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Antagonistic actions of activin A and BMP-2/4 control dorsal lip-specific activation of the early response gene *XFD-1'* in *Xenopus laevis* embryos

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Transcription of the early response gene *XFD-1'* (*XFKH1*) in the dorsal lip (Spemann organizer) of *Xenopus* embryos is activated by dorsal mesoderm inducing factors. Promoter studies revealed the presence of an activin A response element (ARE) which is both necessary and sufficient for transcriptional activation of reporter genes in animal cap explants incubated with activin A. Surprisingly, this ARE is also active within vegetal explants in the absence of exogenously added inducers, but an additional inhibitory response element prevents transcription of the *XFD-1'* gene in the ventral/vegetal region of the embryo *in vivo*. This element is located upstream of the ARE, it responds to bone morphogenic proteins 2 and 4 (BMP-2/4) triggered signals and it overrides the activating properties of the ARE. Expression patterns of BMP-2 and BMP-4 in the late blastula stage embryo and, especially, their absence from the dorsal blastopore lip may thus control the spatial transcription of the *XFD-1'* gene. Accordingly, the temporal activation and the spatial restriction of *XFD-1'* gene activity to the Spemann organizer is regulated by antagonistic actions of two distinct members of the TGF-β family (activin and BMP) which act on different promoter elements.

Keywords: activin response element (ARE)/bone morphogenetic protein/fork head/*Xenopus laevis*/*XFD-1'* (*XFKH1*) promoter

Introduction

Mesoderm induction within the equatorial or marginal zone of amphibian embryos at blastula stage of development depends upon growth factor-like molecules emanating from the vegetal half of the embryo (for reviews see Kessler and Melton, 1994; Dawid, 1994; Slack, 1994; Smith, 1995; Tiedemann *et al.*, 1996). According to the three signal model (Smith and Slack, 1983; Dale and Slack, 1987) two different factors induce the dorsal and the ventral mesoderm and a third factor originating from the dorsal mesoderm subsequently dorsalizes the ventral mesoderm. While this model nicely accounts for the different types of mesodermal derivatives formed, in view of the growing number of factors with inducing activity it needs to be modified in order to account for the diversity and the combinatorial effects of factors being involved in this process. Nevertheless, by the nature of the induced tissue it is still useful to align the activity of a factor to one of these signals. Candidate factors for the dorsalizing signal are noggin (Smith *et al.*, 1993), chordin (Sasai *et al.*, 1994) and Xnor3 (Smith *et al.*, 1995), a distantly related member of the TGF-β family, while the dorsal signal might be due to other members of the TGF-β family, like activin A (Asashima *et al.*, 1990) or Vg-1 (Dale *et al.*, 1993; Thomsen and Melton, 1993), and to certain members of the Wnt family. These dorsal signals evoke within the dorsal blastopore lip, the Spemann organizer, the local activation of genes encoding different types of transcription factors, like *goosecoid* (Cho *et al.*, 1991), *Xlim-1* (Taira *et al.*, 1992) and *XFD-1/XFD-1'* (*pintallavis/XFKH1*) (Dirksen and Jamrich, 1992; Knöchel *et al.*, 1992; Ruiz i Altaba and Jessell, 1992). In the animal cap assay, transcription of these genes can be induced by activin A in the presence of cycloheximide; thereby, they are also regarded as early response genes. Members of the FGF family participate in the induction of ventral and lateral mesoderm but the most potent ventralizing activity is observed with bone morphogenetic proteins 2 and 4 (BMP-2/4), another two members of the TGF-β family which are closely related but differentially regulated (Köster *et al.*, 1991; Dale *et al.*, 1992; Jones *et al.*, 1992; Nishimatsu *et al.*, 1992; Clement *et al.*, 1995). Microinjection of BMP-2 or BMP-4 RNA into dorsal blastomeres at early cleavage stages leads to embryos showing a completely ventralized phenotype, and animal caps from these embryos differentiate to blood cells, the most ventral type of mesodermal derivatives. BMPs are also able to override the dorsalizing activity of activin A. Moreover, expression of dominant negative BMP receptors in the ventral region of embryos inhibits formation of ventral mesoderm and converts the ventral to dorsal mesoderm, eventually resulting in the formation of a secondary body axis (Graff *et al.*, 1994; Suzuki *et al.*, 1994). Thus, it is evident that ventral mesoderm-inducing signals do also override dorsal signals *in vivo*. Besides this ventralizing activity in mesoderm formation or mesodermal patterning, BMPs exert another important function in embryogenesis, i.e. the suppression of neuralization (Hawley *et al.*, 1995; Sasai *et al.*, 1995; Wilson and Hemmati-Brivanlou, 1995; Xu *et al.*, 1995). This is apparently due to the inhibition of neural inducers, like follistatin (Hemmati-Brivanlou *et al.*, 1994), chordin (Sasai *et al.*, 1994) and noggin (Lamb *et al.*, 1993).

The importance of the early response genes activated in the Spemann organizer as developmental control genes is clearly underlined by results obtained with ectopic overexpression of proteins in *Xenopus* or with knockouts of corresponding genes in mouse. While *goosecoid* over-

expression leads to the formation of a second axis (Cho *et al.*, 1991), knockouts of the *Xlim-1* or the *XFD-1/XFD-1′* gene equivalents in mouse result in most severe defects in forebrain or node and notochord formation respectively (Ang and Rossant, 1994; Weinstein *et al.*, 1994; Shawlot and Behringer, 1995). Thus, these factors are essential in performing the organizer's function in dorsal axis formation and neural induction. Therefore, a molecular analysis of the temporal and spatial control mechanisms that govern expression of these genes is very important for an understanding of how the organizer is formed. Recent reports on the inactivation of the *goosecoid* and the *XFD-1/XFD-1′* genes by BMPs (Fainsod *et al.*, 1994; Clement *et al.*, 1995; Re'em-Kalma *et al.*, 1995) have prompted us to analyse the *XFD-1′* promoter for regulatory sequence motifs responding to the signalling of factors of the TGF-β family. *XFD-1′* (*XFKH1*) belongs to the fork head/HNF-3 family of winged helix transcription factors (reviewed in Kaufmann and Knöchel, 1996). The gene is activated in the dorsal blastopore lip and transcripts are subsequently found in the dorsal midline (notochord and neural floor plate) of the embryo until the end of neurulation. Like its pseudo-allele *XFD-1* (*pintallavis*) it is activated in the animal cap assay by activin in the presence of cycloheximide.

We here report on an activin response element (ARE) in the *XFD-1′* promoter which is different from those having recently been described for the Mix.2, the *goosecoid* and the *HNF1α* genes (Huang *et al.*, 1995; Watabe *et al.*, 1995; Weber *et al.*, 1996). The signalling pathway to this ARE operates in the ventral/vegetal region probably by use of endogenous factors but, in the animal cap, it requires incubation with activin A. However, *in vivo* signalling to this ARE is overridden in the ventral/vegetal region by an additional inhibitory signalling which can be mimicked by BMP-2/4. Thus, dorsal lip-restricted transcription of the *XFD-1′* gene is not just the result of local activation; it rather seems to be a direct consequence of inhibitory signalling being absent from the organizer.

Results

An activatory sequence element within the XFD-1′ promoter

To investigate the activation of the dorsal lip-specific early response genes *XFD-1/XFD-1′* (*pintallavis/XFKH1*) we have isolated the two pseudo-allelic genes from a *Xenopus* genomic DNA library. Sequence analysis revealed that these genes are composed of two exons, the winged helix domain being located within exon II (Knöchel *et al.*, 1992). The exons share ~95% identity, and transcription start sites of both genes as mapped by nuclease S1 and 5′-RACE (data not shown) were identified at corresponding positions. The extensive sequence homology within >2 kb of the 5′-region is shown in Figure 1A. Linearity of plot matrix comparison is only interrupted by sequence elements which previously had been reported as vi repeats (Schubiger *et al.*, 1985) or as part of intron I of the ribosomal protein L1 (Loreni *et al.*, 1985). These motifs have probably been inserted as transposable elements; they are frequently found at varying positions in flanking sequences and introns of pseudo-allelic genes in *Xenopus*, but they are not regarded as regulatory elements for

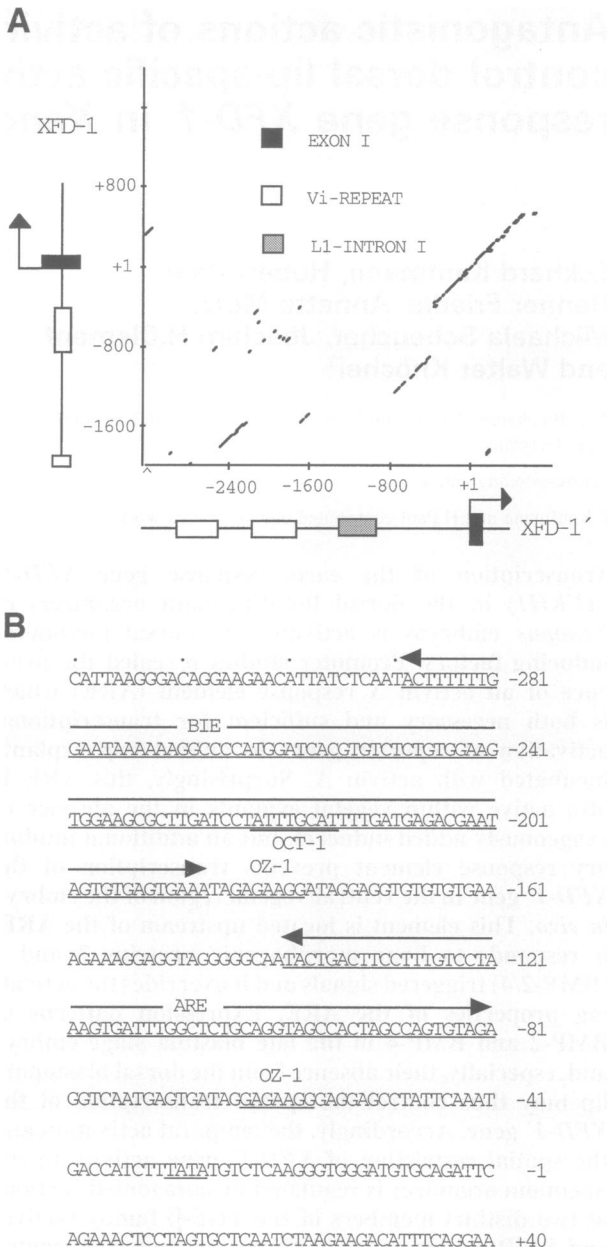

Fig. 1. *XFD-1/XFD-1′* upstream sequences. (**A**) Plot matrix comparison of *XFD-1* (*pintallavis*) and *XFD-1′* (*XFKH1*) upstream regions (sequences are deposited under EMBL accession numbers U65751 and U65750). Minimal match length: 25; number of mismatches: 5. Note that disruption of co-linearity coincides with the integration of putative transposable elements. Arrows at exon I denote transcription initiation sites. (**B**) 320 nucleotides 5′-flanking to the *XFD-1′* gene. TATA box, OCT-1 and OZ-1 binding motifs, activin response element (ARE) and BMP activated inhibitory element (BIE) are underlined.

transcription. Thus, the identical expression patterns of *XFD-1* (= *pintallavis*) and *XFD-1′* (= *XFKH1*) genes (Dirksen and Jamrich, 1992; Knöchel *et al.*, 1992; Ruiz i Altaba and Jessell, 1992) seem to be reflected by an extensive conservation of the putative promoter sequence. Inspection of the 320 bp upstream sequence revealed the presence of an OCT-1 (Mattaj *et al.*, 1985) and two copies of OZ-1-related binding motifs (Ovsenek *et al.*, 1992) (see Figure 1B).

To analyse regulatory elements within the upstream

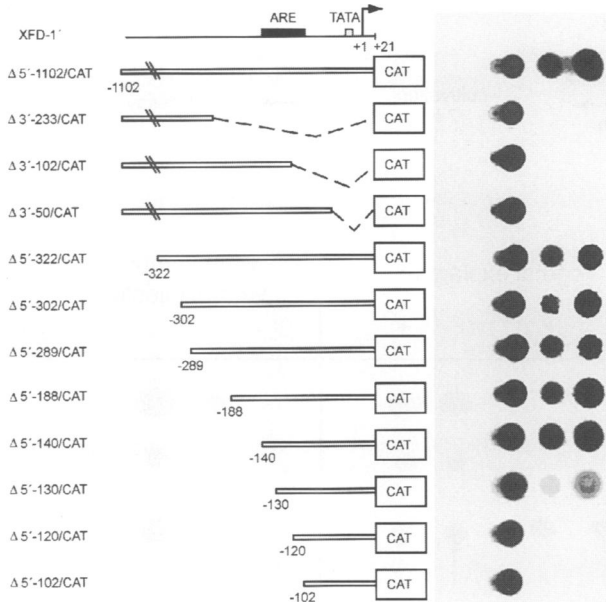

Fig. 2. Analysis of *CAT* expression driven by *XFD-1′* promoter deletion mutants. Δ5′ and Δ3′ mutants of an *Eco*RI–*Dde*I fragment (extending from –1102 to +21; termed Δ5′-1102) were fused to the *CAT* gene (vector: pEU-*CAT*). Each 100 pg DNA of the various constructs was injected at the animal half into both blastomeres of two-cell stage embryos. *CAT* activities were determined at stage 11. Note that all Δ3′-deletions removing the TATA box and all Δ5′-deletions extending more proximal to the transcription initiation site than position –130 inhibit reporter gene expression. Locations of the activin response element (ARE) and TATA box are shown on top.

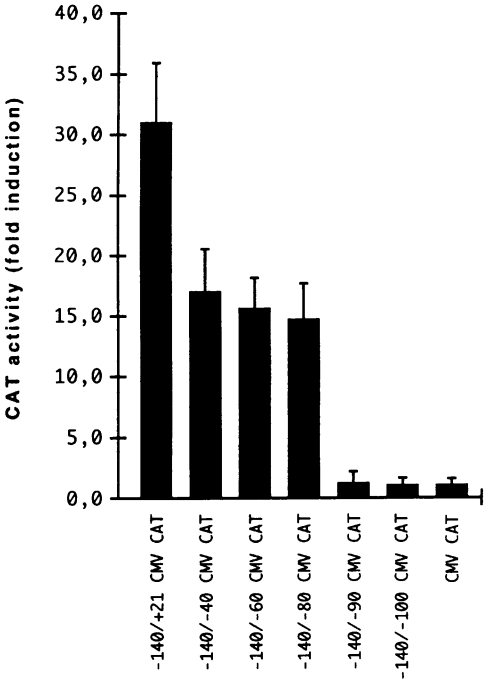

Fig. 3. Identification of the 3′-end of the activatory element. The –140/+21 (Δ5′-140) fragment as well as indicated Δ3′-deletions of this fragment were cloned in front of the basal CMV promoter–*CAT* gene. Injection of these constructs into two-cell stage embryos and determination of *CAT* activity at stage 11 reveals the requirement for the –140 to –80 region. Enzyme activity obtained with the basal CMV promoter–*CAT* gene is set as 1; values are extracted from four different experiments.

region we have cloned an *Eco*R–*Dde*I fragment of the *XFD-1′* gene (extending from –1102 to +21 of transcription start site; designated as Δ5′-1102) in front of the *CAT* gene using the pEU-*CAT* vector (Piaggio and De Simone, 1990). After injection of this construct into both blastomeres of two-cell stage embryos we detect high amounts of *CAT* activity in crude extracts of post-MBT stage embryos (Figure 2). Next we analysed various deletion mutants of this fragment for their regulatory capacity on *CAT* gene expression. While all 3′-deletions of the Δ5′-1102 fragment removing the TATA box clearly show an inhibition of reporter gene activity, all 5′-deletions have no effect except for the Δ5′-130 or more proximal deletions. These results demonstrate the requirement for the TATA box and the presence of an essential sequence motif which starts in the region between –140 and –130. Removal of the OCT-1 and the 5′-located OZ-1-related element which is essential in *LFB1* gene activation (Zapp *et al.*, 1993) does not interfere with reporter gene expression.

To explore the length of the activatory element, we made use of the basal CMV promoter (including a TATA box) fused to the *CAT* gene (Pani *et al.*, 1992) and cloned the –140–*Dde*I (Δ5′-140) fragment as well as 3′-deletions of this fragment in front of the basal CMV promoter. These constructs were injected into two-cell stage embryos and *CAT* activities were determined at stage 11 (Figure 3). Deletion of the region –40 to +21 led to a decrease of activity which might be explained by the loss of the TATA box and adjacent nucleotides of the *XFD-1′* promoter. However, the –40, –60 and –80 3′-deletions are still sufficient to drive reporter gene expression in context with the basal CMV promoter. By the complete inactivation observed with the –90 mutant we conclude that the activatory element requires the –140 to –80 region of the upstream sequence (underlined in Figure 1B). Insertion of the –140 to –60 element in reverse orientation did not reduce *CAT* activity (data not shown), thereby indicating that the orientation of this fragment does not influence interaction with the basal CMV promoter.

The activatory element is an activin response element

Since the *XFD-1′* (*XFKH1*) gene is activated in animal cap explants by activin A (Dirksen and Jamrich, 1992; Knöchel *et al.*, 1992) we have asked whether the activatory element might behave as an activin response element (ARE). To verify this hypothesis we have injected the Δ5′-1102, the Δ5′-140 and the Δ5′-120 promoter–*CAT* gene fusions into the two dorsal (animal side) or into the two ventral blastomeres (vegetal side) of four-cell stage embryos (see Figure 4). Animal or vegetal explants were dissected from blastula stage embryos (stage 8) and incubated for 3 h in the presence or absence of activin A. After additional cultivation for 7 h (without activin) the explants were homogenized and assayed for *CAT* activity. Whereas *CAT* activity in animal caps of dorsally injected control embryos was very low, activin A incubation of caps from embryos previously injected with Δ5′-1102 or Δ5′-140 promoter–*CAT* constructs led to a drastic increase of *CAT* activity. The observed response to activin A is obviously due to the identified regulatory element, because

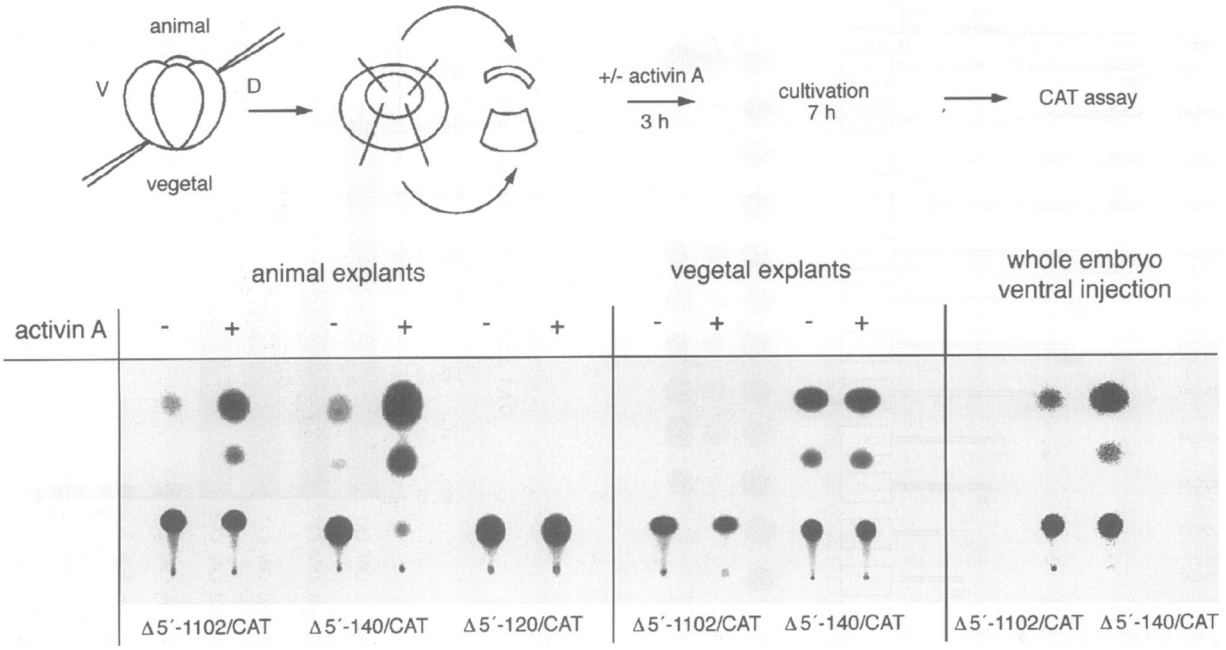

Fig. 4. Influence of activin A on reporter gene activation in animal or vegetal explants. Four-cell stage embryos were injected with indicated promoter–*CAT* constructs into the animal half of the two dorsal blastomeres or into the vegetal half of the two ventral blastomeres. At blastula stage, animal or vegetal explants were dissected, incubated for 3 h in the presence or absense of activin A respectively, and cultivated for another 7 h. Explants were homogenized and assayed for *CAT* activity (see also the scheme). Note that dorsal injections of Δ5′-1102–*CAT* and Δ5′-140–*CAT*, in contrast to Δ5′-120–*CAT*, respond to activin A. Thus, the activatory element behaves as an activin response element (ARE). Analysis of vegetal explants revealed Δ5′-140–*CAT* activity being independent of activin A, whereas Δ5′-1102–*CAT* is not expressed and cannot even be activated by activin A. In support of this, ventral injection of Δ5′-1102–*CAT* and Δ5′-140–*CAT* followed by analysis of *CAT* activity from whole embryos (stage 11) demonstrates reporter gene activity for the short but not for the long promoter fragment.

the Δ5′-120 fragment is not sufficient for this type of induction. We therefore refer to this sequence motif as an activin response element (ARE).

Surprisingly, injection into the ventral blastomeres with subsequent dissection of vegetal explants led to completely different results. While the Δ5′-140 mutant was already active without activin A treatment, the Δ5′-1102 mutant was negative and could even not be activated by incubation with activin A (see Figure 4). This implies that activin or an activin-like inducing factor as well as all necessary components of the signalling transduction pathway are present within the vegetal half of blastula stage embryos. However, an inhibitory element located further upstream of the ARE but being present in the Δ5′-1102 fragment of the *XFD-1′* promoter prevents expression of this dorsal lip-specific gene. This conclusion is supported by analysis of *CAT* activity in whole embryos at stage 11 after ventral injection of the Δ5′-1102 or the Δ5′-140–*CAT* fusions (Figure 4). While the short fragment leads to an activation, the long fragment obviously inhibits reporter gene activity. In contrast, animal cap explants seem to be rather devoid of the inducing factor and do therefore require exogenous activin A. However, as shown by the activin-induced Δ5′-1102 fragment, they do not contain an inhibitory factor which acts on the inhibitory sequence element. In this respect, animal caps behave like whole embryos injected into the dorsal blastomeres (see below).

An inhibitory sequence element controls the spatial expression of the XFD-1′ gene

Observed differences of reporter gene activation in animal and vegetal explants and different results obtained by using longer or shorter promoter fragments imply a more complex mechanism for the spatial activation of the *XFD-1′* gene than would be expected by a simple activation process. To analyse the spatial activity of the promoter we have fused the Δ5′-1102 fragment to a β-gal reporter and injected this construct in dorsal and ventral blastomeres of four-cell and 32-cell stage embryos. When embryos had reached the gastrula stage they were analysed for β-gal activity (Figure 5). While in the case of dorsal injection the reporter gene is expressed in a pattern very similar to that of the *XFD-1′* gene (compare Figure 5A and G with E), ventral injections either show no or only minor amounts of enzyme activity (Figure 5B). This means that the promoter fragment used is nearly sufficient to render a pattern which genuinely corresponds to that of the wild-type gene. Activation is observed in the dorsal but not in the ventral part of the developing embryo and the expression seems to be confined to the dorsal midline. In contrast, the Δ5′-140 fragment leads to an activation of the reporter gene not only after dorsal but also after ventral injections (Figure 5C and D). Simultaneously, it was observed in the majority of injected embryos that the dorsal expression becomes broadened and is no longer restricted to the midline. These results demonstrate that an inhibitory sequence element located between –140 and –1102 confines expression of the *XFD-1′* gene to the dorsal cell derivatives which form the dorsal midline and prevents transcription within the ventral part of the embryo.

Transcriptional inactivation by the inhibitory sequence element depends upon BMP signalling

XFD-1/*XFD-1′* gene expression is inhibited both by BMP-2 and by BMP-4 (Clement *et al.*, 1995; Re'em-Kalma

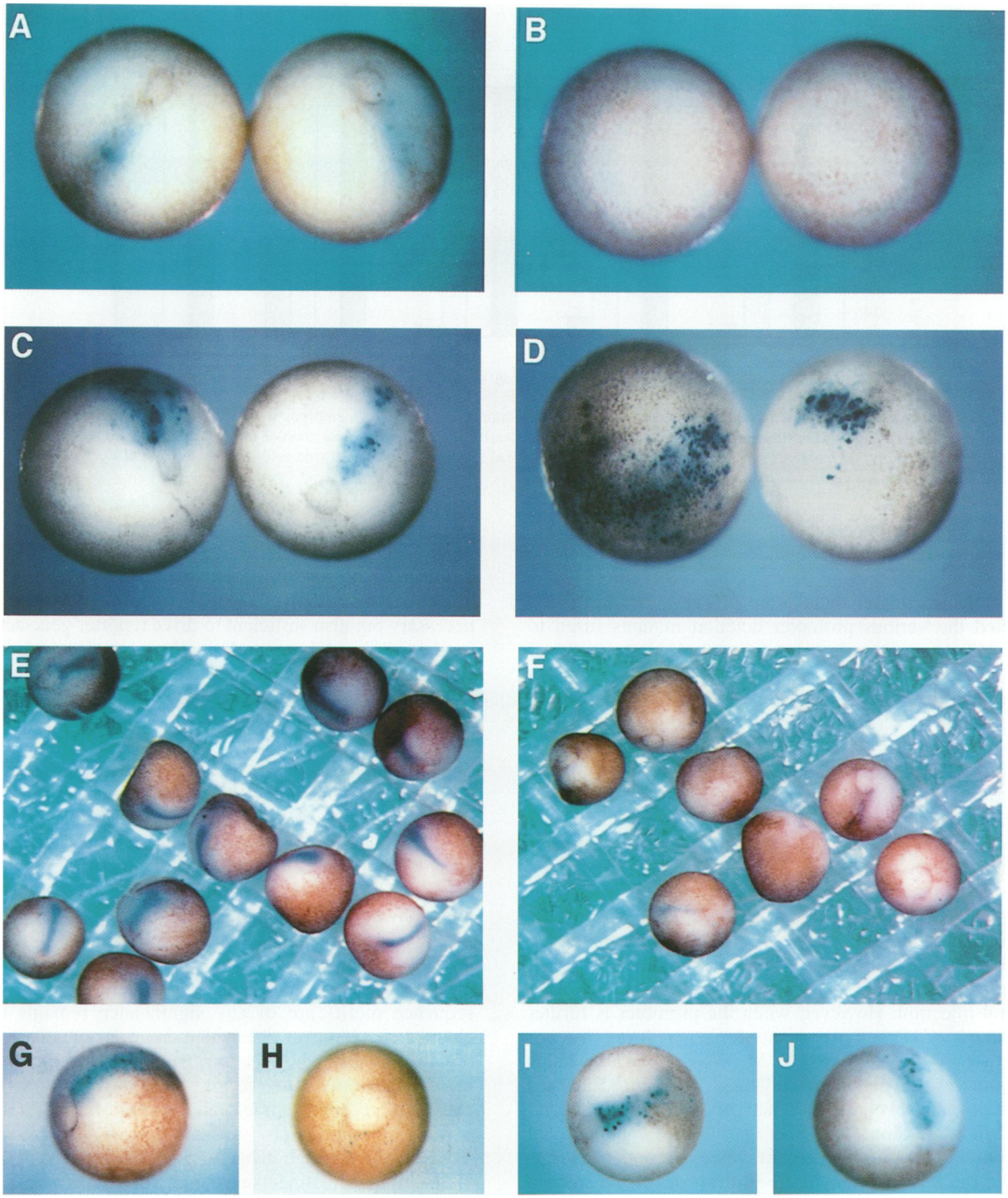

Fig. 5. *XFD-1′* promoter deletion mutant directed β-gal expression in whole embryos. Δ5′-1102–β-gal (**A** and **B**) and Δ5′-140–β-gal (**C** and **D**) constructs were injected into the B1 (A and C) or into the A4 (B and D) blastomeres of 32-cell stage embryos. Enzyme activity was determined at late gastrula stage. Whole mount *in situ* hybridization for *XFD-1′* transcripts is shown for normal embryos (**E**) and embryos injected at the four-cell stage each with 1 ng BMP-4 mRNA into both dorsal blastomeres (**F**). Δ5′-1102–β-gal (**G** and **H**) and Δ5′-140–β-gal (**I** and **J**) constructs were injected into the two dorsal blastomeres of four-cell stage embryos. (G) and (I): without BMP-4 mRNA; (H) and (J): co-injection with BMP-4 mRNA.

et al., 1995; Jones *et al.*, 1996). This is also documented by a comparison of whole mount *in situ* hybridizations of wild-type embryos with embryos previously injected with BMP-4 mRNA (Figure 5E and F), the latter showing no *XFD-1′* transcripts (weak expression observed in a minority of cases is probably due to an insufficient translation of BMP mRNA in these embryos). Therefore, we have analysed whether the inhibitory sequence element might be involved in or even be necessary for the inhibition by BMPs. Injections of Δ5′-1102 and Δ5′-140 promoter fragments fused to the β-gal reporter into dorsal blastomeres of four-cell stage embryos demonstrate that the Δ5′-1102 but not the Δ5′-140 promoter fragment is completely inactivated by co-injection with BMP-4 mRNA (Figure

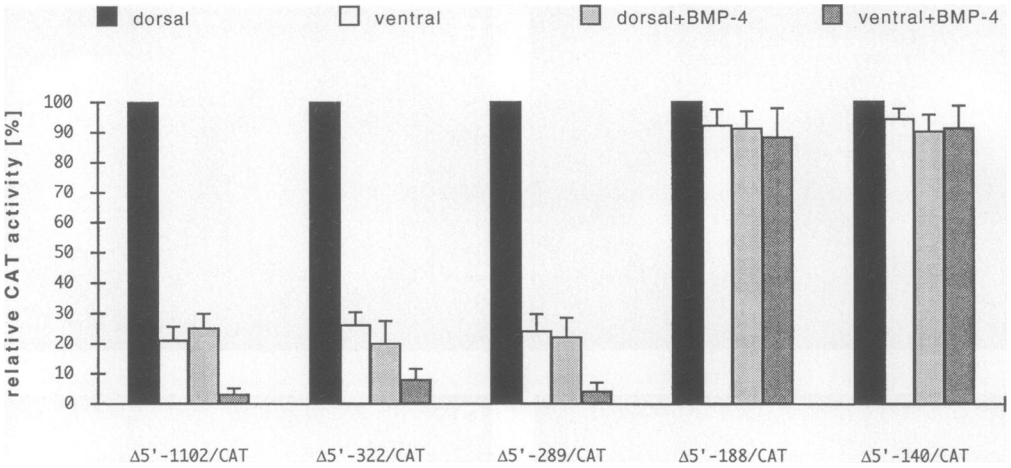

Fig. 6. Interaction of BMP-4 signalling with *XFD-1′* promoter deletion mutants. Four-cell stage embryos were either injected with 20 pg of the indicated promoter–*CAT* DNA constructs or co-injected with 1 ng BMP-4 mRNA into the marginal zone of the two dorsal or the two ventral blastomeres respectively. *CAT* activities were determined when embryos had reached stage 11. Enzyme activities obtained after dorsal injection were set as 100%; data are averaged from five independent experiments.

5G–J). This result points towards a BMP-responsive element located between positions –1102 and –140 of the *XFD-1′* promoter.

To localize this element more precisely, we have first injected the various promoter deletion mutants fused to the *CAT* gene into dorsal and ventral blastomeres of four-cell stage embryos. Reporter gene activities were determined at stage 11. Figure 6 demonstrates that all 5′-deletions up to Δ5′-289 are active after dorsal, but rarely after ventral injections. However, further deletions (Δ5′-188 and Δ5′-140) raise the *CAT* activity also after ventral injections to a level comparable with that obtained after dorsal injections. Thus, the inhibitory element is localized between positions –289 and –188 and it confers the dorsal/ventral difference in *XFD-1′* gene expression. Secondly, we have co-injected the promoter deletion mutants together with *in vitro* transcribed BMP-4 mRNA. The results demonstrate that all dorsally injected deletion mutants up to position –289 are substantially inhibited by BMP-4; simultaneously, these mutants show only low activity after ventral injection. However, when the promoter is further shortened, *CAT* activity is no longer reduced by co-injection with BMP-4 RNA. Moreover, this also holds true for ventral injection of these mutants. Embryos maintain high levels of *CAT* activity and do not respond to BMP expression. From these results we conclude that the inhibitory sequence element which prevents transcription of the *XFD-1′* gene in the ventral part of the embryo is identical with a sequence motif required for gene silencing by BMP-4. We suggest that this element be referred to as a BMP-activated inhibitory element (BIE).

Discussion

How many AREs?

Previous work has shown that activation of dorsal lip-specific genes, like *XFD-1′* (*XFKH1*), *Xlim-1* or *goosecoid*, can be induced by incubating animal caps with activin A in the absence of *de novo* protein synthesis (Cho *et al.*, 1991; Dirksen and Jamrich, 1992; Tadano *et al.*, 1993). This suggests a TGF-β-like signalling mechanism in which the ultimate step is realized by the activation of pre-existing transcription factor(s). Here we report on the identification of an activin response element (ARE) which is located between –140 and –80 upstream of the *XFD-1′* gene transcription start site. This promoter element is necessary but also sufficient to drive reporter gene activity in the whole embryo and, in the animal cap assay, it strongly responds to activin A. Surprisingly, a comparison of this ARE and other recently reported AREs from *Xenopus* promoters, like *Mix.2* (Huang *et al.*, 1995), *goosecoid* (Watabe *et al.*, 1995), *HNF1α* (Weber *et al.*, 1996) or the mesoderm-specific M region of the *Xsna* promoter (Mayor *et al.*, 1993), and those from the mouse and zebrafish *goosecoid* promoters (Watabe *et al.*, 1995; Joore *et al.*, 1996) reveals no or only very limited sequence homologies. At present, this discrepancy might be best explained by use of different signalling pathways or by one pathway which finally activates different transcription factors. The best fit is obtained with the *Mix.2* and the *HNF1α* elements (see Figure 7A), but whether this finding or the apparent homologies between distinct, short sequence motifs are of any significance remains to be elucidated. Moreover, these elements vary greatly in their reported lengths, thus leaving enough space for binding more than only one factor. Gel shift studies using embryonic protein extracts and AREs of *Mix.2* (Huang *et al.*, 1995) and *XFD-1′* promoters (unpublished results) show several retardation bands, only one of them appearing after activin A treatment and thereby behaving as an activin response factor (ARF). This points towards cooperative effects between constitutively binding and transiently activated transcriptional regulators, and it will be interesting to learn about the chemical nature of these factors. The outcome of these studies will probably also shed light on the requirements of FGF signalling components reported for activin signalling (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994) and of MAPKKK mediating TGF-β signal transduction (Yamaguchi *et al.*, 1995).

The ARE responds within the vegetal half

Injections of promoter deletion mutants into ventral blastomeres and subsequent incubation of isolated vegetal

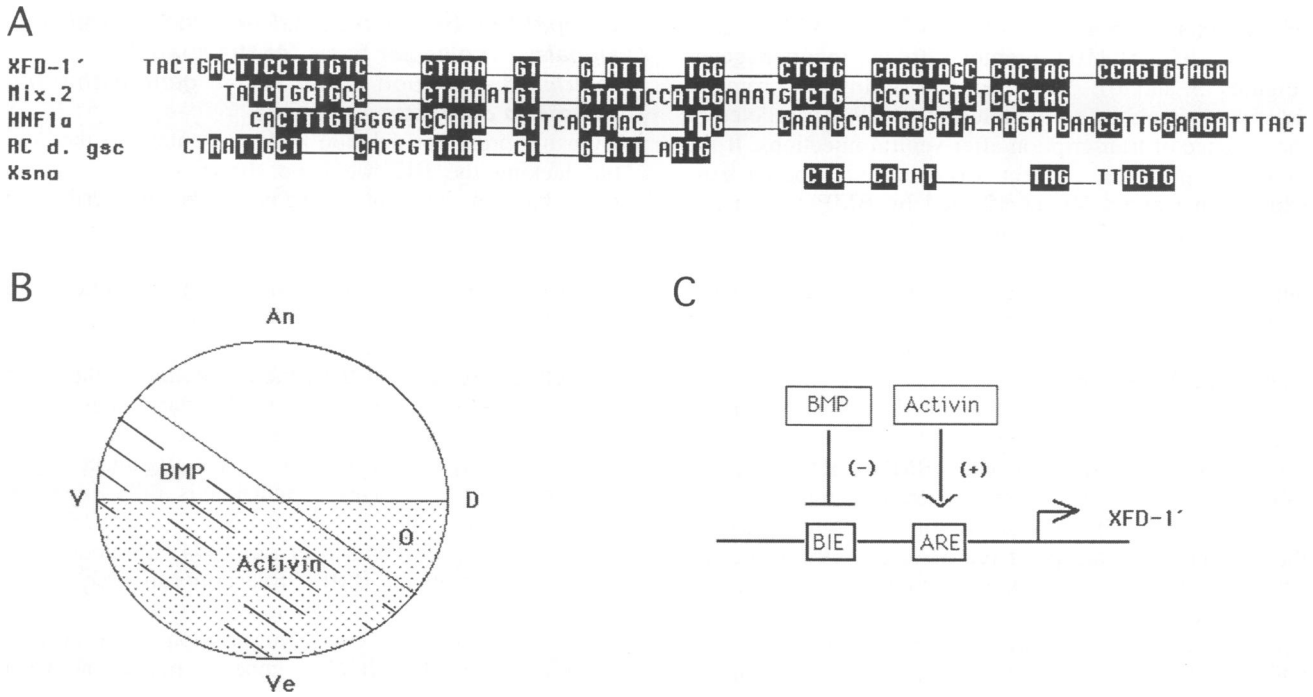

Fig. 7. Spatial activity of the *XFD-1′* gene is controlled by two distinct promoter elements. (**A**) AREs from *XFD-1′*, *Mix.2* (Huang *et al.*, 1995), *HNF1α* (Weber *et al.*, 1996) and *goosecoid* (reverse complement of distal element: Watabe *et al.*, 1995) genes and the M region of the *Xsna* gene (Mayor *et al.*, 1993) are aligned for the best fit. Minimal match length: 6; number of mismatches: 1. Identical positions are shadowed, deletions are shown by lines. (**B**) Late blastula/early gastrula stage embryo showing the endogenous distribution of BMP-2/4 and of a general mesoderm inducing factor (activin or Vg-1-like factor). Note that the organizer (O) is devoid of BMP-2/4. (An) animal pole, (Ve) vegetal pole, (D) dorsal, (V) ventral. (**C**) Scheme of the antagonistic actions of BMP-2/4 and activin A on the *XFD-1′* promoter. Note that two different TGF-β-like factors are signalling through different DNA targets and that the effect of the inhibitory element (BIE) overrides that of the activin response element (ARE).

explants in the absence or presence of activin A demonstrate that short promoter (–140) driven reporter gene expression is independent of exogenous inducers (Figure 4). This means that an endogenous signal (probably activin A or Vg-1) and all components of the signalling pathway are present in the vegetal half of blastula stage embryos. This conclusion supports the recent findings obtained with the distal element of the *goosecoid* promoter showing an endogenous signalling activity to be present after injection into all C and D tiers of 32-cell stage embryos (Watabe *et al.*, 1995). However, we do not detect any reporter gene activity even after application of activin A when using the long (–1102) promoter fragment, which demonstrates the presence of some inhibitory mechanism within the ventral/vegetal half acting upstream of position –140 of the *XFD-1′* promoter.

A BMP-responsive inhibitory element suppresses XFD-1′ transcription

As shown by injections into the B1 blastomeres of 32-cell stage embryos or into the two dorsal blastomeres of four-cell stage embryos, use of the –1102 promoter fragment is sufficient to mimic expression of the wild-type gene (Figure 5). However, it has to be noted that additional sequence elements are certainly required to regulate this gene within the embryo. Indeed, the observation of low reporter gene activity after injection into the ventral blastomeres (Figure 6) or of low expression found in animal cap explants lacking activin A treatment (Figure 4) is incompatible with the *in vivo* situation and suggests additional inhibitory mechanisms acting on sequence elements which are not present on the promoter fragment used. By extending the upstream region for another 2.2 kb we were not able to compensate for this failure (data not shown); therefore, if these additional elements exist, they are not present within 3.3 kb located 5′-flanking to the gene. However, the results shown in the present work clearly demonstrate that an inhibitory sequence element suppresses *XFD-1′* transcription in the ventral/vegetal region of the embryo and that removal of this element leads to transcriptional activation of reporter genes in cells derived from early ventral blastomeres (Figures 5 and 6). This is clearly documented by the increase of *CAT* activity after injection of Δ5′-188–*CAT* constructs into the ventral blastomeres of four-cell stage embryos and by injection of Δ5′-140–β-gal constructs into the A4 blastomeres of 32-cell stage embryos. In the latter case we observe expression in the ectoderm which is not monitored when using the Δ5′–1102 promoter fragment. The inhibitory sequence element is located downstream of position –289, it is completely or partially removed at position –188 and, although the functional significance is not known, it obviously co-localizes with an OCT-1 binding site found at position –223 to –216.

Recent reports have shown that BMP-2 and BMP-4 prevent transcription of *XFD-1/XFD-1′* genes in *Xenopus* embryos or in animal cap explants induced by activin A (Clement *et al.*, 1995; Re'em-Kalma *et al.*, 1995; Jones *et al.*, 1996). In search of candidate molecules signalling to the inhibitory element, we have tested whether BMP expression might be relevant to the inhibition observed. First, it became clear that microinjection of BMP-4 RNA

inhibits expression not only of the wild-type *XFD-1'* gene but also of Δ5'-1102 promoter-driven reporter genes (Figures 5 and 6). Moreover, the ability of promoter deletion mutants to be inhibited by BMP-4 paralleled to the absence of transcription after ventral injections. If the inhibitory promoter element was removed, reporter gene expression was no longer affected by BMP-4. Thus, in contrast to the Δ5'-289 mutant, the Δ5'-188–*CAT* construct is efficiently expressed after ventral injection and, simultaneously, it is not inhibited after either dorsal or ventral injection with BMP-4. We therefore conclude that the inhibitory element is essential in BMP-induced suppression of the *XFD-1'* gene.

Additional experiments (data not shown) have demonstrated that injection of BMP-2 RNA leads to the same effects as those obtained with BMP-4 RNA, i.e. an inhibition of the same promoter deletion mutants. This may be explained by the observation that both factors can bind to the same receptors (Graff *et al.*, 1994), thereby activating the same signal transduction pathway. Whether the features of the inhibitory sequence element reported here reflect a direct or an indirect response to this signalling pathway remains to be investigated. Therefore, at the present stage we prefer to designate the sequence motif found as a BMP signalling-triggered inhibitory element (BIE) rather than as a BMP response element. Our current experiments are designed in order to define its exact length and to isolate factors binding to the BIE. There are several reports suggesting an involvement of Ras/Raf/AP-1 (Xu *et al.*, 1996), MAPKKK (Yamaguchi *et al.*, 1995) or MAPKK pathways (Northrop *et al.*, 1995) in BMP signalling, but very recent work has clearly shown that Mad proteins are pivotal as mediators of the BMP signal transduction pathway (Graff *et al.*, 1996; Hoodless *et al.*, 1996; Liu *et al.*, 1996). Moreover, it became evident that distinct Mad proteins transduce signals for different members of the TGF-β superfamily. Our present results support and extend these findings because we provide direct evidence that BMP-2/4 and activin A are signalling through different DNA targets.

BIE overrides ARE activity

Reporter gene expressions driven by *XFD-1'* promoter deletion mutants show that BMP signalling inhibits all those mutants containing the BIE. Only short promoter mutants comprising the ARE but lacking the BIE respond within the embryo to activatory signals independently of BMPs. This finding provides a plausible explanation for previous findings that BMP signalling overrides the signalling activity of activin or an activin-like endogenous inducer (Dale *et al.*, 1992; Jones *et al.*, 1992; Graff *et al.*, 1994; Maeno *et al.*, 1994; Suzuki *et al.*, 1994; Clement *et al.*, 1995). Simultaneous incubation of animal cap explants with activin A and BMP-4 converts the expression from dorsal to ventral marker genes; BMP-2/4-injected embryos show a ventral phenotype and expression of dominant-negative BMP receptor mutants converts ventral to dorsal mesoderm. All these observations are fully compatible with our results obtained with the *XFD-1'* promoter: BMP inhibits, and removal of BMP or BMP signalling components leads to, an activation of this dorsal lip-specific early response gene.

Competition between dorsalizing and ventralizing signals: a molecular basis for the spatially restricted activation of the XFD-1' gene within the Spemann organizer

By using promoter deletion mutants containing the ARE but lacking the BIE we have shown that the *XFD-1'* gene has an inherent capacity to be transcribed in vegetal explants and in the ventral/vegetal region of whole embryos. This means that an endogenous activin-like factor and all necessary signalling components are present within the vegetal half of the embryo (Figure 7B). This raises the question of the *in vivo* mechanism restricting activation of the gene to the dorsal blastopore lip. The results of the present work clearly suggest that endogenous signalling to the BIE prevents transcription of the wild-type gene outside of the organizer region (Figure 7B and C). This conclusion is fully supported by the spatial transcription of BMP-2 and BMP-4 in *Xenopus* embryos (Fainsod *et al.*, 1994; Clement *et al.*, 1995). While maternal BMP-2 transcripts are ubiquitously distributed in early cleavage stage embryos and disappear rapidly after MBT, zygotic transcription starts only by neurula stages. The *BMP-4* gene is mainly activated after MBT and transcripts appear in the animal half and the ventral/lateral marginal zone. Therefore, at late blastula/early gastrula stages the dorsal blastopore lip is essentially devoid of BMP-2/4 transcripts. This deficiency may be further facilitated or maintained by BMP-antagonizing molecules produced in the organizer-like chordin or noggin (Re'em-Kalma *et al.*, 1995; Sasai *et al.*, 1995). In our opinion, the best candidate for suppressing dorsal genes in the ventral/vegetal region at midblastula stage is BMP-2 which might subsequently be replaced by BMP-4. The two proteins have already been identified in early embryos, but their localization still needs to be established (Ueno *et al.*, 1992; Shoda *et al.*, 1993). This concept would also imply that these two differentially regulated signals bind to the same receptor (Graff *et al.*, 1994) or, at least, that they both activate a common factor binding to the BIE. It should be noted however, that other members of the BMP family are known to be transcribed within the organizer. While the anti-dorsalizing morphogenetic protein (ADMP) may be responsible for a fine tuning of gene activity in the dorsal lip (Moos *et al.*, 1995), the functional role of BMP-7 has been thoroughly investigated for the inhibition of neural induction and neural patterning (Hawley *et al.*, 1995), but its influence on the expression of early response genes in the organizer, especially on the *XFD-1'* gene, remains to be investigated.

In summary, promoter analysis of the early response gene *XFD-1'* led to the identification of an ARE which responds to activin signalling within dorsal and ventral regions of the *Xenopus* embryo by transcriptional activation. However, this activation is not only antagonized but even overridden by an inhibitory element (BIE) which is activated directly or indirectly by BMP-2/4 signalling. Lack of BMP-2 and BMP-4 in the organizer region allows transcription of the *XFD-1'* gene and, probably, of other early response genes encoding products which additionally inhibit BMP activity. Thus, the antagonistic actions of activin and BMP-2/4 signalling

control the spatial activation of the *XFD-1'* gene within the Spemann organizer.

Materials and methods

Reporter gene constructs

A *Xenopus XFD-1'* genomic clone has been previously described (Knöchel *et al.*, 1992). Sequence comparisons were performed with MacMolly software (Soft Gene, Berlin). Various deletions of the 5'-flanking region were generated either by limited hydrolysis with exonuclease III (Sambrook *et al.*, 1989) or by generating distinct promoter subfragments by PCR employing appropriate primers containing restriction sites for *Hin*dIII–*Nco*I at the 3'-end and for *Bam*HI–*Sal*I at the 5'-end; this facilitated integration into the pEU-*CAT* and β-galactosidase reporter plasmids, respectively. The pEU-*CAT* vector was designed in order to detect very weak promoter activities and has a very low autoactivity by itself (Piaggio and De Simone, 1990). The β-galactosidase reporter, *phs3–lacZA*, contains the entire gene for LacZ in front of a mouse-derived heat-shock promoter (Kothary *et al.*, 1989). This heat shock element was removed by digestion with *Nco*I–*Sal*I prior to the construction of the various deletion constructs.

For promoter analysis of the transcriptional activity of various deletions in the context of a basal TATA box, we employed a construct kindly provided by R.Costa (Chicago, IL), which contains the basal CMV promoter in front of the *CAT* gene (Pani *et al.*, 1992). The deleted promoter elements were inserted into the *Xba*I site of this vector.

Embryos, microinjections and blastula explants

Xenopus laevis embryos were obtained by artificial fertilization, dejellied in 2% cysteine hydrochloride in 0.1× MBSH (adjusted to pH 7.5) [1× MBSH: 88 mM NaCl, 10 mM KCl, 2.4 mM $NaHCO_3$, 0.8 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.4 mM $CaCl_2$ and 20 mM HEPES pH 7.35] and staged according to Nieuwkoop and Faber (1975). For injections, embryos were placed into 4% Ficoll (Pharmacia) in 1× MBSH. Indicated amounts of plasmid DNA were injected into each blastomere at the two-cell stage, the two dorsal or the two ventral blastomeres at the four-cell stage or into the A4 or B1 blastomeres at the 32-cell stage (Dale and Slack, 1987) respectively. Embryos were cultured at 18°C in 0.1×MBSH until the desired stage. Animal or vegetal explants were dissected at stage 8, incubated with 66 ng/ml activin A (kindly provided by M.Asashima, Tokyo) in 0.1% albumin/Barth solution [44 mM NaCl, 0.5 mM KCl, 0.41 mM $MgSO_4$, 0.17 mM $Ca(NO_3)_2$, 2 mM $CaCl_2$, 0.6 mM $NaHCO_3$, 1 mM Na_2HPO_4, 0.07 mM KH_2PO_4 and 5 mM HEPES pH 7.35] for 3 h and subsequently cultured for 7 h in Barth solution at 18°C.

Synthesis and microinjection of BMP-4 mRNA

mRNA for microinjections was prepared by *in vitro* transcription of an *Eco*RI–*Xho*II fragment of BMP-$4_{I'}$ cDNA L2 (Köster *et al.*, 1991) cloned into the *Bgl*II site of the pSP64T expression vector using the TransProbe SP6 transcription kit (Pharmacia) and 1 μg *Bam*HI-linearized DNA. One nanogram of BMP-4 mRNA was co-injected with 20 pg Δ5'-*XFD-1'* promoter deletion–pEU-*CAT* constructs into the marginal zones of both dorsal and ventral blastomeres of *Xenopus* embryos at the four-cell stage.

Whole mount in situ hybridization

XFD-1' transcripts in *Xenopus* embryos were detected by using the whole mount *in situ* hybridization technique (Harland, 1991) with some modifications. After puncturing the blastocoel at the animal half of the embryo, they were fixed in MEMPFA (0.1 M MOPS pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$ and 4% paraformaldehyde) at room temperature for 90 min and stored at –20°C in ethanol. *In situ* hybridization was performed with digoxygenin-labelled antisense RNA transcribed from *XFD-1'* cDNA (Knöchel *et al.*, 1992). The colour reaction of the antibody-conjugated alkaline phosphatase was carried out by using BM purple substrate (Boehringer Mannheim). The embryos were finally fixed in MEMPFA for at least 1 h and dehydrated in methanol.

β-galactosidase detection in phs3–lacZA-injected Xenopus embryos

Δ5' *XFD-1'* promoter deletion mutants in phs3–lacZA vector were analysed by the β-gal assay (Detrick *et al.*, 1990). Embryos were washed three times in 1× PBS (137 mM NaCl, 2.7 mM KCl, 8.5 mM Na_2HPO_4 and 1.5 mM KH_2PO_4 pH 7.3), and fixed at 4°C for 1 h in 2% paraformaldehyde, 0.2% glutaraldehyde, 0.02% Nonidet P40, 0.1% Na-deoxycholate in 0.1× PBS. After washing another three times in 1× PBS the embryos were placed in the β-gal staining solution (5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.01% Na-deoxycholate, 0.02% Nonidet P40 and 1 mg/ml β-D-galactopyranoside) for 24 h at 37°C. After another washing and fixing embryos were photographed in methanol.

Preparation of protein extracts from embryos or explants

Whole cell extracts were made on ice by homogenization with 30 μl/embryo or 4 μl/explant 250 mM Tris–HCl pH 7.8. After centrifugation at 13 000 *g* at 4°C for 15 min, the resulting supernatant was carefully taken up without lipids and stored at –20°C.

CAT assay

CAT enzyme assays were performed according to Gorman *et al.* (1982) with slight modifications. One hundred and twenty microlitres of whole embryo or 40 μl of explant extract were preincubated with 25 nCi or 5 nCi respectively of D-threo-[dichloroacetyl-1-^{14}C]chloramphenicol (Amersham) at 37°C. The reaction was initiated by adding 2 μl 40 mM acetyl-CoA. After 0.5–2 h incubation chloramphenicol derivatives were extracted with ethylacetate, which was removed by drying. The residue was resuspended in 10 μl ethylacetate and spotted on silica thin layer chromatography plates (95% chloroform, 5% methanol, 1 h run). Plates were dried and processed with a Fuji phosphoimager BAS 1500 using MacBas 2.2 software.

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