XSmad2 directly activates the activin-inducible, dorsal mesoderm gene XFKH1 in Xenopus embryos

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Introduction

During embryogenesis, signals produced by one group of cells often change the fate of others. The transforming growth factor-β (TGF-β) superfamily is a key group of growth and differentiation factors that constitute such signals. These secreted signalling molecules control embryonic development by regulating cell proliferation, differentiation, survival and migration (reviewed by Hogan et al., 1994; Kingsley, 1994; Massagué and Weis-Garcia, 1996) and, as a result, play a central role in establishing the basic body plan in flies and vertebrates. The ability of TGF-β superfamily members to regulate such diverse processes is at least in part due to their morphogenic properties, where the signalling molecules act over relatively large distances, and different concentrations of ligand mediate different downstream responses (Green and Smith, 1990; Ferguson and Anderson, 1992; Green et al., 1992; Gurdon et al., 1994; Lecuit et al., 1996; Nellen et al., 1996).

The mechanism of receptor activation is now well understood and is similar for the three best-studied family members, TGF-β1, activin and the bone morphogenetic proteins (BMPs) (Massagué and Weis-Garcia, 1996). The recent discovery of the Smad family of intracellular molecules (for a review, see Massagué et al., 1997) has shed some light on the mechanism by which signals are transduced from the activated receptors to the nucleus, and there is some indication that the specificity of the transcriptional responses for different TGF-β family members is a consequence of differential Smad activation (Baker and Harland, 1996; Graff et al., 1996; Lagna et al., 1996; Zhang et al., 1997). However, it is still very unclear how the Smads function in the nucleus, and how signalling regulates transcription. Moreover, nothing is known about the molecular mechanism by which TGF-β family members invoke different responses in a concentration-dependent manner.

In early Xenopus embryos, a number of different TGF-β family members have been implicated in the specification and patterning of mesoderm, in particular activin, Vg-1, the BMPs and nodal-related genes Xnr1–3 (for reviews, see Smith, 1993, 1995; Lemaire and Kodjabachian, 1996; Thomsen, 1997). While all of these factors have mesoderm-inducing activity, the specific roles of each family member have not been firmly established. The earliest zygotic response to endogenous mesoderm-inducing signals is the up-regulation of regulatory genes in an immediate-early fashion (Dawid, 1992). These genes encode transcription factors and signalling molecules, and it is presumably the precise concentrations of these factors and the complex interactions between them that govern particular mesodermal cell fate (Dawid, 1992; Beddington and Smith, 1993). A small number of these early response genes have been characterized and shown to be induced directly by activin, which is a very potent inducer of mesoderm, capable of inducing a full range of ventral and dorsal mesodermal cell types in prospective ectoderm (animal caps), in a concentration-dependent manner (Green and Smith, 1990; Green et al., 1992). XFKH1 (XFD-1) and its pseudo-allele pintallavis (XFD-1), which encode transcription factors of the forkhead/winged-helix family (Kaufmann and Knöchel, 1996), are such activin-inducible early response genes that are first expressed in dorsal...
mesoderm (Dirksen and Jamrich, 1992; Knöchel et al., 1992; Ruiz i Altaba and Jessell, 1992). Studying the transcriptional regulation of XFKH1/pintallavis therefore provides an excellent opportunity to discover the molecular mechanism of activin-responsive transcription, and ultimately to gain insights into the molecular basis of mesoderm induction.

Here we demonstrate that the XFKH1 gene contains two regions that mediate activin-responsive transcription in *Xenopus* embryos: one upstream of the transcription start site, the other in the first intron. These two regions are functionally distinct, since that in the intron is a direct target of the activin signalling pathway, whilst that in the 5' flanking sequences responds to activin indirectly. We go on to show that the region from the XFKH1 intron acts as a direct activin-inducible enhancer. Of the Smad family members, Smad2 has been implicated in activin signalling (Baker and Harland, 1996; Graff et al., 1996; Lagna et al., 1996), and we show that *Xenopus* Smad2 (XSmad2) can activate XFKH1 expression. The two activin-responsive sequences we have identified can be distinguished by their ability to be activated by overexpression of a constitutively active derivative of XSmad2: the intron enhancer is induced independently of new protein synthesis, whilst the upstream response element is induced via an indirect mechanism.

This work therefore establishes the XFKH1 intron enhancer as a direct nuclear target of the activin signalling pathway in *Xenopus* embryos, and also provides strong new evidence that XSmad2 is a component of the activin signalling pathway. Moreover, because this enhancer bears little sequence similarity to other known direct activin-responsive sequences, we infer that the activin signalling pathway can target transcription factors with distinct DNA-binding specificities in different contexts.

**Results**

**Transcription of a XFKH1–globin fusion gene is strongly induced by activin independently of new protein synthesis**

We first isolated and characterized a XFKH1 genomic clone. The start of transcription was identified by primer extension and RNase protection (data not shown), and was in agreement with that identified by Kaufmann et al. (1996). To investigate the activin-induced regulation of the XFKH1 gene in *Xenopus* embryos, we developed an RNase protection assay, which would enable us to measure transcriptional activation directly at the level of RNA synthesis. This assay has advantages over enzyme reporter assays in that it is possible to distinguish between direct and indirect effects of activin by assayign transcription in the absence or presence of a protein synthesis inhibitor.

We therefore constructed a fusion gene in which the upstream sequences of XFKH1 together with the first exon and intron were fused to the human β-globin gene (~2850I-globin; Figure 1A). This was injected as supercoiled DNA into single cell embryos with a reference globin fusion gene driven by the constitutively active EF-1α promoter, which acts as an internal control (REF-globin; Figure 1A). In initial experiments, we also tested a promoterless globin gene as a control for spurious transcription, often detected in *Xenopus* embryos. Animal cap explants were dissected from injected and un.injected embryos at stage 8 and cultured in the presence or absence of activin. For each condition, 15 explants were used to average differences between embryos resulting from mosaic expression. Transcriptional activation of these fusion genes was monitored by RNase protection using a human β-globin probe (Figure 1A and B).

This XFKH1–globin gene (~2850I-globin) was strongly induced by activin in this assay (Figure 1B, compare lanes 1 and 2). The activin-induced transcription was specific for the XFKH1 sequences, since the promoterless globin gene was only very weakly transcribed, and was unaffected by activin (Figure 1B, lanes 5 and 6). In addition, activin-induced transcription of ~2850I-globin was only marginally affected by pre-incubation of the animal caps with cycloheximide (Figure 1B, lanes 1–4), indicating that...
this transcriptional activation does not require new protein synthesis. As expected, the induction of the endogenous \textit{XFKH1} gene by activin was also unaffected by cycloheximide (data not shown; Dirksen and Jamrich, 1992; Ruiz i Altaba and Jessell, 1992), whereas the activin induction of \textit{chordin}, which requires new protein synthesis (Sasai et al., 1994), was almost entirely inhibited (data not shown; see also Figure 2B, compare lanes 7 and 8 with 10 and 11, demonstrating that this concentration of cycloheximide efficiently inhibits protein synthesis in this assay. Thus the XFKH1–globin fusion gene, –2850I-globin, contains sequences sufficient to mediate a direct transcriptional response to activin.

\textbf{XSmad2 transcriptionally activates \textit{XFKH1} via a direct mechanism}

Since the transcriptional activation of –2850I-globin by activin was direct, we next addressed whether XSmad2, which has been implicated in the activin signalling pathway (Massagué et al., 1997), could also activate transcription of the endogenous \textit{XFKH1} gene and –2850I-globin. Overexpression of XSmads was achieved by injecting the relevant mRNA into single cell embryos. As expected, overexpression in animal caps of full-length XSmad2, but not XSmad1, was sufficient to induce \textit{XFKH1} expression (data not shown); XSmad2 has been shown to be specific for the induction of dorsal mesodermal genes, of which \textit{XFKH1} is one (Baker and Harland, 1996; Graff et al., 1996; Lagna et al., 1996; Zhang et al., 1997). Moreover, an N-terminally truncated derivative of XSmad2 (XSmad2C) induced transcription of \textit{XFKH1} more efficiently than the full-length protein and accumulated to higher levels (Figure 2A; data not shown; see also Baker and Harland, 1996). XSmad2C was therefore used in all subsequent assays.

Overexpression of XSmad2C in the animal cap activated the XFKH1–globin fusion gene –2850I-globin, and the endogenous \textit{XFKH1} gene at least as efficiently as activin (Figure 2B, lanes 1–3 and 7–9). To determine whether this really was a direct effect on the regulatory regions of the \textit{XFKH1} gene, or whether XSmad2C worked indirectly through its ability to induce other mesoderm-specific genes, we exploited the fact that no transcription occurs in \textit{Xenopus} embryos before the mid-blastula transition (Newport and Kirschner, 1982), although injected mRNAs are translated from the time of injection. XSmad2C protein accumulated efficiently in mRNA-injected embryos cultured to stage 8 (Figure 2C, lanes 1 and 3). Animal caps were then dissected from these embryos and incubated in cycloheximide to prevent further protein synthesis, but allowing transcription to proceed normally, thereby allowing us to determine the effect of the accumulated XSmad2C protein. Treatment of animal caps with cycloheximide at stage 8 had no effect on the ability of XSmad2C to activate transcription of –2850I-globin or the endogenous \textit{XFKH1} gene, but severely inhibited its ability to activate \textit{chordin} transcription (Figure 2B, lanes 4–6 and 10–12). We could demonstrate that all the transcriptional activation resulting from overexpression of XSmad2C occurred after cycloheximide was added, since in control experiments no transcription of –2850I-globin or \textit{XFKH1} was detected in stage 8 embryos expressing XSmad2C (data not shown).

\textbf{Fig. 2. Overexpression of XSmad2C mimics activin induction.}

(A) Overexpression of XSmad2 and XSmad2C transcriptionally activates \textit{XFKH1}. Animal caps dissected from stage 8 embryos injected with REF-globin alone (lane 1) or together with mRNAs encoding myc-tagged XSmad2C (lane 2) or XSmad2 (lane 3) were cultured until control embryos had reached stage 11. Total RNA was analysed by RNase protection using probes against \textit{Xbra}, \textit{XFKH1} and \textit{EF-1α} mRNA. (B) XSmad2C can transcriptionally activate \textit{XFKH1} and –2850I-globin via a direct mechanism. Animal caps were dissected from stage 8 embryos injected with –2850I-globin and REF-globin alone (lanes 1, 2, 4 and 5) or together with mRNA encoding myc-tagged XSmad2C (lanes 3 and 6), or from embryos injected with REF-globin alone (lanes 7, 8, 10 and 11) or together with mRNA encoding myc-tagged XSmad2C (lanes 9 and 12). They were cultured with or without activin in the absence (lanes 1–3 and 7–9) or presence (lanes 4–6 and 10–12) of cycloheximide. Total RNA was analysed by RNase protection, using probes against globin mRNA (lanes 1–6) or against \textit{chordin}, \textit{XFKH1} and \textit{EF-1α} mRNA (lanes 7–12). In (A) and (B), protected fragments for globin mRNA were as described in Figure 1; \textit{Xbra}, 214 nt; \textit{XFKH1}, 166 nt; \textit{chordin}, 188 nt; \textit{EF-1α}, 94 nt. The \textit{XFKH1} probe also protects an ~112 nt fragment of \textit{pintaliopsis} mRNA. (C) Expression of myc-tagged XSmad2C. Total protein extracts prepared from stage 8 and stage 11 embryos injected with globin fusion genes and mRNA encoding myc-tagged XSmad2C as indicated were fractionated by SDS–PAGE. Myc-tagged XSmad2C was detected by immunoblotting with 9E10 antibody.
Fig. 3. The intron of XFKH1 can mediate a direct transcriptional response to activin signalling. (A) Schematic of XFKH1–globin fusion genes. XFKH1 sequences are denoted as in Figure 1; globin sequences, open-ended unshaded rectangle. (B) The intron of XFKH1 contributes greatly to the activin inducibility of –2850I-globin. Animal caps were dissected from stage 8 embryos injected with XFKH1–globin fusion genes as indicated, together with REF-globin, and cultured in the absence (odd-numbered lanes) or presence (even-numbered lanes) of activin. (C) The intron of XFKH1 responds to activin induction independently of new protein synthesis. Animal caps were dissected from stage 8 embryos injected with –165-globin or –102I-globin, together with the REF-globin internal control as indicated and were cultured with or without activin in the absence or presence of cycloheximide as indicated. In (B) and (C), globin transcripts were detected by RNase protection; protected fragment sizes are given in Figure 1. The gels were quantitated as described in Materials and methods.

The XFKH1–globin fusion gene, –2850I-globin, therefore contains sequences that mediate a transcriptional response to XSmad2C independently of new protein synthesis.

The first intron of XFKH1 contains a direct activin response element

To localize the activin-responsive regions of XFKH1, we compared the activin inducibility of six XFKH1–globin fusion genes containing different amounts of upstream sequences, with or without the first exon and intron (Figure 3A). The results indicated that the presence of the first exon and intron was critical for strong activin-induced transcription. All the fusion genes that contained the XFKH1 exon and intron were strongly induced by activin in animal cap assays regardless of the amount of upstream sequences (Figure 3B, lanes 3, 4, 7, 8, 11 and 12). In contrast, fusion genes containing only upstream sequences were either weakly inducible by activin (–2850-globin and –1192-globin; Figure 3B, lanes 5, 6, 9 and 10) or were completely uninducible (–102-globin; Figure 3B, lanes 13 and 14). Analysis of 5’ and 3’ deletion mutants of –1192-globin in the same assay located the upstream activin-responsive element more precisely (Table I). A construct with just 128 bp upstream of the start of transcription was still induced by activin, indicating that the 5’ boundary of this element is downstream of –128 (Table I). The 3’ boundary probably lies upstream of –106, since a construct that contains 1192 bp of upstream sequences with an internal deletion of –103 to –106 was activin inducible, whereas an analogous construct with a larger deletion (–103 to –152) was uninducible (Table I).

Taken together, these results suggest that there is a region in the first intron, or conceivably the first exon, which is sufficient to confer strong activin inducibility onto an uninducible promoter. They also indicate there is an activin-responsive region in the 5’ flanking sequences located between –128 and –106 relative to the start of transcription.

Table I. Mapping the activin-responsive element in the 5’ flanking region of XFKH1

<table>
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<th>5’ Deletionb</th>
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<tr>
<td>–1192</td>
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<td>–673</td>
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<td>–497</td>
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<td>–128</td>
<td>11.3</td>
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<td>–102</td>
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<th>Internal deletionsc</th>
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<td>–103/–106</td>
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<td>–103/–152</td>
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aRelative to the start of transcription as +1. All fusion genes assayed have 79 bp of XFKH1 5’ UTR fused to human β-globin.

bData are calculated as the ratio between activin-treated and untreated animal caps, and are the averages from up to four independent experiments. Standard deviations were on average 30% of the means shown here.

cNumbers given are inclusive. The XFKH1 sequences extend to the 5’ endpoint –1192.
this fusion gene contains the sequences that act as a direct target of the activin signalling pathway.

The most likely explanation for the activin inducibility of –102I-globin was the presence of an activin-responsive enhancer in the first intron of XFKH1. To test this, we assayed the activin inducibility of a fusion gene, I-102-globin, in which residues 24–1585 of the intron were placed upstream of –102-globin. This construct, the sequences of the intron are now in the opposite orientation relative to the transcription start site. Animal caps dissected from stage 8 embryos injected with –102I-globin, –102-globin or I-102-globin, together with the REF-globin internal control as indicated, were cultured in the absence (lanes 1, 3 and 5) or presence (lanes 2, 4 and 6) of activin. Globin transcripts were detected by RNase protection; protected fragment sizes are given in Figure 1. The gel was quantitated as described in Materials and methods.

The most likely explanation for the activin inducibility of –102I-globin was the presence of an activin-responsive enhancer in the first intron of XFKH1. To test this, we assayed the activin inducibility of a fusion gene, I-102-globin, in which residues 24–1585 of the intron were placed upstream of –102-globin. In this construct, the sequences of the intron act as an enhancer. The injected constructs are schematized as in Figure 3. In I-102-globin, sequences 24–1585 from the intron (IVS 1) are placed upstream of –102-globin. In this construct, the sequences of the intron act as an enhancer. In I-102-globin, sequences 24–1585 from the intron (IVS 1) are placed upstream of –102-globin. In this construct, the sequences of the intron act as an enhancer. In I-102-globin, sequences 24–1585 from the intron (IVS 1) are placed upstream of –102-globin. In this construct, the sequences of the intron act as an enhancer. In I-102-globin, sequences 24–1585 from the intron (IVS 1) are placed upstream of –102-globin. In this construct, the sequences of the intron act as an enhancer. In I-102-globin, sequences 24–1585 from the intron (IVS 1) are placed upstream of –102-globin. In this construct, the sequences of the intron act as an enhancer. In I-102-globin, sequences 24–1585 from the intron (IVS 1) are placed upstream of –102-globin. In this construct, the sequences of the intron act as an enhancer. In I-102-globin, sequences 24–1585 from the intron (IVS 1) are placed upstream of –102-globin. In this construct, the sequences of the intron act as an enhancer. In I-102-globin, sequences 24–1585 from the intron (IVS 1) are placed upstream of –102-globin. In this construct, the sequences of the intron act as an enhancer. In I-102-globin, sequences 24–1585 from the intron (IVS 1) are placed upstream of –102-globin. In this construct, the sequences of the intron act as an enhancer. In I-102-globin, sequences 24–1585 from the intron (IVS 1) are placed upstream of –102-globin. In this construct, the sequences of the intron act as an enhancer. In I-102-globin, sequences 24–1585 from the intron (IVS 1) are placed upstream of –102-globin. In this construct, the sequences of the intron act as an enhancer. In I-102-globin, sequences 24–1585 from the intron (IVS 1) are placed upstream of –102-globin. In this construct, the sequences of the intron act as an enhancer.

The most likely explanation for the activin inducibility of –102I-globin was the presence of an activin-responsive enhancer in the first intron of XFKH1. To test this, we assayed the activin inducibility of a fusion gene, I-102-globin, in which residues 24–1585 of the intron were placed upstream of –102-globin. The fusion gene demonstrated above to have a very low basal activity and to be completely uninducible by activin. The fusion gene I-102-globin was strongly induced by activin, at least as efficiently as –102I-globin, which contains the intron in its natural context (Figure 4, compare lanes 1, 2, 5 and 6). We consistently observed that the presence of the intron sequences upstream of –102-globin resulted in suppression of the basal level of transcription (Figure 4, lanes 3 and 5; see below).

Taken together, these data suggest that the XFKH1 intron contains an activin-inducible enhancer that can activate transcription directly in response to activin, regardless of its location (or orientation) relative to the start of transcription.

Deletion analysis of the XFKH1 intron
To locate the activin-inducible enhancer in the XFKH1 intron, we analysed a series of deletion mutants of –102I-globin. 5′ and 3′ deletions were made in the intron from restriction enzyme sites that were internal to the splice donor and acceptor sites to avoid interfering with splicing (Figure 5A and B). First, a broad series of 5′ deletion mutants that covered the intron were tested in animal cap assays for their ability to be transcriptionally induced by activin. This analysis suggested that the 5′ boundary of the activin-responsive region lay somewhere between nucleotides 644 and 954 (relative to the first base pair of the intron; Figure 5A, compare lanes 7 and 8 with 9 and 10). The 5′ boundary of the activin-responsive region was mapped more precisely by analysing further deletion mutants (Figure 5B). The activin inducibility of the fusion gene with the internal deletion of 28–881 was still relatively strong, whilst the fusion gene with an internal deletion of 28–923 was only marginally activated by activin, and deletion to 954 resulted in the fusion gene that was essentially uninducible. We therefore concluded that the 5′ boundary of the activin-responsive region lay downstream of residue 881 (Figure 5B). The 3′ boundary was similarly mapped. Deletion of nucleotides 990–1242 left the activin inducibility relatively unimpaired, whereas deletion of nucleotides 921–1242 resulted in the abolition of activin inducibility (Figure 5B). We concluded therefore that the 3′ boundary of the activin-responsive region lay upstream of nucleotide 990.

A small region of the XFKH1 intron is sufficient to confer activin inducibility onto an uninducible minimal promoter
We next addressed whether the region of the intron identified in the deletion analysis above was sufficient to confer activin inducibility onto an uninducible minimal
M. Howell and C. S. Hill

Fig. 6. The region of the XFKH1 intron (887–993) acts as an activin-inducible enhancer. (A) Schematic representation of globin reporter genes controlled by regions of the XFKH1 intron. XFKH1 and globin sequences are schematized as in Figure 3. (B) Analysis of the activin inducibility of a series of globin reporter genes controlled by regions of the XFKH1 intron. Animal caps dissected from 8 embryo injected with the series of globin reporter genes together with the REF-globin internal control as indicated were cultured with or without activin. (C) The region of the XFKH1 intron (887–993) is activated by overexpression of XSmad2C via a direct mechanism. Animal caps were dissected from 8 embryo injected with (887–993)2-globin and REF-globin (lanes 1–6), (907–963)2-globin and REF-globin (lanes 7–12) or –165-globin and REF-globin (lanes 13–18) either alone (lanes 1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16 and 17) or together with mRNA encoding myc-tagged XSmad2C (lanes 3, 6, 9, 12, 15 and 18). They were cultured with or without activin in the absence or presence of cycloheximide as indicated. The activin concentration for the samples in lanes 2, 5, 14 and 17 was 20 ng/ml; that used for samples in lanes 8 and 11 was 80 ng/ml. We reproducibly found that (887–993)2-globin was induced over a large range of activin concentrations; in contrast (907–963)2-globin was only induced by high levels of activin. Globin transcripts were detected by RNase protection; protected fragment sizes are given in Figure 1. The gels were quantitated as described in Materials and methods.

We constructed globin reporter genes in which single or double copies of small DNA segments derived from this region of the XFKH1 intron were placed upstream of the minimal uninducible promoter of –102-globin (Figure 6A). A single copy of the region from the XFKH1 intron spanning nucleotides 887–993 was sufficient to confer activin inducibility onto the minimal promoter (Figure 6B, compare lanes 3 and 4, and 11 and 12), comparable with that observed with –102I-globin which contains the entire XFKH1 intron in its natural context (Figure 6B, compare lanes 1 and 2, and 3 and 4). A reporter gene driven by two copies of this 107 bp region was considerably more activin inducible, mainly as a result of a repression of transcription in the absence of activin signalling (Figure 6B, lanes 5 and 6). To narrow down further the region required for activin-induced transcription, we tested globin reporter genes driven by smaller DNA fragments derived from the 107 bp region in the animal cap assay. Two copies of a fragment spanning nucleotides 907–963 could clearly elicit an activin induction, although considerably less efficiently than two copies of 887–993, due primarily to the higher uninduced level of transcription (Figure 6B, lanes 5–8); further 5′ and 3′ deletion resulted in a DNA fragment that was completely inactive (Figure 6B, lanes 9 and 10).

The region of the XFKH1 intron (887–993) is activated directly by overexpression of XSmad2C

Finally, we examined whether the small regions of the XFKH1 intron identified above could be activated by overexpression of the constitutively active derivative of XSmad2 (XSmad2C), and, more importantly, whether the effects of activin signalling and XSmad2C expression could occur in the absence of new protein synthesis. Transcriptional activation of the globin reporter gene driven by two copies of 887–993 was strongly induced...
Activin signalling and transcription

Fig. 7. Activin signalling to the XFKH1 gene. The XFKH1 gene is denoted as in Figure 1. Oval, intron enhancer that responds directly to activin (solid arrow), and in addition may also be targeted indirectly (dashed arrow); triangle, sequences in the upstream region that respond indirectly to activin signalling (dashed arrow). CHX, cycloheximide. Our experiments have revealed the presence of sequences within the intron enhancer that can repress basal transcription in the absence of an activin signal. Below, sequence comparison of the activin-inducible enhancer from the XFKH1 intron (887–993) and the analogous region from the pintallavis gene (XFD-1; Kaufmann et al., 1996). Identities are shown by vertical lines. The smaller regions tested for activin inducibility are indicated. Arrows denote sequences related to the ‘AAATGT’ motifs found in the ARE from the Mix.2 promoter which are critical for binding the activin-responsive complex (Chen et al., 1996). Note, one of the motifs in the XFKH1 enhancer is in the opposite orientation to those in the Mix.2 promoter. The dotted line indicates a region of eight consecutive base pairs that is identical to one of the Al-rich repeats found in the DE from the goosecoid promoter (Watabe et al., 1995).

Discussion

XFKH1 contains two distinct activin-responsive regions

The zygotic genes first expressed in the mesoderm of early-gastrula Xenopus embryos are of profound importance for the further development of the organism. The XFKH1 gene, which is the focus of this study, is one such mesodermal gene which encodes a transcription factor of the forkhead/winged-helix family (Kaufmann and Knöchel, 1996). By analogy with the function of the close family member HNF-3β in mouse embryos, it is likely to be involved in the formation of the organizer and notochord (Ruiz i Altaba et al., 1993; Weinstein et al., 1994). Analysis of the activin-induced transcriptional regulation of XFKH1 has afforded us insights into both the regulation of mesoderm induction and the molecular mechanism of activin signalling.

We have demonstrated that the activin inducibility of the Xenopus XFKH1 gene is controlled by two independent regulatory regions which differ in their responses to activin. The region in the XFKH1 intron responds directly, indicating that transcriptional activation is mediated by transcription factors already present in the cell when it is stimulated with activin (Figure 7). Moreover, this region of the intron acts as an activin-inducible enhancer, in that it can function out of its natural context, can operate in

by activin and by overexpression of XSmad2C in animal cap assays (Figure 6C, lanes 1–3), and was completely unaffected by incubating the stage 8 animal cap explants in the protein synthesis inhibitor, cycloheximide (Figure 6C, compare lanes 1–3 and 4–6). In contrast, the weaker activin and XSmad2C inductions of the globin reporter gene driven by two copies of 907–963 were completely abolished by cycloheximide (Figure 6C, compare lanes 7–9 and 10–12). To investigate whether there was a perfect correlation between sequences that responded directly to activin, and those that responded directly to XSmad2C, we tested whether the XFKH1–globin fusion gene, −165-globin which contains the upstream indirect activin-responsive region of the XFKH1 gene, was also dependent on protein synthesis for its ability to be induced by XSmad2C. The data shown in Figure 6C (lanes 13–18) indicate that activation of this fusion gene by XSmad2C was indeed abolished by cycloheximide.

The data presented here demonstrate that the region from the XFKH1 intron spanning nucleotides 887–993 is the smallest fragment that can mediate a direct transcriptional response to activin signalling and XSmad2C overexpression in Xenopus embryos, and that for all the responsive regions tested, the behaviour of overexpressed XSmad2C faithfully mimics the effects of the activin signalling pathway.
either orientation relative to the transcription start site and is sufficient to confer direct activin inducibility onto a minimal uninducible promoter. The other activin-responsive region in XFKH1 lies upstream of the transcription start site and approximately corresponds to that identified in the recent study of Kaufmann et al. (1996). However, this region does not respond directly, since in this case activin-induced transcription can be abolished by pre-treatment of animal cap explants with cycloheximide, indicating that it is mediated by transcription factors whose synthesis is controlled by the activin signalling pathway (Figure 7). Our analysis therefore indicates that the expression of a single gene can be controlled both directly and indirectly by one signal transduction pathway.

The presence of two classes of activin-responsive region in XFKH1 is likely to be significant for its regulation in vivo, although we have yet to confirm their relative contributions in the context of the entire gene. The intron enhancer is presumably responsible for the initial up-regulation of the gene in response to the activin-like signal, whereas the promoter-proximal region may play a role in maintaining XFKH1 expression. We have demonstrated that expression of the XFKH1–globin fusion gene –2850I-globin in early Xenopus embryos faithfully recapitulates the spatial and temporal expression pattern of the endogenous XFKH1 gene (M.Howell, C.S.Hill, S.Drayton and E.Amaya, unpublished data). Analysis of the expression pattern of a number of the constructs reported here should allow us to determine the minimal sequences required to achieve the expression pattern of the endogenous XFKH1 gene, as well as indicating the domains of the embryo competent to respond to the endogenous activin-like signal. The early expression pattern of the endogenous gene is likely to be a consequence of multiple interacting signalling pathways, and may involve Wnt and BMP pathways in addition to the activin-related one (Watabe et al., 1995; Kaufmann et al., 1996).

The activin-responsive XFKH1 intron enhancer

Our analysis of the activin-induced transcription of XFKH1 has allowed us to identify an enhancer that is targeted directly by the activin signalling pathway. In our assays where the transcripts of XFKH1 and its pseudo-allele pintallavis can be distinguished, no differences in the regulation of the two genes have been detected. We therefore presume that the sequences essential for the activin inducibility of XFKH1 should be conserved in pintallavis. Figure 7 shows an alignment of the sequence of the 107 bp XFKH1 activin-responsive enhancer with the analogous region from pintallavis (Kaufmann et al., 1996). Indeed, much of the enhancer sequence is very well conserved; only the region downstream of residue 967 is divergent. Work currently is in progress to test whether this less conserved region is indeed dispensable for activin-responsive transcription.

Although our data indicated that the fragment spanning residues 887–993 was the smallest region sufficient to mediate direct activin-responsive transcription, a reporter gene driven by two copies of a smaller fragment (907–963) could respond indirectly to activin (Figures 6 and 7). We do not yet know the significance of this for the function of the entire enhancer. It is possible that the factors that bind this central region, which may be necessary but are certainly not sufficient for the direct activin induction, are present in the cells before they receive an activin signal, and are also synthesized in response to activin induction. Our data also suggest that activin-induced transcription may be the combined result of deactivating transcriptional repression and stimulating transcriptional activation, since we observe that transcription mediated by the intron enhancer is actively repressed in unstimulated animal cap explants. This repression was most marked when the enhancer was taken out of its natural context, or duplicated, and therefore the significance of this activity of the enhancer in its native context remains to be established.

Identification of all the factors that bind the enhancer that are responsible for activin-induced transcription, including those that act negatively, is obviously going to be very informative for understanding how these early response genes are activated by the mesoderm-inducing signals. We currently are using band-shift analysis and footprinting to identify transcription factors that bind this enhancer and to determine which, out of all the factors that can bind, are those responsible for mediating activin-induced transcription. Our initial results indicate that there are multiple protein complexes that bind this enhancer, and a number of these are up-regulated between blastula and mid-gastrula stage embryos in a protein synthesis-independent manner (unpublished).

The role of XSmad2

In this study, we have shown that activin signalling can be faithfully mimicked by overexpressing an N-terminally deleted version of XSmad2 (XSmad2C). Where the activin response is direct, as in the induction of (887–993)2-globin or the XFKH1 gene, so is the response to XSmad2C; conversely, where the activin response is indirect, as in the transcriptional activation of –165-globin or the chordin gene, the same is true for the response to XSmad2C. Thus XSmad2C acts specifically. The perfect correspondence between the activity of the activin signalling pathway and the activity of XSmad2C provides new evidence that Smad2 is a component of the activin signalling pathway in Xenopus embryos (see also Baker and Harland, 1996; Graff et al., 1996; Lagna et al., 1996).

While the mechanism of Smad activation upon stimulation is becoming clearer, in that different Smads are known to be directly phosphorylated by different receptors for TGF-β family members (Macias-Silva et al., 1996; Kretzschmar et al., 1997), and form hetero-olimeric complexes which then translocate to the nucleus (Lagna et al., 1996; Hata et al., 1997; Zhang et al., 1997), their function in the nucleus is not yet clear. They may themselves be involved in transcriptional regulation since, in the context of GAL4 fusion proteins, the C-terminal domains of Smads 1, 2 and 4 can constitutively activate transcription (Liu et al., 1996; Massagué et al., 1997; Meersseman et al., 1997; see also Wu et al., 1997). However, the role of the endogenous Smads in regulated transcription remains to be defined. Evidence is emerging for the involvement of Smads in complexes at regulatory elements with site-specific DNA-binding proteins (Chen et al., 1996), and a recent report indicated that the N-terminal domain of the Drosophila family member MAD can itself bind DNA (Kim et al., 1997). However, since full-length
MAD does not bind DNA, the Smads may either only interact with DNA in vivo as activated complexes, and/or always require other DNA-binding proteins.

Our data provide the first example of a Smad directly activating transcription via a defined regulatory sequence, and therefore provide a starting point for elucidating the actual role of Smads in transcriptional activation. Our data indicate that the C-terminal region of XSmad2 is sufficient for activation, in agreement with the work of Baker and Harland (1996). XSmad2C, which is constitutively nuclear (Baker and Harland, 1996; our unpublished data), may therefore function in the absence of an upstream signal by targeting a complex of Smads and/or other co-factors to regulatory elements of the directly inducible genes. To determine whether this is the case, we currently are investigating the interaction of XSmad2C with other nuclear factors and with the XFKH1 intron enhancer.

**Direct activin response elements in other mesodermal genes**

Two other activin-responsive elements have been identified in other *Xenopus* mesodermal genes. The distal element (DE) from the goosecoid promoter mediates activin-responsive transcription in the absence of new protein synthesis (Watabe et al., 1995), while the activin response element (ARE) from the Mix.2 promoter has been shown to bind a transcription factor complex very rapidly upon activin stimulation (Huang et al., 1995; Chen et al., 1996; see above). It has not yet been demonstrated categorically that the ARE can stimulate transcription in response to activin in the absence of new protein synthesis. These elements bear little resemblance to each other, and are also clearly distinct from the XFKH1 intron enhancer described here. Visual inspection of the XFKH1 enhancer sequence reveals some weak similarities to regions of both the ARE and DE (Figure 7), and we currently are investigating the functional significance of this. However, these short regions of similarity themselves are not sufficient for activity, since the region of our enhancer (917–950) that contains an 8 bp match to one of the AT-rich repeats of the DE and contains an ‘AAATGT’ motif of the ARE is transcriptionally inactive (Figure 6B). The region required to mediate activin-induced transcription is much more extensive and, in fact, since all three identified activin-responsive regions are rather large, multiple DNA-binding factors may be involved in activin-induced transcription in all cases.

The observation that these three activin-responsive sequences (the DE, the ARE and the XFKH1 enhancer) are so dissimilar suggests that the activin signalling pathway can target transcription factors with different DNA-binding specificities. Since the activin signal seems to be transduced to the nucleus by a specific complex of Smads, it is possible that the Smads are capable of interacting with different transcription factors at different regulatory sequences, perhaps via specific interaction domains distinct from DNA-binding domains. Alternatively, these mapped elements may respond to different doses of activin, which might induce the formation of different complexes of Smads, each capable of activating distinct sequence elements. In this regard, it is interesting to note that the critical dependence on the dose of activin for the induction of particular mesodermal genes can be recapitulated by overexpressing different amounts of mRNA encoding Xenopus Smad2 (Baker and Harland, 1996; Graff et al., 1996).

The intron enhancer we have identified in the XFKH1 gene is directly activated in response to activin, and as such defines the bottom of the activin signalling pathway. It should provide a very valuable tool for elucidating the mechanism by which activin signals lead to transcriptional regulation and for determining the role played in this by the Smad proteins.

**Materials and methods**

**Isolation of a XFKH1 genomic clone**

Approximately $7 \times 10^7$ plaques of a *Xenopus* genomic library in λEMBL4 (a gift from L.Dawid) were screened with a random primed probe corresponding to nucleotides 19–849 of the *pintallavisa* cDNA (Ruiz i Altava and Jessell, 1992) as previously described (Howe et al., 1995). Twelve, plaque-pure, identical positive clones were identified; all subsequent work was carried out on a single clone. Although a *pintallavisa* probe was used, the genomic clone we obtained was that of the pseudo-alien XFKH1 (Dirksen and Jamrich, 1992), also called XFD-1* (Knöchel et al., 1992). The clone was characterized by restriction mapping, PCR, primer extension and sequencing, and was shown to contain –2850 bp of upstream sequence relative to the start of transcription, a 166 bp first exon comprising a 97 bp 5′ UTR and 69 bp of coding sequence, and a 1585 bp intron. The region downstream of the intron has not been sequenced but is estimated to be ~7.7 kb and, by analogy with the virtually identical XFD-1* clone (Kauffmann et al., 1996), assumed to contain a second exon encoding 376 amino acids, a 3′ UTR and 3′ flanking sequences. Since there are a number of small differences between our sequence and that of XFD-1* whose sequence was deposited in the EMBL database (U65750) by W.Knochel and colleagues while this work was in progress, we have retained our own sequence numbering and deposited our sequence (from –1192 upstream of the transcription start site to 1751 bp downstream) in the EMBL database (Accession number AJ000764). The sizes of unsequenced regions of our clone were estimated by agarose gel electrophoresis.

**Construction of XFKH1-β-globin fusions**

All DNA manipulations were carried out by standard techniques, and the plasmid structures were verified by sequencing and restriction analysis. All XFKH1–β-globin fusion constructs were in pUC18. Constructs –2850–globin, –1192–globin and –102–globin contain the indicated XFKH1 upstream sequences and 79 bp of XFKH1 5′ UTR fused 5′ to the human β-globin gene (nucleotides +50 to +2161) immediately upstream of the authentic globin translation initiation codon (Figure 1). Constructs –2850I-globin, –1192I-globin and –102I-globin (where ‘I’ denotes the presence of the XFKH1 intron) contain XFKH1 sequences –2850 to +1751, –1192 to +1751 and –102 to +1751 respectively, fused in-frame to nucleotides +55 to +2161 of the human β-globin gene. The fusion point was at the last base pair of the XFKH1 intron. The construct I-102-globin was derived from –102–globin and contained the region spanning nucleotides 24–1585 from the XFKH1 intron placed upstream of –102–globin. REF-globin consisted of a human β-globin reporter with a 50 bp deletion in exon 2 (Mohen et al., 1989) placed downstream of the human EF-1α promoter region. The promoterless globin construct contained just the β-globin sequences in pUC18. Deletions in the upstream XFKH1 promoter region and first intron were created using the exonucleases Exo VII (Amersham) and Exo III (Gibco-BRL). The construct –102SN-globin was created from –102–globin by the insertion of a linker oligonucleotide (top strand, 5′–ACTAGTGAATGCTAGCGAGCT–3′) upstream of position –102 in a polynucleotide linker to introduce SpeI and Nhel restriction sites. Fragments corresponding to specific regions of the XFKH1 intron were obtained by PCR using oligonucleotides terminating with Nhel and SpeI restriction sites. PCR products were digested with Nhel and SpeI, dimerized in *vitro* and ligated into the same sites of –102SN-globin to create the constructs (887–993)N-globin, (907–963)N-globin and (917–950)N-globin.

**Embryos, microinjection and animal cap assays**

*Xenopus* embryos were obtained by *in vitro* fertilization, dejellied with 2% cysteine hydrochloride pH 8, maintained in 1/15× normal amphibian...
medium (NAM; Peng, 1991) and staged according to Nieuwkoop and Faber (1967). Embryos for injection were transferred to 3/4× NAM, 4% Ficoll 400 and injected at the one-cell stage into the animal pole with ~5.5 nl of supercoiled DNA comprising 80 pg of XFKH1–globin fusion plasmid and 50 pg of REF-globin plasmid. Where stated, the total injection volume of ~5.5 nl also contained 1.5 ng of synthetic RNA encoding XSmad2 or XSmad2C (see below). After injection, embryos were maintained at 14°C, removed to 3/4× NAM at the 8- to 16-cell stage and cultured until stage 8. Embryos cultured beyond stage 8 were transferred into 1/10 NAM. Animal caps were dissected in 10% NAM/90% CMFM containing 0.1% bovine serum albumin (BSA) (Peng, 1991) from injected or un.injected stage 8/8.5 embryos; 15 caps were used per condition. They were incubated at 21°C in 500 μl of 1× NAM containing 0.1% BSA in the presence or absence of activin until control embryos had reached stage 11, then snap-frozen in a minimal volume of buffer prior to RNA isolation. Activin was either human recombinant activin A at a concentration of 9–20 ng/ml unless otherwise stated, or from conditioned medium (Figures 1 and 3B) used at a concentration of 32 U/ml as described (Green et al., 1992). The level of activation of the endogenous XFKH1 gene with activin from these two sources was identical.

For experiments in which protein synthesis was inhibited by cycloheximide, animal caps were dissected from stage 8 embryos and incubated in 10% NAM/90% CMFM containing 0.1% BSA and 5 μg/ml cycloheximide. After incubation for 30 min at 21°C, the caps were transferred to 1× NAM/0.1% BSA containing 5 μg/ml cycloheximide in the presence or absence of activin for 30 min, then transferred to the same solution but without cycloheximide and cultured until control embryos had reached stage 11.

**RNA isolation and RNase protections**

For RNA protection assays, RNA was isolated essentially as described (Hill et al., 1995) except that the DNase step was omitted and tRNA (50 μg) was added as carrier. One hundred and fifty μl of guanidinium isothiocyanate lysis solution was used per 15 caps and 400 μl was used for 10 embryos; all other additions were proportional. Fifteen caps were analysed in the RNase protection, and the equivalent of two embryos was used. RNA protection mapping was performed exactly as described (Treisman, 1985) except that RNase T1 (Calbiochem) was used alone at a concentration of 26 U/ml.

Nitrocellulose RNase protection gels were quantitated using a PhosphorImager. The amount of mRNA generated from the XFKH1–globin fusion genes was normalized with respect to that generated from the internal reference (REF-globin). Activator-induced stimulations are expressed as the ratio of activin- or XSmad2C-treated and untreated animal caps (fold inductions). The actual values for the fold inductions for a given XFKH1–globin fusion gene varied between experiments due to differences in inductions). The actual values for the fold inductions for a given XFKH1–globin fusion gene varied between experiments due to differences in 

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Acitivin signalling and transcription


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