP59, 5'-TCCTCTGACTGC-GATTCTCTTCTTTTTCTGAAGCAGAACTTG-3'; EP60, 5'-TGCGATTCTTCTTTTTCTGAAGCAGAACTTG-TAGTAAATAATAGGG-3'; EP61, 5'-CTTTTTCTGAA-GCAGAACTTGTAGTAAATAATAGGGAGTGCCAGG-CAC-3'; 10 pmol of each primer was end-labelled and purified (Sambrook et al., 1989), and 10⁶ cpm (~1 pmol) was used per primer extension reaction.

4.5. Electrophoretic mobility shift analysis

FAST proteins were produced in vitro from cDNA plasmids (Bourillot and Gurdon, in preparation) using the TnT system (Promega). DNA probes were generated from F1μ, F2μ, F1F2μ and 5.6 kb (WT) *Eomes-globin* constructs by excision of a 253 bp *PstI-NcoI* fragment which constitutes approximately the 3' half of the *Eomes* ARE, containing both FAST2 sites, F1 and F2, and both 43 bp repeats (Fig. 5B). Digests were de-phosphorylated using calf intestinal alkaline phosphatase (Boehringer). These 253 bp *Eomes* probes were end-labelled with ³²P using standard methodology (Sambrook et al., 1989), purified on an acrylamide gel and eluted in DNA elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% SDS). Probe eluates were ethanol (2.5 vol) precipitated and dissolved in 10–20 μl TE (to ~30 000 cpm/μl). Binding reactions were carried out in 20 μl each in binding buffer (20 mM HEPES (pH 7.9), 8% glycerol, 2 mM EDTA, 2 mM DTT, 7.2 mM MgCl₂), to which was added (in order) 2 μl [³⁵S]FAST2 protein, 0.1 mg/ml final concentration (Fc) poly-[dIdC-dIdC] (Pharmacia; average size 500 bp), 0.5 mg/ml Fc BSA, and DNA probe (30 000 cpm per reaction). Samples were gently mixed and binding was allowed to occur at room temperature (24°C) for 30 min. A 4.5% non-denaturing polyacrylamide gel (14 cm × 18 cm × 1 mm) was made in and pre-run in 0.2× TBE for 30 min at 150 V. After pre-running, reactions were electrophoresed at

150 V with blue dyes (0.1% xylene cyanol, 0.1% Bromophenol Blue, 5% glycerol) run in a side lane, until the xylene cyanol had moved 3/4 gel length (~3.5 h). The gel was dried and exposed to X-ray film overnight.

Acknowledgements

We thank Tim Mohun and Caroline Hill for helpful advice and discussions, and Natasha MacDowell, Kazuya Shimizu, Henrietta Standley and Aaron Zorn for a critical reading of the manuscript. We thank Malcolm Whitman and Jeffrey Wrana for *FAST1* and *FAST2* plasmids, respectively, Gerry Thomsen for the *activin βb* plasmid, and Caroline Hill for *β-globin* plasmids. This work was sponsored by the Cancer Research Campaign.

References

- Amaya, E., Musci, T.J., Kirschner, M.W., 1991. Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in *Xenopus* embryos. *Cell* 66, 257–270.
- Artinger, M., Blitz, I., Inoue, K., Tran, U., Cho, K.W.Y., 1997. Interaction of *goosecoid* and *brachyury* in *Xenopus* mesoderm patterning. *Mech. Dev.* 65, 187–196.
- Cascio, S., Gurdon, J.B., 1987. The initiation of new gene transcription during *Xenopus* gastrulation requires immediately preceding protein synthesis. *Development* 100, 297–305.
- Chen, X., Rubock, M.J., Whitman, M., 1996. A transcriptional partner for MAD proteins in TGF-β signalling. *Nature* 383, 691–696.
- Chen, X., Weisberg, E., Fridmacher, V., Watanabe, M., Naco, G., Whitman, M., 1997. Smad4 and FAST-1 in the assembly of activin-responsive factor. *Nature* 389, 85–89.
- Ciruna, B.G., Rossant, J., 1999. Expression of the T-box gene *Eomesodermin* during early mouse development. *Mech. Dev.* 81, 199–203.
- Cornell, R.A., Musci, T.J., Kimelman, D., 1995. FGF is a prospective competence factor for early activin-type signals in *Xenopus* mesoderm induction. *Development* 121, 2429–2437.
- Derynck, R., Gelbart, W.M., Harland, R.M., Heldin, C.H., Kern, S.E., Massague, J., Melton, D.A., Mlodzik, M., Padgett, R.W., Roberts, A.B., Smith, J., Thomsen, G.H., Vogelstein, B., Wang, X.F., 1996. Nomenclature: vertebrate mediators of TGF-β family signals. *Cell* 87, 173.
- Dyson, S., Gurdon, J.B., 1997. Activin signalling has a necessary function in *Xenopus* early development. *Curr. Biol.* 7, 81–84.
- Dyson, S., Gurdon, J.B., 1998. The interpretation of position in a morphogen gradient as revealed by occupancy of activin receptors. *Cell* 93, 557–568.
- Feinberg, A.P., Vogelstein, B., 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132, 6–13.
- Feng, X.-H., Zhang, Y., Wu, R.-Y., Derynck, R., 1998. The tumor suppressor Smad4/DPC4 and transcriptional adaptor CBP/p300 are coactivators for Smad3 in TGF-β-induced transcriptional activation. *Genes Dev.* 12, 2153–2163.
- Friedle, H., Rastegar, S., Paul, H., Kaufmann, E., Knöchel, W., 1998. *Xvent-1* mediates BMP-4-induced suppression of the dorsal-lip-specific early response gene *XFD-1'* in *Xenopus* embryos. *EMBO J.* 17, 2298–2307.
- Graff, J.M., Bansal, A., Melton, D.A., 1996. *Xenopus* Mad proteins transduce distinct subsets of signals for the TGF-β superfamily. *Cell* 85, 479–487.
- Green, J.B.A., New, H.V., Smith, J.C., 1992. Responses of embryonic *Xenopus* cells to activin and FGF are separated by multiple dose thresholds and correspond to distinct axes of the mesoderm. *Cell* 71, 731–739.
- Hancock, S.N., Agulnik, S.I., Silver, L.M., Papaioannou, V.E., 1999. Mapping and expression analysis of the mouse ortholog of *Xenopus* *Eomesodermin*. *Mech. Dev.* 81, 205–208.
- Hemmati-Brivanlou, A., Melton, D.A., 1992. A truncated activin receptor inhibits mesoderm induction and formation of axial structures in *Xenopus* embryos. *Nature* 359, 609–614.
- Henry, G.L., Brivanlou, I.H., Kessler, D.S., Hemmati-Brivanlou, A., Melton, D.A., 1996. TGF-β signals and a pattern in *Xenopus laevis* endodermal development. *Development* 122, 1007–1015.
- Howell, M., Hill, C.S., 1997. *XSmad2* directly activates the activin-inducible, dorsal mesoderm gene *XFKH1* in *Xenopus* embryos. *EMBO J.* 16, 7411–7421.
- Huang, H.-C., Murtaugh, L.C., Vize, P.D., Whitman, M., 1995. Identification of a potential regulator of early transcriptional responses to mesoderm inducers in the frog embryo. *EMBO J.* 14, 5965–5973.
- Janknecht, R., Wells, N.J., Hunter, T., 1998. TGF-β-stimulated cooperation of Smad proteins with the coactivators CBP/p300. *Genes Dev.* 12, 2114–2119.
- Kimura, N., Nakashima, K., Ueno, M., Kiyama, H., Taga, T., 1999. A novel mammalian T-box-containing gene, *tbr2*, expressed in mouse developing brain. *Brain Res. Dev. Brain Res.* 115, 183–193.
- Labbé, E., Silvestri, C., Hoodless, P.A., Wrana, J.L., Attisano, L., 1998. Smad2 and Smad3 positively and negatively regulate TGF-dependent transcription through the forkhead DNA-binding protein FAST2. *Mol. Cell* 2, 109–120.
- Lagna, G., Hata, A., Hemmati-Brivanlou, A., Massague, J., 1996. Partnership between DPC4 and SMAD proteins in TGF-β signalling pathways. *Nature* 383, 832–836.
- Latinkic, B.V., Unbhauer, M., Neal, K.A., Lerchner, W., Smith, J.C., Cunliffe, V., 1997. The *Xenopus* *Brachyury* promoter is activated by FGF and low concentrations of activin and suppressed by high concentrations of activin and by paired-type homeodomain proteins. *Genes Dev.* 11, 3265–3276.
- Mohun, T.J., Taylor, M.V., Garrett, N., Gurdon, J.B., 1989. The CARG promoter sequence is necessary for muscle-specific transcription of the cardiac actin gene in *Xenopus* embryos. *EMBO J.* 8, 1153–1161.
- Moon, R.T., Kimelman, D., 1998. From cortical rotation to organizer gene expression: toward a molecular explanation of axis specification in *Xenopus*. *BioEssays* 20, 536–545.
- Pouponnot, C., Jayaraman, L., Massagué, J., 1998. Physical and functional interactions of Smads and p300/CBP. *J. Biol. Chem.* 273, 22865–22868.
- Rebbert, M.L., Dawid, I.B., 1997. Transcriptional regulation of the *Xlim-1* gene by activin is mediated by an element in intron I. *Proc. Natl. Acad. Sci. USA* 94, 9717–9722.
- Russ, A.P., Wattler, S., Colledge, W.H., Aparicio, S.A.J.R., Carlton, M.B.L., Pearce, J.J., Barton, S.C., Surani, M.A., Ryan, K., Nehls, M.C., Wilson, V., Evans, M.J., 2000. *Eomesodermin* is required for mouse trophoblast development and mesoderm formation. *Nature* 404, 95–99.
- Ryan, K., Garrett, N., Mitchell, A., Gurdon, J.B., 1996. *Eomesodermin*, a key early gene in *Xenopus* mesoderm differentiation. *Cell* 87, 989–1000.
- Ryan, K., Butler, K., Bellefroid, E., Gurdon, J.B., 1998. *Eomesodermin* is expressed in neural differentiation. *Mech. Dev.* 75, 155–158.
- Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. *Molecular Cloning, a Laboratory Manual*, 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 10.59–10.61.
- Sasai, Y., Lu, B., Steinbeisser, H., Geissert, D., Gont, L.K., DeRobertis, E.M., 1994. *Xenopus chordin*: a novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell* 79, 779–790.
- Shi, Y., Wang, Y.F., Jayaraman, L., Yang, H., Massague, J., Pavletich, N.P., 1998. Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF-β signaling. *Cell* 94, 585–594.
- Shimizu, K., Gurdon, J.B., 1999. A quantitative analysis of signal transduc-

- tion from activin receptor to nucleus and its relevance to morphogen gradient interpretation. Proc. Natl. Acad. Sci. USA 96, 6791–6796.
- Slack, J.M.W., 1991. From Egg to Embryo, Cambridge University Press, Cambridge, pp. 76–80.
- Slack, J.M., Darlington, B.G., Gillespie, L.L., Godsave, S.F., Isaacs, H.V., Paterno, G.D., 1989. The role of fibroblast growth factor in early *Xenopus* development. Development 107 (Suppl.), 141–148.
- Smith, J.C., Yaqoob, M., Symes, K., 1988. Purification, partial characterization and biological effects of the XTC mesoderm-inducing factor. Development 103, 591–600.
- Smith, J.C., Cooke, J., Green, J.B., Howes, G., Symes, K., 1989. Inducing factors and the control of mesodermal pattern in *Xenopus laevis*. Development 107 (Suppl.), 149–159.
- Smith, J.C., Price, B.M.J., Green, J.B.A., Weigel, D., Herrmann, B.G., 1991. Expression of a *Xenopus* homolog of *Brachyury (T)* is an immediate-early response to mesoderm induction. Cell 67, 79–87.
- Stennard, F., Ryan, K., Gurdon, J.B., 1997. Markers of vertebrate mesoderm induction. Curr. Opin. Genet. Dev. 7, 620–627.
- Stroschein, S.L., Wang, W., Luo, K., 1999. Cooperative binding of Smad proteins to two adjacent DNA elements in the Plasminogen Activator Inhibitor-1 promoter mediates transforming growth factor β -induced Smad-dependent transcriptional activation. J. Biol. Chem. 274, 9431–9441.
- Tada, M., Casey, E.S., Fairclough, L., Smith, J.C., 1998. *Bix1*, a direct target of *Xenopus* T-box genes, causes formation of ventral mesoderm and endoderm. Development 125, 3997–4006.
- Thomsen, G., Woolf, T., Whitman, M., Sokol, S., Vaughan, J., Vale, W., Melton, D.A., 1990. Activins are expressed early in *Xenopus* embryogenesis and can induce axial mesoderm and anterior structures. Cell 63, 485–493.
- Vize, P.D., 1996. DNA sequences mediating the transcriptional response of the *Mix.2* homeobox gene to mesoderm induction. Dev. Biol. 177, 226–231.
- Watabe, T., Kim, S., Candia, A., Tothbächer, U., Hashimoto, C., Inoue, K., Cho, K.W.Y., 1995. Molecular mechanisms of Spemann's organizer formation: conserved growth factor synergy between *Xenopus* and mouse. Genes Dev. 9, 3038–3050.
- Wattler, S., Russ, A., Evans, M., Nehls, M., 1998. A combined analysis of genomic and primary protein structure defines the phylogenetic relationship of new members of the T-box family. Genomics 48, 24–33.
- Wotton, D., Lo, R.S., Lee, S., Massagué, J., 1999. A Smad transcriptional corepressor. Cell 97, 29–39.
- Wu, R.Y., Zhang, Y., Feng, X.H., Derynck, R., 1997. Heteromeric and homomeric interactions correlate with signaling activity and functional cooperativity of Smad3 and Smad4/DPC4. Mol. Cell. Biol. 17, 2521–2528.
- Zawel, L., Dai, J.L., Buckhaults, P., Zhou, S., Kinzler, K.W., Vogelstein, B., Kern, S.E., 1998. Human Smad3 and Smad4 are sequence-specific transcription activators. Mol. Cell 1, 611–617.
- Zhang, Y., Derynck, R., 1999. Regulation of Smad signalling by protein associations and signalling crosstalk. Trends Cell Biol. 9, 274–279.
- Zorn, A.M., Butler, K., Gurdon, J.B., 1999. Anterior endomesoderm specification in *Xenopus* by Wnt/ β -catenin and TGF- β signalling pathways. Dev. Biol. 209, 282–297.