The EMBO Journal Vol.16 No.24 pp.7411–7421, 1997

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

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Transforming growth factor (TGF)-β family members play a central role in mesoderm induction during early embryogenesis in *Xenopus*. Although a number of target genes induced as an immediate-early response to activin-like members of the family have been described, little is known about the molecular mechanisms involved. Our systematic analysis of the activin induction of the target gene *XFKH1* reveals two regions that mediate activin-responsive transcription: one, in the first intron, is targeted directly by the activin-signalling pathway; the other, in the 5′ flanking sequences, responds to activin indirectly, possibly being required for maintenance of gene expression. We demonstrate that a 107 bp region of the *XFKH1* first intron acts as an enhancer and confers activin inducibility onto a minimal uninducible promoter in the absence of new protein synthesis. It bears little sequence similarity to other activin responsive sequences. We further demonstrate that overexpression of a constitutively active derivative of *Xenopus* Smad2 (XSmad2), which has been implicated as a component of the activin signalling pathway, is sufficient for direct activation of transcription via this enhancer. Moreover, we show that XSmad2 acts indirectly on the proximal promoter element induced by activin via an indirect mechanism. These results establish the *XFKH1* intron enhancer as a direct nuclear target of the activin signalling pathway in *Xenopus* embryos, and provide strong new evidence that XSmad2 is a transducer of activin signals.

Keywords: activin-responsive enhancer/Smad/transcriptional activation/*Xenopus*/*XFKH1*

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).

TGF-β family members signal via serine–threonine kinase receptors at the cell surface, and elicit their biological effects by signalling to the nucleus and regulating the transcriptional activation of target genes (reviewed by Hill, 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 assaying 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 uninjected 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).

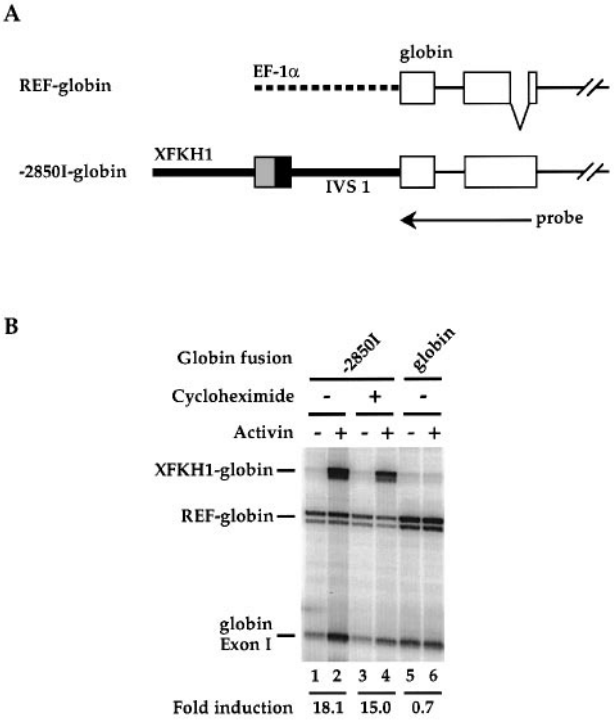

Fig. 1. Activin inducibility of the XFKH1–globin fusion gene –2850I-globin. (**A**) Schematic representation of the globin fusion genes, REF-globin and –2850I-globin. Dotted line, EF-1α promoter region; unshaded boxes, globin exons, with an intron between them. All globin fusion genes contain three exons of human β-globin including the 3′ UTR and two β-globin introns. The 50 bp deletion in the second globin exon in REF-globin allows discrimination between REF-globin and XFKH1–globin transcripts. In –2850I-globin, the black line denotes 5′ flanking sequences and the intron (IVS 1) of *XFKH1*; grey box, *XFKH1* 5′ UTR; black box, coding sequences of *XFKH1* exon 1; arrow, antisense RNase protection probe. (**B**) The XFKH1–globin fusion gene –2850I-globin is induced by activin independently of new protein synthesis. Animal caps were dissected from stage 8 embryos injected with –2850I-globin and REF-globin (lanes 1–4) or promoterless globin and REF-globin (lanes 5 and 6), and cultured with or without activin in the absence or presence of cycloheximide as indicated. Globin transcripts were analysed by RNase protection. Protected fragments were as follows: exon II from mRNA generated from –2850I-globin or promoterless globin, 208 nt; exon II from mRNA generated from REF-globin, 158 nt; exon I (common to mRNA generated from all globin plasmids), 94 nt. Protected fragments of injected DNA or unspliced RNA from –2850I-globin and REF-globin are 434 and 384 nt respectively (not shown). The RNase protection gel was quantitated as described in Materials and methods.

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 *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 *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.

XSmad2 transcriptionally activates 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 *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 *XFKH1* expression (data not shown); XSmad2 has been shown to be specific for the induction of dorsal mesodermal genes, of which *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 *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 *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 *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 *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 *XFKH1* gene, but severely inhibited its ability to activate *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 *XFKH1* was detected in stage 8 embryos expressing XSmad2C (data not shown).

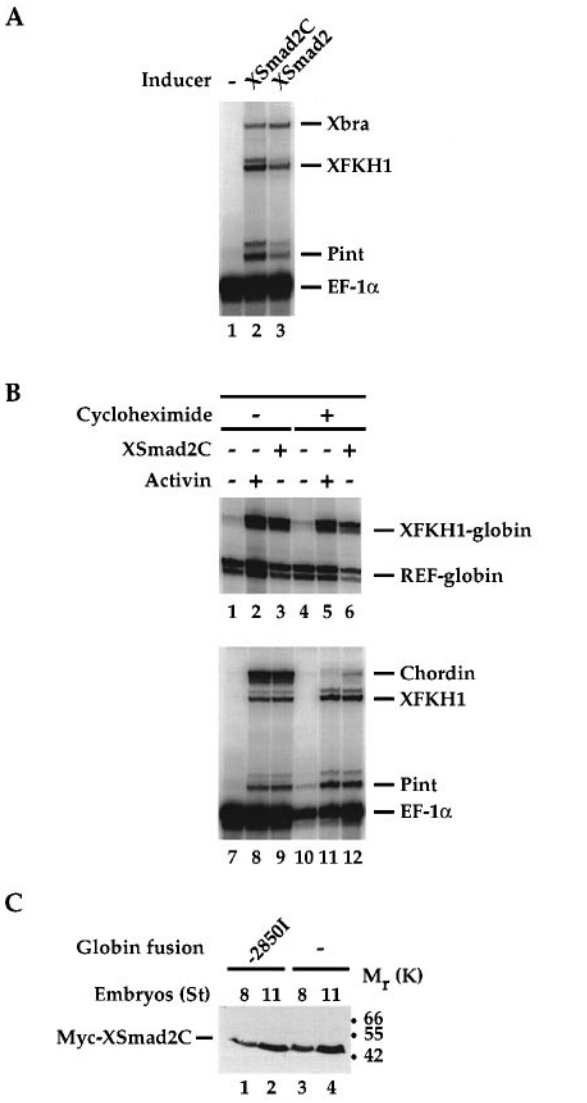

Fig. 2. Overexpression of XSmad2C mimics activin induction. (**A**) Overexpression of XSmad2 and XSmad2C transcriptionally activates *XFKH1*. Animal caps dissected from stage 8 embryos injected with REF-globin alone (lane 1) or together with mRNAs encoding myc-tagged XSmad2C (lane 2) or XSmad2 (lane 3) were cultured until control embryos had reached stage 11. Total RNA was analysed by RNase protection using probes against *Xbra*, *XFKH1* and *EF-1*α mRNA. (**B**) XSmad2C can transcriptionally activate *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 *chordin*, *XFKH1* and *EF-1*α mRNA (lanes 7–12). In (A) and (B), protected fragments for *globin* mRNA were as described in Figure 1; *Xbra*, 214 nt; *XFKH1*, 166 nt; *chordin*, 188 nt; *EF-1*α, 94 nt. The *XFKH1* probe also protects an ~112 nt fragment of *pintallavis* 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.

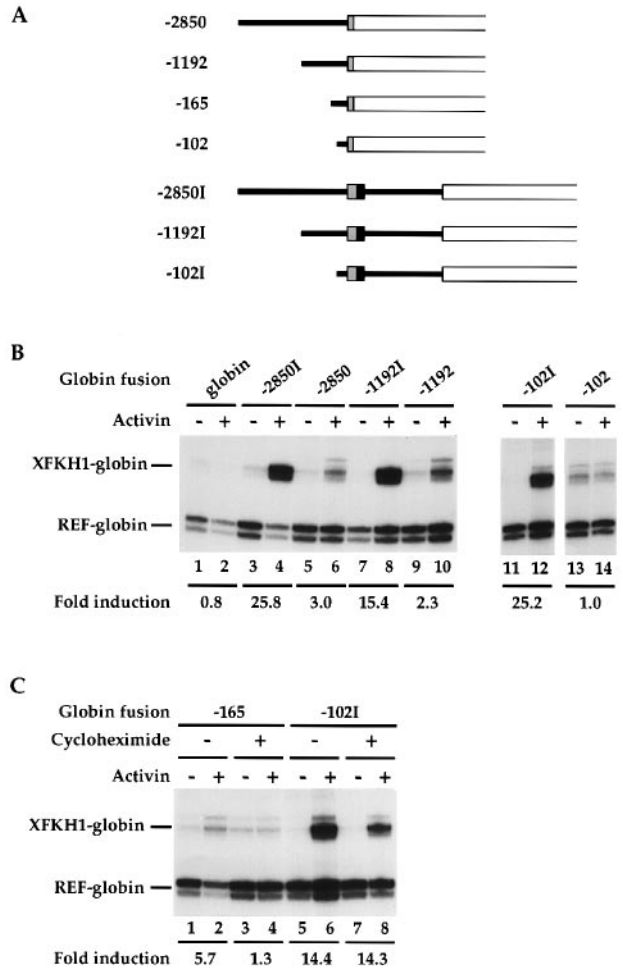

Fig. 3. The intron of *XFKH1* can mediate a direct transcriptional response to activin signalling. (**A**) Schematic of XFKH1–globin fusion genes. *XFKH1* sequences are denoted as in Figure 1; globin sequences, open-ended unshaded rectangle. (**B**) The intron of *XFKH1* contributes greatly to the activin 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).

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

5′ Deletion[a]	Activin stimulation[b]
–1192	2.2
–673	2.9
–497	3.1
–165	5.9
–148	3.6
–128	11.3
–102	1.2
Internal deletions[c]	
–103/–106	2.6
–103/–152	1.0

[a]Relative to the start of transcription as +1. All fusion genes assayed have 79 bp of *XFKH1* 5′ UTR fused to human β-globin.
[b]Data 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.
[c]Numbers given are inclusive. The *XFKH1* sequences extend to the 5′ endpoint –1192.

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.

To determine which of these regions was responsible for the direct activin-induced transcription elicited by –2850I-globin and endogenous *XFKH1*, we compared the activin inducibility of two XFKH1–globin fusion genes, –102I-globin and –165-globin, in animal cap assays in the presence or absence of cycloheximide (Figure 3C). Activin-induced transcription of –165-globin, which contains only the upstream activin-responsive region, was inhibited completely by cycloheximide (Figure 3C, lanes 1–4; see also Figure 6C, right-hand panel), indicating that it occurs via an indirect mechanism. In contrast, the activin induction of the XFKH1–globin fusion gene, –102I-globin which contains a minimal uninducible promoter with the first exon and intron of *XFKH1* was unaffected by cycloheximide (Figure 3C, lanes 5–8), demonstrating that

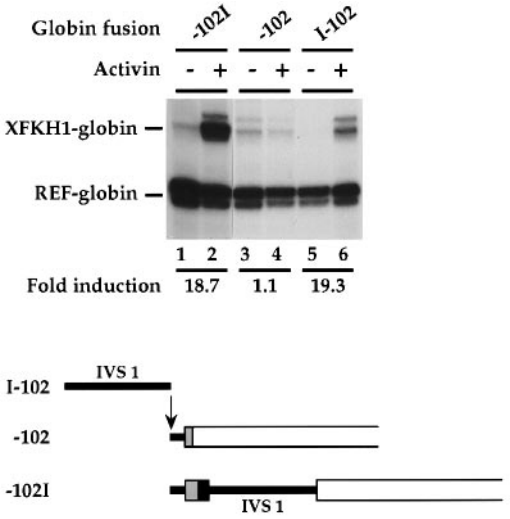

Fig. 4. The intron of *XFKH1* acts as an enhancer. The injected constructs are schematized as in Figure 3. In I-102-globin, sequences 24–1585 from the intron (IVS 1) are placed upstream of –102-globin. In this construct, the sequences of the intron are now in the opposite orientation relative to the 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.

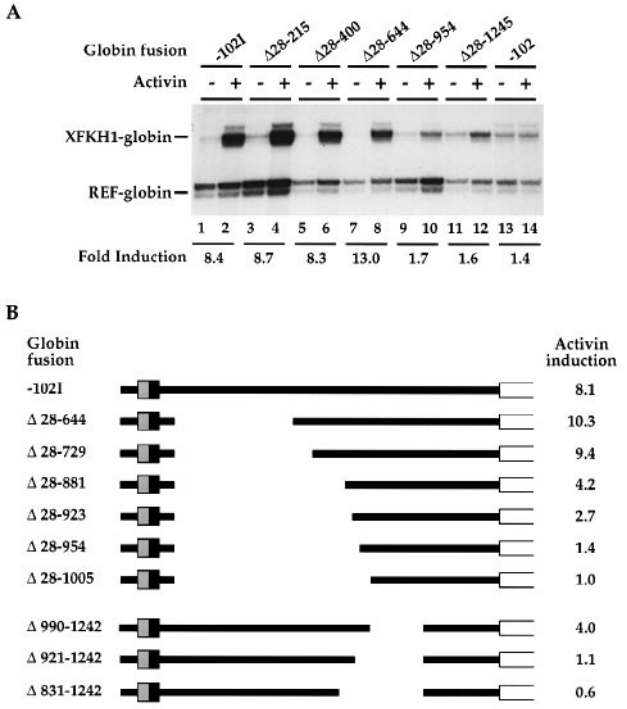

Fig. 5. Deletion analysis of the *XFKH1* intron. (**A**) and (**B**) Animal caps were dissected from stage 8 embryos injected with a series of intron deletion mutant XFKH1–globin fusion genes together with the REF-globin internal control as indicated, and incubated with or without activin. All intron deletion clones contain –102 bp of 5′ flanking *XFKH1* sequence, 166 bp *XFKH1* first exon, and the intron minus the amount deleted; the numbering of the deleted residues is inclusive and is relative to the start of intron. 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. In (B), the injected constructs are schematized as in Figure 3. The data in (B) are the averages of up to six independent experiments. Standard deviations were on average 28% of the means shown here.

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, 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 a 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

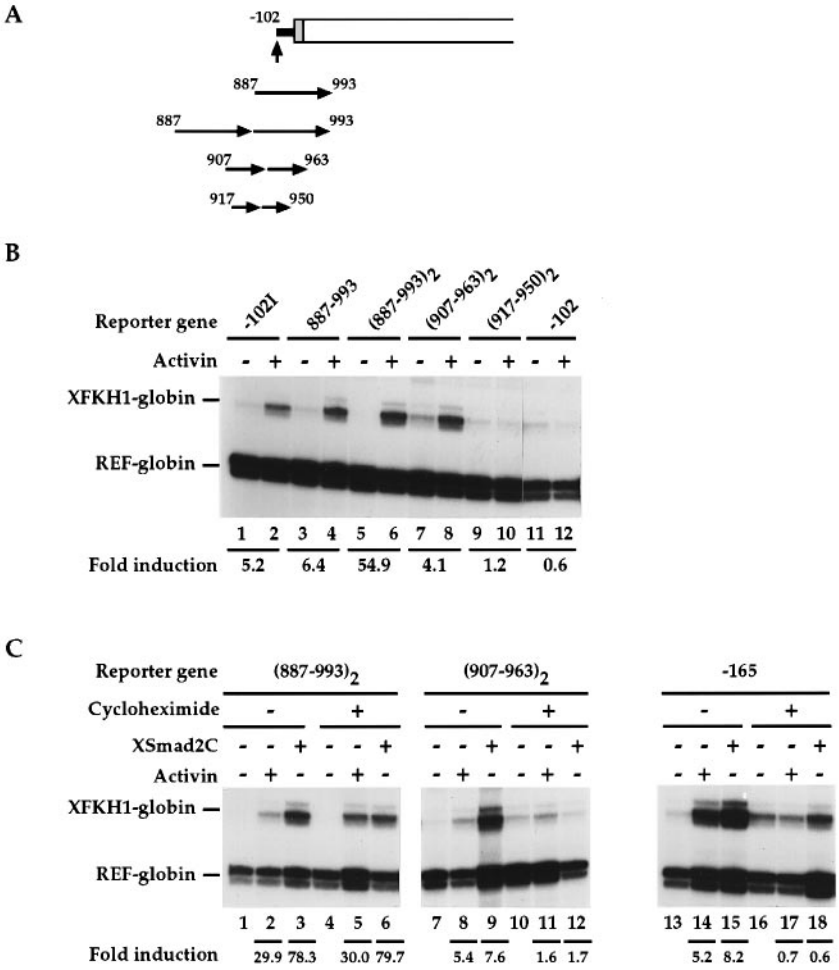

Fig. 6. The region of the *XFKH1* intron (887–993) acts as an activin-inducible enhancer. (**A**) Schematic representation of globin reporter genes controlled by regions of the *XFKH1* intron. *XFKH1* and globin sequences are schematized as in Figure 3. (**B**) Analysis of the activin inducibility of a series of globin reporter genes controlled by regions of the *XFKH1* intron. Animal caps dissected from stage 8 embryos 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 stage 8 embryos 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.

promoter. 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

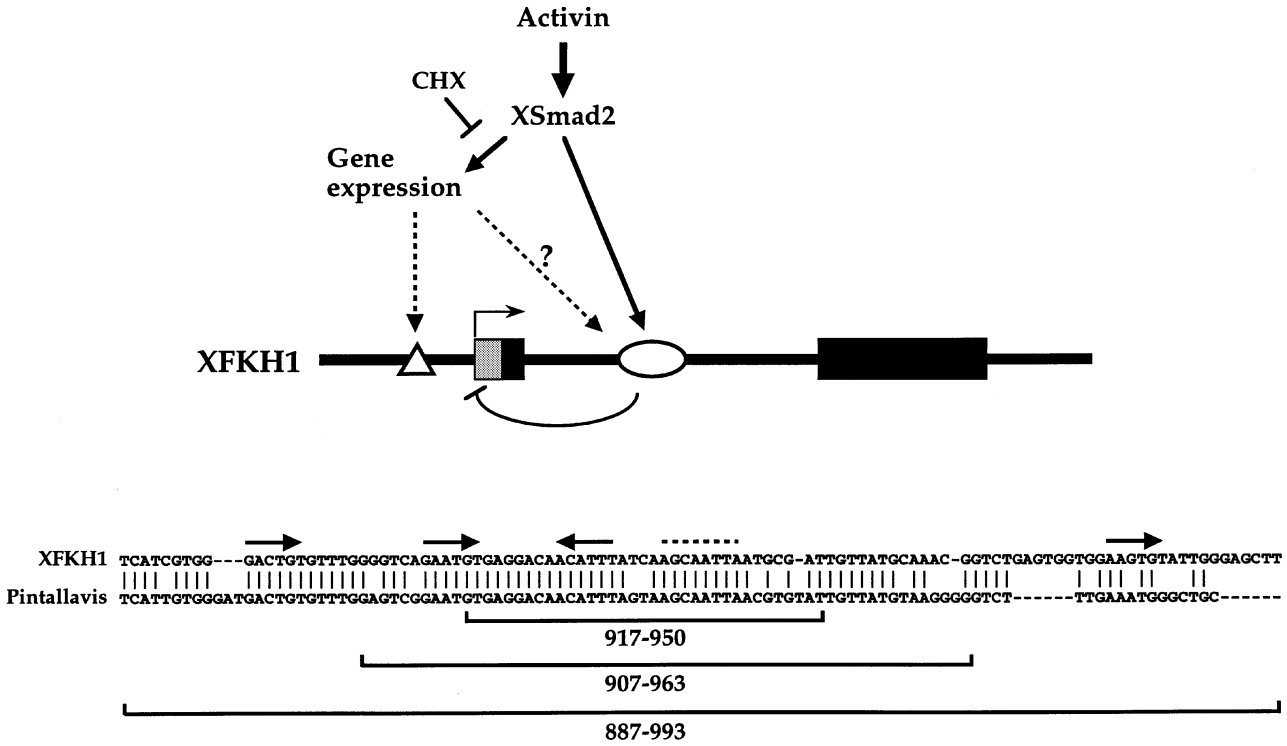

Fig. 7. Activin signalling to the *XFKH1* gene. The *XFKH1* gene is denoted as in Figure 1. Oval, intron enhancer that responds directly to activin (solid arrow), and in addition may also be targeted indirectly (dashed arrow); triangle, sequences in the upstream region that respond indirectly to activin 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 AT-rich repeats found in the DE from the *goosecoid* promoter (Watabe *et al.*, 1995).

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.

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

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 pretreatment 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 (Macías-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 7×10^5 plaques of a *Xenopus* genomic library in λEMBL4 (a gift from I.Dawid) were screened with a random primed probe corresponding to nucleotides 19–849 of the *pintallavis* cDNA (Ruiz i Altaba 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 *pintallavis* probe was used, the genomic clone we obtained was that of the pseudoallele *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 (Kaufmann *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.Knöchel 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–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 (Mohun *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 polylinker region to introduce *Spe*I and *Nhe*I restriction sites. Fragments corresponding to specific regions of the *XFKH1* intron were obtained by PCR using oligonucleotides terminating with *Nhe*I and *Spe*I restriction sites. PCR products were digested with *Nhe*I and *Spe*I, dimerized *in vitro* and ligated into the same sites of –102SN-globin to create the constructs $(887–993)_2$-globin, $(907–963)_2$-globin and $(917–950)_2$-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 uninjected 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 RNase 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 denaturing 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. RNase protection mapping was performed exactly as described (Treisman, 1985) except that RNase T1 (Calbiochem) was used alone at a concentration of 26 U/ml.

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 between 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 batches of embryos. Results within a given experiment, however, were always consistent. Fusion genes that exhibit an induction <2-fold have been considered uninducible.

RNA probes were synthesized using the method of Melton *et al.* (1984). Antisense probes for *Xbra* and *EF-1*α were as described (Smith *et al.*, 1991). The antisense *XFKH1* probe was generated by cloning a *Pst*I–*Hin*dIII fragment (containing all of the first exon and part of the upstream and intron sequences) of *XFKH1* into pGEM4Z (Promega) and linearizing with *Xba*I. T7 RNA polymerase generated an antisense probe of 319 nt which protected a fragment of 166 nt (and also protects a fragment of ~112 nt of *pintallavis* mRNA). For the β-globin probe, an *Nco*I–*Bam*HI fragment from human β-globin, encompassing the first exon, first intron and most of the second exon of the human β-globin gene was cloned into pGEM4Z and linearized with *Nco*I. Transcription with T7 RNA polymerase generated an antisense probe of 470 nucleotides which protects mRNA fragments as described in the legend to Figure 1. For the *chordin* probe, a fragment of the *chordin* gene corresponding to the region encoding amino acids 155–293 (Sasai *et al.*, 1994) was isolated from a stage 17 cDNA library (from C.Kintner) by PCR and cloned into pBSK II (Stratagene). After digestion with *Bam*HI, T7 RNA polymerase generated an antisense probe of 242 nt which protected a fragment of 188 nt.

Synthesis of XSmad2 and XSmad2C mRNA

Capped synthetic mRNA encoding XSmad2 or XSmad2C with an N-terminal Myc tag was synthesized *in vitro* (Melton *et al.*, 1984) from plasmids FTX5-XSmad2 or FTX5-XSmad2C respectively, linearized downstream of a plasmid-encoded poly(A) tail. FTX5-XSmad2 and FTX5-XSmad2C are derivatives of FTX5 which encode MIEQKLISEEDLAMAGS/XSmad2 (amino acids 1–467) and MIEQKLISEEDLAM/XSmad2 (amino acids 198–467) respectively. FTX5 was derived from T7Plink (Dalton and Treisman, 1992) and contains the human β-globin 5′ UTR, nucleotides encoding the 9E10 epitope (underlined) and a 3′ UTR from RSRFR2 [nucleotides 1495–1601 plus a poly(A) tail of ~150 nucleotides (Pollock and Treisman, 1991)].

Protein extracts and immunoblotting

Frozen embryos were lysed in lysis buffer (Howe *et al.*, 1995) and 20 µg of total protein was fractionated on an SDS–15% polyacrylamide gel. After electrophoresis, protein was transferred to nitrocellulose and immunoblotted with 9E10 antibody using standard techniques.

Acknowledgements

We thank Igor Dawid and Chris Kintner for libraries, Tim Mohun, Jonathan Graff and Douglas Melton for plasmids, and Gerard Evan for 9E10 antibody. We thank John Cadera for maintaining the *Xenopus* colony, Jim Smith for plasmids, conditioned media from CHO cells expressing the human βA activin gene and much helpful advice, and Richard Treisman for useful discussions. We are grateful to Jeremy Brockes, Parmjit Jat, Tim Mohun, Elly Tanaka and Richard Treisman for very helpful comments on the manuscript. C.S.H. would like to thank Hazel Sive and Rob Grainger for running the extremely useful Cold Spring Harbor course on early *Xenopus* development. We thank the NHPP, NIDDK, NICHHD and the US Department of Agriculture for human recombinant activin A [lot 15365–36(1)]. C.S.H. is a Royal Society University Research Fellow, and this work was supported by the Ludwig Institute for Cancer Research and the Royal Society.

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Received on July 10, 1997; revised on August 22, 1997