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Regulation of the zebrafish *goosecoid* promoter by mesoderm inducing factors and Xwnt1

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Abstract

Goosecoid is a homeobox gene that is expressed as an immediate early response to mesoderm induction by activin. We have investigated the induction of the zebrafish *goosecoid* promoter by the mesoderm inducing factors activin and basic fibroblast growth factor (bFGF) in dissociated zebrafish blastula cells, as well as by different wnts in intact embryos. Activin induces promoter activity, while bFGF shows a cooperative effect with activin. We have identified two enhancer elements that are functional in the induction of the *goosecoid* promoter. A distal element confers activin responsiveness to a heterologous promoter in the absence of de novo protein synthesis, whereas a proximal element responds only to a combination of activin and bFGF. Deletion experiments show that both elements are important for full induction by activin. Nuclear proteins that bind to these elements are expressed in blastula embryos, and competition experiments show that an octamer site in the activin responsive distal element is specifically bound, suggesting a role for an octamer binding factor in the regulation of *goosecoid* expression by activin. Experiments in intact embryos reveal that the proximal element contains sequences that respond to *Xwnt1*, but not to *Xwnt5c*. Furthermore, we show that the distal element is active in a confined dorsal domain in embryos and responds to overexpression of activin in vivo, as well as to dorsalization by lithium. The distal element is to our knowledge the first enhancer element identified that mediates the induction of a mesodermal gene by activin.

Keywords: Goosecoid; Activin; Wnt; bFGF; Promoter; Zebrafish; Danio rerio

1. Introduction

The induction and subsequent patterning of embryonic mesoderm is a strictly regulated and complex process, driven by the concerted action of signaling molecules during blastula and gastrula stages (recently reviewed in Kessler and Melton, 1994). An important function of these signaling molecules is the transcriptional induction of genes involved in the establishment of the identity of mesodermal cells. Therefore, the understanding of the inductive properties of mesoderm inducing signals, as well as of the transcriptional mechanisms they employ to induce mesodermal genes is crucial to elucidate the molecular basis of mesoderm induction.

Basic FGF (bFGF) was the first purified growth factor demonstrated to induce mesoderm in *Xenopus* animal cap explants, producing only ventrolateral mesoderm (Slack et al., 1987; Green et al., 1992). Consistent with a role for bFGF in the induction of ventro-posterior development, expression of a dominant-negative FGF receptor in *Xenopus* embryos causes defective trunk and posterior development, without affecting anterior development (Amaya et al., 1991). In 1990, Asashima and co-workers showed that activin, a member of the TGFβ superfamily of growth factors, was a powerful mesoderm inducer in *Xenopus* animal caps (Asashima et al., 1990). In contrast to bFGF, activin is capable of inducing the most dorsal types of mesoderm, such as notochord (Green et al., 1990; Sokol et al., 1990). Overexpression of a dominant-negative activin receptor results in a complete disruption of mesoderm formation in *Xenopus* embryos, suggesting

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that signaling through activin receptors or closely related receptors plays an important role in mesoderm induction in vivo (Hemmati-Brivanlou and Melton, 1992; Hemmati-Brivanlou and Melton, 1994).

In addition to a partial overlap in inductive properties, activin and FGF signals interact in different ways. Low doses of bFGF lower the concentration of activin required for the maximal induction of muscle actin in dissociated and reaggregated animal cap cells (Green et al., 1992). Expression of a dominant-negative FGF receptor blocks activin-induced expression of mesodermal marker genes, such as *Xbra*, *Xnot* and *Mix1*, and to a lesser extent *gsc* and *Xlim1*, in *Xenopus* animal caps, suggesting that FGF is required for activin mediated mesoderm induction (LaBonne and Whitman, 1994; Cornell and Kimelman, 1994). Inhibition of Ras activity, a downstream component of the FGF signaling pathway, inhibits both activin and FGF mediated mesoderm induction in *Xenopus* animal caps (Whitman and Melton, 1992). A dominant inhibitory Raf-kinase, which acts downstream of Ras, blocks activin-induced muscle actin expression in *Xenopus* animal caps (LaBonne and Whitman, 1994). Recently, it was shown that inhibition of MAP-kinase, by overexpression of a MAP-kinase inactivating phosphatase, blocks mesoderm induction in response to activin and bFGF in *Xenopus* animal caps, confirming the requirement for FGF signaling in the process of activin mediated mesoderm induction (Gotoh et al., 1995; LaBonne et al., 1995; Umbhauer et al., 1995). It must be noted that the identity of the endogenous mesoderm inducing signals is not known. In addition to activin and bFGF, several other candidate molecules have been identified, most notably Vg1 and bone morphogenetic proteins (reviewed in Kessler and Melton, 1994), the latter of which have been implied in the specific induction of ventral mesoderm and in the repression of dorsal mesoderm induction (reviewed by Harland, 1994).

In addition to factors that induce mesoderm directly, a divergent class of molecules, designated as competence modifiers, has been identified that modulate the nature of the response of embryonic cells to mesoderm inducing signals (Moon and Christian, 1992), such as members of the wnt family (Moon and Christian, 1992), and *noggin* (Smith and Harland, 1992). *Xwnt8*, which has no intrinsic mesoderm inducing capacity, is able to convert bFGF induced ventrolateral mesoderm into dorsal mesoderm in *Xenopus* animal caps (Christian et al., 1992). Microinjected *Xwnt8* mRNA rescues a complete dorsal axis in UV ventralized embryos (Smith and Harland, 1991; Sokol et al., 1991), a property which it shares with the mouse and *Xenopus wnt1* genes (McMahon and Moon, 1989; Sokol et al., 1991). Similarly, *noggin* does not induce mesoderm, but can convert ventral to dorsal mesoderm (Smith et al., 1993).

The understanding of transcriptional mechanisms underlying the induction of mesodermal genes, requires the study of genes that are targets of mesoderm inducing signals by direct induction. The search for early mesodermal markers has led to the identification of several genes that are induced by activin in the absence of de novo protein synthesis. Among these are *Mix1* (Rosa, 1989), the frog homologue of the mouse *Brachyury T* gene (*Xbra*) (Smith et al., 1991), *Xlim1* (Taira et al., 1992), XFKH1/XFD1/*pintallavis* (Dirksen and Jamrich, 1992; Ruiz i Altaba and Jessell, 1992; Knöchel et al., 1992), *Xnot* (von Dassow et al., 1993) and *goosecoid* (Cho et al, 1991). *Goosecoid* (*gsc*) is a homeobox gene that is expressed exclusively in the organizer region of *Xenopus*, mouse, chicken and zebrafish (Cho et al., 1991; Blum et al., 1992; Izpisúa-Belmonte et al., 1993; Stachel et al., 1993; Schulte-Merker et al., 1994). Its expression is induced by concentrations of activin that result in the induction of the most dorsal type of mesoderm (Green et al., 1992, Gurdon et al., 1994). Inhibition of FGF signaling decreases the immediate induction of *gsc* transcripts by activin in *Xenopus* animal caps (LaBonne and Whitman, 1994). Furthermore, injected *Xwnt8* mRNA induces ectopic *gsc* expression in *Xenopus* embryos (Steinbeisser et al., 1993). These results suggest that *gsc* expression is regulated by mesoderm inducers, as well as by competence modifiers, rendering the *gsc* gene an excellent model for the study of the transcriptional regulatory mechanisms involved in mesoderm induction.

To study the transcriptional mechanisms underlying *gsc* expression, we cloned the zebrafish *gsc* promoter. In this paper we describe the delimitation of two enhancer elements involved in activin and bFGF mediated regulation of the zebrafish *gsc* promoter, as studied in dispersed blastula cells, as well as in intact embryos. The activity of a distally located enhancer element is induced by activin in the absence of de novo protein synthesis. A more proximally located element, surprisingly, responds only to the combination of activin and bFGF in dispersed blastula cells, as well as to *Xwnt1* in intact embryos. The proximal element is required for the activin mediated induction of the promoter. Binding of nuclear proteins to these elements has been analyzed, and the spatial transcriptional activity of the distal element in embryos has been established.

2. Results

2.1. Molecular cloning and spatial activity of a zebrafish gsc promoter

The zebrafish *gsc* promoter was cloned as described in Section 4. Molecular analysis of the cloned promoter region revealed a structure as shown in Fig. 1A. The transcription start site was mapped 29 bp downstream of the TATA box using RNase protection assays (data not shown, indicated as +1 in Fig. 1A). To investigate whether the cloned fragment contained *gsc* regulatory

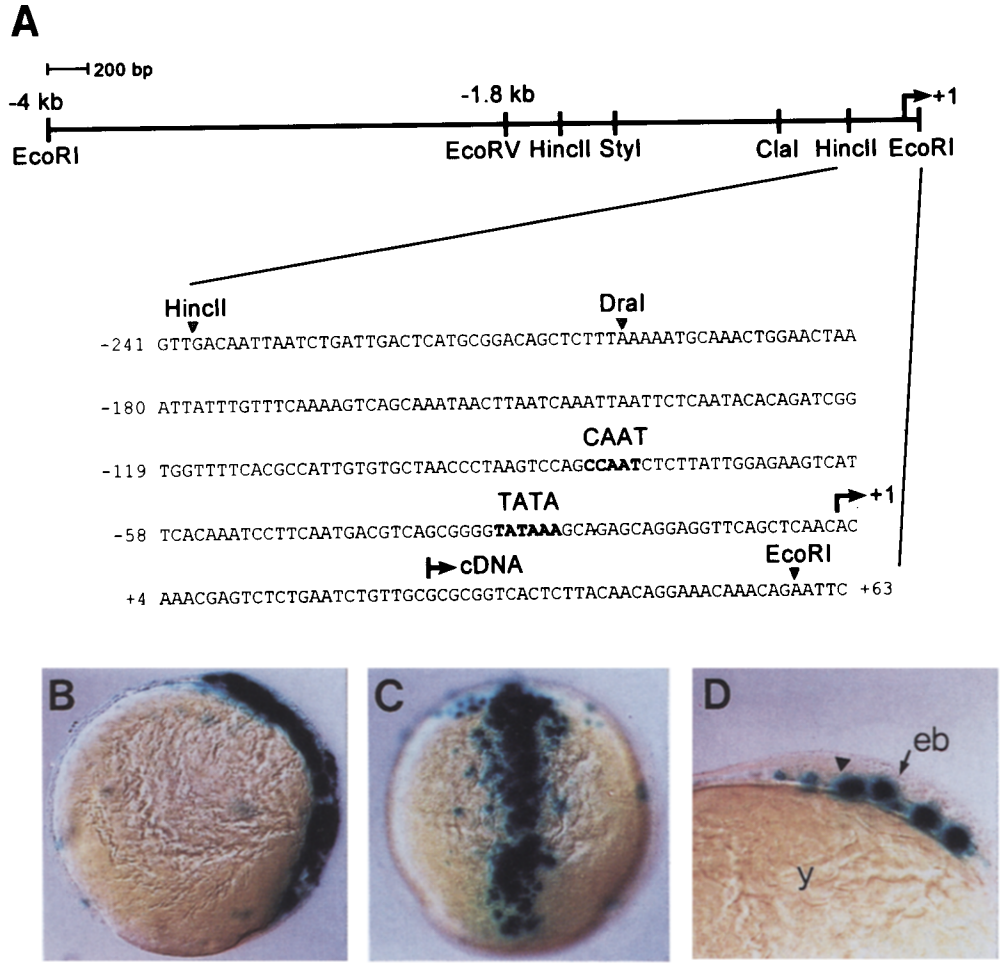


Fig. 1. (A) Schematic representation of the 4 kb zebrafish *gsc* promoter EcoRI fragment. Sites are indicated in the 1.8 kb EcoRV-EcoRI fragment. The sequence represents the proximal promoter region, extending from the HincII site at position −238 to the EcoRI site at position +63. CAAT-box and TATA-box consensus sequences are indicated. The DraI site was used to generate antisense RNA probes in order to map the approximate location of the transcription initiation site (indicated as +1) using RNase protection assays. A protected fragment of approx. 63 bp was observed in RNA from 6 hpf and not in RNA from 24 hpf embryos (data not shown). The first basepair of the cDNA clone pGSC211 is indicated at position +29. (B,C,D) In vivo LacZ analysis of *gsc* promoter activity. Three picograms of a LacZ reporter construct driven by a 1.8 kb EcoRV-EcoRI *gsc* promoter fragment were injected at the interface between the yolk and the blastomere of one cell stage embryos. Injected embryos were fixed and stained for β-galactosidase activity at 8.5 hpf. (A) Lateral view showing *gsc* promoter activity along the entire dorsal hypoblast and parts of the epiblast. Dorsal is to the right. (B) Dorsal view of the same embryo showing that *gsc* promoter activity is confined to the dorsal midline region. (C) Detail of the anterior hypoblast of another embryo showing *gsc* promoter activity in the anteriormost involuted cells. Activity is confined to the hypoblast in this embryo. The arrowhead indicates the border between the epiblast and the hypoblast. Abbreviations: EB (epiblast), Y (yolk cell). Anterior is to the left.

sequences, a 1.8 kb EcoRV-EcoRI fragment (Fig. 1A) was cloned into a LacZ reporter vector, injected into zebrafish embryos, which were stained for β-galactosidase activity at 80% epiboly (8.5 h post fertilization (hpf)). Promoter activity was detected in an elongated stripe along the dorsal midline of the embryo (Fig. 1B,C). In a minor portion of the embryos, activity was confined to hypoblast cells only (Fig. 1D). Similar results were obtained with the 4 kb EcoRI promoter fragment (data not shown).

The pattern of activity of the 1.8 kb *gsc* promoter LacZ construct strongly resembles the distribution of *gsc* transcripts, indicating that regulatory elements important for specific expression in the dorsal midline region of gastrula embryos are contained within this promoter fragment.

2.2. *Identification and delimitation of activin and activin/bFGF responsive elements in the goosecoid promoter*

Activin rapidly and strongly induces endogenous *gsc* expression in dissociated zebrafish blastula cells and this induction is enhanced by cotreatment of the cells with bFGF, whereas bFGF alone has no effect (Joore, 1995). To assess the localization of growth factor response elements within the zebrafish *gsc* promoter, promoter-reporter constructs were injected into zebrafish embryos, and promoter activity was determined in dissociated blastula cells, incubated with activin, bFGF or a combination of activin and bFGF (see Section 4). The activity of EVELuc, containing 1820 bp of upstream sequences is induced 5.0-fold by activin, 11.4-fold by activin plus

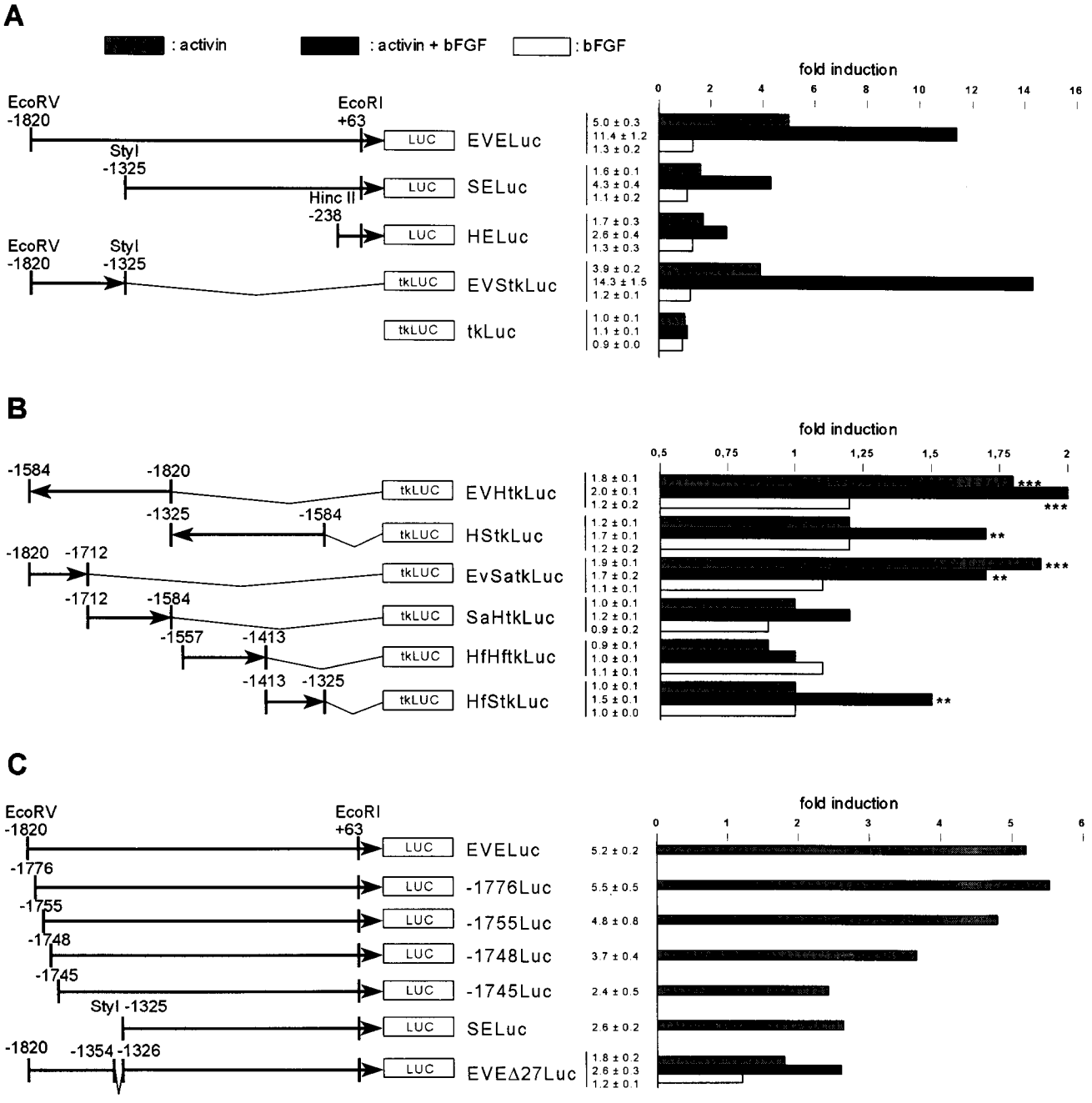


Fig. 2. Effects of activin and bFGF on the activity of gsc promoter-luciferase constructs in dissociated zebrafish blastula cells, treated with activin (grey bars), activin + bFGF (black bars) or bFGF (open bars). Schematic representations of promoter constructs are shown on the left. Numbers indicate basepair positions relative to the transcription start site at +1. Arrows indicate the orientation of the promoter fragments in the construct. The HSV thymidine kinase promoter is indicated by tk. Results are expressed as fold induction relative to samples treated with vehicle ± SEM. Experimental procedures are described in Section 4. Briefly, 20–80 embryos were injected with 30 pg supercoiled construct per embryo and subsequently dissociated at 4 hpf. The dispersed cells were treated with growth factors for 2 h, followed by determination of luciferase activity. Activin A and bFGF were used at a concentration of 20 ng/ml. (A) A region important for induction by activin and bFGF resides between −1820 and −1325 on the *gsc* promoter. Values represent the means of at least 12 individual determinations from at least 3 independent experiments. (B) Separate elements mediate the responses to activin and to activin + bFGF. Values represent the means of at least 12 individual determinations from at least 3 independent experiments. Statistically significant inductions are indicated by asterisks (Student's *t*-test, $***P < 0.001\%$, $**P < 0.005\%$). (C) Effects of deletions in the regions conferring responsiveness to activin and the combination of activin and bFGF on the induction of gsc promoter constructs. Values represent the means of at least 12 individual determinations from at least 3 independent experiments.

bFGF and 1.3-fold by bFGF (Fig. 2A). Deletion of the distal 495 bp (SELuc) results in a significant reduction of the response to activin (1.6-fold), as well as of the response to activin plus bFGF (4.3-fold, Fig. 2A). A construct containing 238 bp of upstream sequences (HELuc) is induced 1.7-fold by activin, 2.6-fold by activin plus bFGF and 1.3-fold by bFGF. Subsequently, the region between −1820 and −1325 bp was cloned into a luciferase vector driven by the herpes simplex virus thymidine kinase promoter (EVStkLuc). This construct was induced 3.9-fold by activin and 14.3-fold by activin plus bFGF (Fig. 2A). No effects were observed on the activity of the empty tkLuc vector. These results indicate that the region between −1820 and −1325 bp contains elements important for activin induction of the *gsc* promoter. Although bFGF alone had no significant effect on the activity of the *gsc* promoter, a strong cooperative effect with activin was observed, increasing the induction by activin over 3-fold.

To localize the responses to growth factors more precisely, a series of constructs was made containing fragments of the region between positions −1820 to −1325 in a tkLuc reporter vector. EVHtkLuc (−1820 to −1584), was induced 1.8-fold by activin, and 2.1-fold by activin plus bFGF, whereas HStkLuc (−1583 to −1325) showed no significant response to activin (1.2-fold), but was significantly induced by activin plus bFGF (1.7-fold) (Fig. 2B). These results are striking in two ways. First, the levels of induction of each fragment separately are lower than those observed for the entire region (−1820 to −1325). Second, separate responsive regions appear to exist for activin and the combination of activin and bFGF. Subsequently, smaller regions were tested. EVSatkLuc (−1820 to −1712) showed a response to activin, as well as to activin plus bFGF (1.9- and 1.7-fold, respectively), whereas SaHtkLuc (−1712 to −1584) and HfHftkLuc (−1557 to −1413) exhibited no significant response. The construct HfStkLuc (−1413 to −1325) showed a response only to activin plus bFGF (1.5-fold) (Fig. 2B). These results show that an element responsive to activin is located between −1820 and −1712 in the *gsc* promoter and an element responding only to the combination of activin and bFGF is located between −1413 and −1325. Our data demonstrate that both regions function in an orientation independent manner. Since bFGF has no cooperative effect on the induction of the activin responsive region, these experiments dissect the induction by activin and the cooperativity of bFGF into apparently separate regulatory pathways. In addition, the lower inductions of EVSatkLuc and HfStkLuc as compared to EVStkLuc suggest a high degree of cooperativity between the different enhancer elements.

To test the function of the activin responsive region between −1820 and −1712 in the context of EVELuc (−1820 to +63), unilateral 5′ deletion constructs of EVELuc were made and their induction by activin was compared to EVELuc, as well as to SELuc (−1325 to +63). Deletions up to −1755 had no significant effect on the induction by activin (Fig. 2C, −1776Luc and −1755Luc). However, deletion of an additional 7 basepairs (−1748 Luc) resulted in a lower induction as compared to the full length construct, whereas a deletion of the next 3 basepairs (−1745Luc) reduced the induction to the level of SELuc (Fig. 2C). This further delimits the activin responsive region to positions −1755 to −1712 (43 bp), designated the distal element. Subsequently, a construct with a 27 bp deletion in the region responsive to activin plus bFGF was tested (EVEΔ27Luc). As is shown in Fig. 2C, this construct is induced 1.8-fold by activin and 2.6-fold by activin plus bFGF, which is significantly lower than the wild type construct. Furthermore, the ratio between induction by activin plus bFGF and by activin is 1.4-fold for EVEΔ27Luc and 2.3 for EVELuc, indicating that an important part of the synergistic effect of bFGF is lost as a result of the deletion in EVEΔ27Luc. This supports our observation that the region between −1413 and −1325 (88 bp) of the *gsc* promoter, which we will call the proximal element, mediates a response to the combination of activin and bFGF.

2.3. The distal element responds to activin in the absence of de novo protein synthesis

Goosecoid is an immediate early response gene to activin in *Xenopus* animal caps (Cho et al., 1991; Tadano et al., 1993). To test whether the activin responsive distal element functions in the absence of protein synthesis, induction was determined in the presence of the protein synthesis inhibitor cycloheximide. A construct containing two copies of the region between −1820 and −1712 of the zebrafish *gsc* promoter in the tkLuc vector (EVSa2tkLuc) was induced 5.8-fold by activin (Fig. 3A). Costimulation with bFGF did not result in a significantly higher induction, completely in agreement with our results with EVSatkLuc (Fig. 2B). This construct was injected into embryos and dispersed blastula cells were preincubated with cycloheximide prior to growth factor treatment. Parallel samples were assayed for luciferase activity or luciferase mRNA expression, using a lysate RNase protection assay (Gillespie et al., 1992; Haines and Gillespie, 1992). In these experiments, activin induced luciferase activity 2.4-fold (Fig. 3B, lane 5), closely in agreement with luciferase mRNA induction (2.0-fold, lane 5). Induction levels are lower compared to the results shown in Fig. 3A, as a consequence of the higher amount of injected DNA and the preincubation period (Joore, unpublished observations). In the presence of cycloheximide (lanes 6 and 7), luciferase activity is decreased and not induced by activin, showing that protein synthesis is effectively inhibited. By contrast, in the presence of cycloheximide, luciferase mRNA is readily induced by activin. The induction of luciferase mRNA by cycloheximide

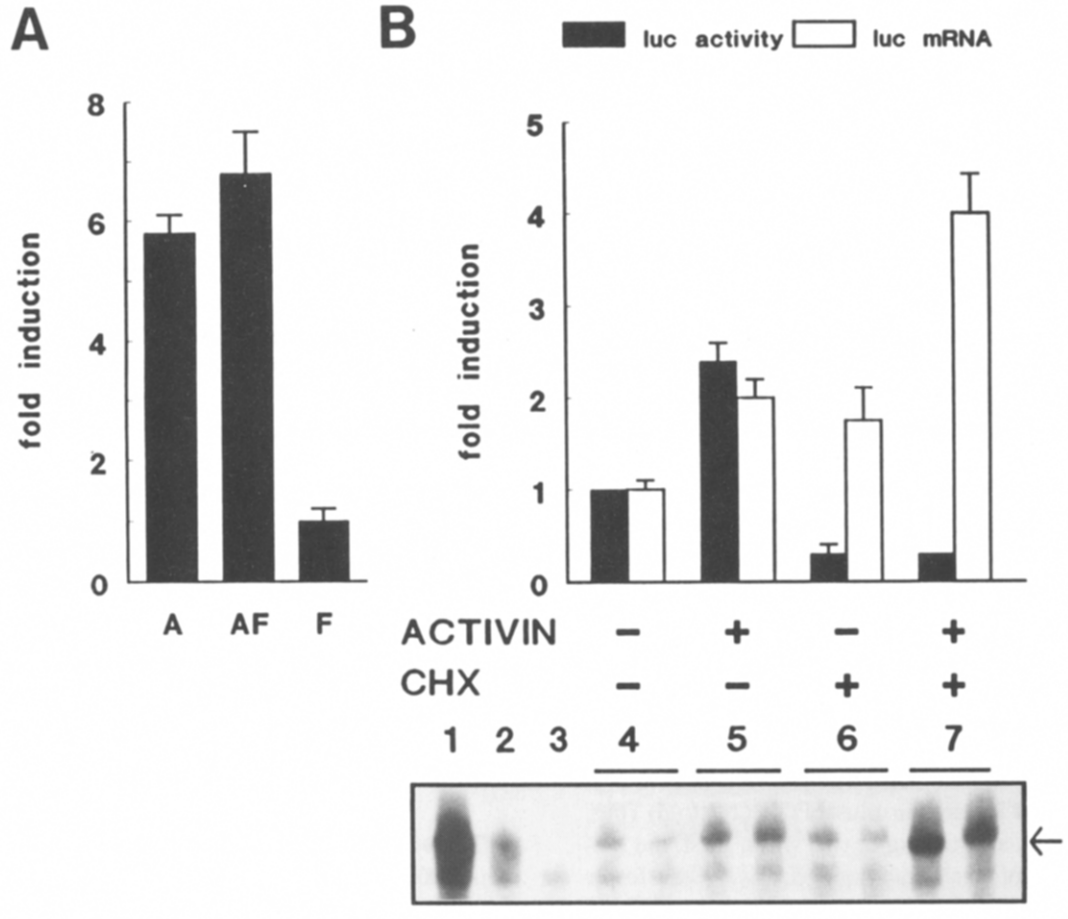


Fig. 3. Effects of cycloheximide on the induction of the distal element by activin. (A) Induction by activin (20 ng/ml), bFGF (20 ng/ml) or a combination of both (indicated as A, F and AF, respectively) of EVSa2tkLuc, containing two copies of the distal element region, in dissociated blastula cells. Experimental procedures are described in Section 4. Graphs represent the means of 8 individual determinations from 2 independent experiments, expressed as fold induction over untreated samples. Bars indicate SEM. (B) EVSa2tkLuc is induced by activin in the absence of de novo protein synthesis in dissociated blastula cells. The construct was injected at 60 pg per embryo. Dissociated blastula cells from injected embryos were preincubated with 10 μg/ml cycloheximide for 30 min or left untreated, followed by a 2 h incubation with 20 ng/ml activin or vehicle. Parallel samples were processed for luciferase activity determination or for luciferase mRNA expression using a lysate RNase protection assay (see Section 4). The lower panel shows a representative autoradiogram of an RNase protection gel. The arrow indicates the protected luciferase probe fragment. Lanes 1 and 2 show RNase protections on 10 and 1 pg of luciferase sense RNA, respectively. Lane 3 shows that luciferase mRNA is absent in blastula cells from uninjected embryos. Lanes 4–7 show luciferase mRNA expression in duplicate samples of blastula cells from injected embryos, treated with vehicle (lane 4), 20 ng/ml activin (lane 5), cycloheximide (lane 6) or 20 ng/ml activin and cycloheximide (lane 7). The graph shows the means of the results of 2 independent experiments. Black bars represent luciferase activity induction over vehicle treated samples (means of 6 determinations ± SEM). Open bars represent luciferase mRNA levels as quantified on a PhosphorImager, expressed as fold induction over vehicle treated samples (means of 4 determinations ± SEM).

alone is possibly a result of the downregulation of a labile factor involved in messenger degradation. These results demonstrate that induction of the distal element by activin is protein synthesis independent and is therefore, most likely, a result of direct modifications of transcription factors by the activated activin signaling cascade.

2.4. Sequences of the proximal element respond to Xwnt1, but not to Xwnt5c

Expression of *goosecoid* during gastrulation is closely linked to the organizer region in zebrafish, *Xenopus*, mouse and chicken (Cho et al., 1991; Blum et al., 1992; Izpisúa-Belmonte et al., 1993; Stachel et al., 1993; Schulte-Merker et al., 1994). Several classes of genes have been shown to induce ectopic organizer activity, resulting in axis duplications, among which are members of the wnt family (Sokol et al., 1991 and references therein). RNA overexpression of *Xenopus wnt1* induces the formation of secondary axes (McMahon and Moon, 1989), whereas overexpression of *Xwnt5c* has no such capacity (J.G. Koster, B. Stegeman and O.H.J. Destrée, unpublished data). As described above, a deletion of nucleotides −1353 to −1327 strongly decreases the induction of EVELuc by activin and bFGF (Fig. 2C). To test whether these sequences are involved in regulation by

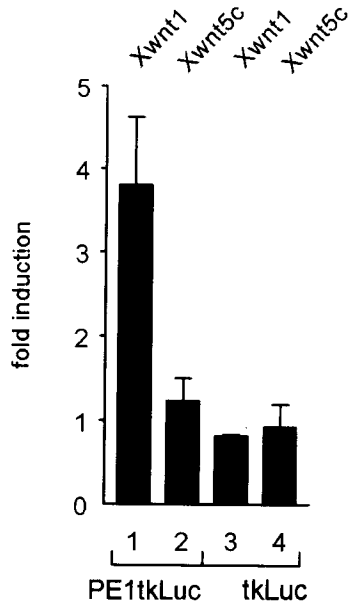


Fig. 4. Sequences from the proximal element are induced in vivo by coexpression of *Xenopus wnt1*, but not by *wnt5c*. Ten picograms of a construct containing a single copy of the region between −1384 to −1335 of the *gsc* promoter in tkLuc (PE1tkLuc, lanes 1 and 2) or 10 pg of the empty tkLuc reporter (lanes 3 and 4) was coinjected with 10 pg of pSG5-Xwnt1 (lanes 1 and 3), pSG5-Xwnt5c (lanes 2 and 4) or the empty vector pSG5 into the blastomeres of 2–4 cell stage embryos. Embryos were allowed to develop to 7 hpf, harvested in groups of 10 and luciferase activity was determined. The results are expressed as fold induction over pSG5 coinjected control samples. Lanes 1 and 2 are the means of three independent experiments, representing at least 60 injected embryos each, lanes 3 and 4 show the means of duplicate samples of a representative experiment. Bars indicate SEM.

wnts, a reporter construct containing nucleotides −1384 to −1335 (PE1tkLuc) was coinjected with expression constructs expressing *Xwnt1* (Noordermeer et al., 1989) or *Xwnt5c* (J.G. Koster, B. Stegeman and O.H.J. Destrée, unpublished data). Coinjection with pSG5-Xwnt1 induces PE1tkLuc activity 3.9-fold, whereas coinjection of pSG5-Xwnt5c has no effect (Fig. 4). Similar results were obtained with HfStkLuc, as well as with EVELuc constructs (data not shown). These results demonstrate that sequences of the proximal element located between positions −1384 and −1335 respond to overexpression of a wnt gene with axis inducing activity, whereas a wnt gene that lacks this activity has no effect, thus substantiating the notion that wnts are positive regulators of *gsc* expression in vivo.

2.5. Binding of nuclear proteins to the distal and proximal elements

The sequence of the distal activin responsive element reveals several potential binding sites for nuclear proteins. A CAAT-box (Santoro et al., 1988) is present between −1754 and −1750, and a perfect octamer consensus sequence (reviewed in Staudt and Leonardo, 1991) resides between −1726 and −1719 (Fig. 5A). To determine whether proteins binding to the distal element are present in embryos, nuclear extracts were prepared from dissociated blastula cells derived from 4 hpf embryos. In gel retardation assays, using a 108 bp EcoRV-SauIIIA fragment (Fig. 5A) as a probe, two major retarded complexes, and several weaker complexes were observed (Fig. 5B). Competition with 100-fold molar excess of the unlabelled fragment showed that these complexes were specific, whereas an unrelated promoter fragment did not compete. To gain insight into the localization of the complexes on the fragment, competitions with a 100-fold molar excess of a series of oligonucleotides were performed (oligonucleotides indicated in Fig. 5A). Oligonucleotide ARR1, encompassing the CAAT-box did not compete; neither did ARR2 (Fig. 5C). However, ARR3, encompassing the CAAT-box and the octamer site efficiently competed for all observed complexes, whereas an oligonucleotide containing a consensus octamer site competed for the upper major complex only. An unrelated oligonucleotide did not compete, showing the specificity of this assay.

A similar analysis was carried out with the proximal element. As a probe, an 88 bp HinfI-StyI fragment (−1413 to −1325) was used on 4 hpf dissociated blastula cell nuclear extracts (Fig. 5D). Three major retarded complexes were observed, one of which showed very low mobility (Fig. 5, lane 2). Competition with 100-fold molar excess of unlabelled probe efficiently competed for binding of the upper complex, whereas competition of the two complexes with higher mobility was less prominent, but detectable (lane 3). No competition was observed with an unrelated promoter fragment (lane 4).

We conclude that proteins which specifically bind to the distal and proximal elements are present in blastula stage embryos. Competition experiments on the distal element suggest that proteins bind to the octamer site in this element (Fig. 5A). Furthermore, sequences between −1724 and −1714, encompassing the octamer site appear to be important for the binding of all complexes to this region, suggesting a role for an octamer site binding protein in the regulation of the distal activin responsive element.

2.6. In vivo spatial activity of the distal element

The activity of the distal activin response element was investigated in intact embryos. A construct containing two copies of the region between −1820 and −1712, coupled to an adenovirus E1b TATA box driving the LacZ reporter gene (EVSa2TLac) was injected into embryos. Activity of enhancerless constructs driven by an E1b TATA box is undetectable in embryos (data not shown), therefore, expression of this construct in the embryo is determined exclusively by enhancing elements that activate transcription from the E1b TATA box.

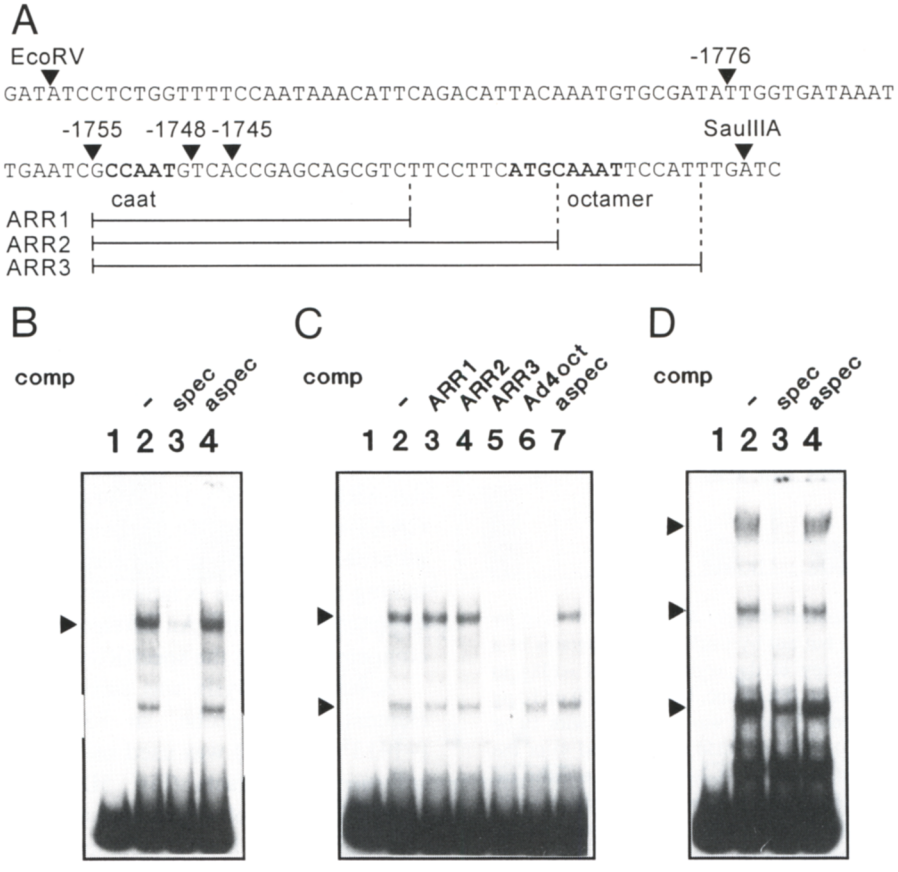


Fig. 5. Binding of nuclear proteins to the proximal and distal elements of the *gsc* promoter. Ten micrograms of nuclear extract prepared from dissociated blastula cells was incubated with 20 000 cpm of end-labeled probe and analyzed by gel retardation. Protein–DNA complexes were resolved on a 5% non-denaturing polyacrylamide gel and exposed for autoradiography for 3 days at −80°C with an intensifying screen. Arrowheads in (B), (C) and (D) indicate the major specific complexes. (A) Sequence of the distal element region of the zebrafish *gsc* promoter, used as a probe in gel retardation experiments. The CAAT and OCTAMER sites are indicated, as well as the oligonucleotides used in competition experiments. Numbers indicate the 5′ ends of the promoter deletion constructs shown in Fig. 5A. The minimal region required for activin induction (distal element) extends from position −1755 to the SauIIIA site at −1712. (B) Binding to the distal element using an EcoRV-SauIIIA fragment (−1820 to −1712) as a probe. Lane 1 shows the free probe, lane 2 incubation with nuclear extract, lanes 3 and 4 competition with a 100-fold molar excess of unlabeled probe and of an aspecific fragment (see Section 4), respectively. (C) Competition experiment with a 100-fold molar excess of unlabeled synthetic oligonucleotides. Lane 1 shows the free probe, lane 2 incubation with extract, lane 3 competition with ARR1 (−1755 to −1734), lane 4 with ARR2 (−1755 to −1724), lane 5 with ARR3 (−1755 to −1714), lane 6 with an oligonucleotide harboring the Adenovirus 4 octamer site (Ad4oct, Verrijzer et al., 1990), lane 7 with an aspecific oligonucleotide (PE1, see Section 4). (D) Binding to the proximal element using an HinfI-StyI fragment (−1413 to −1325) as a probe. Lane 1 shows the free probe, lane 2 incubation with extract, lanes 3 and 4 competition with a 100-fold molar excess unlabeled probe and aspecific fragment (see Section 4), respectively.

At 4.3 hpf, a late blastula stage, distal element activity is detected in a localized patch at the blastoderm margin on one side of the embryo (Fig. 6A,B). In an early gastrula stage embryo (6 hpf) the activity is detected exclusively in the region of the embryonic shield, at the dorsal side of the embryo, where involution is most prominent (Fig. 6C,D). At 75% epiboly (8 hpf, midgastrula stage) activity is detected in a wide region at the dorsal side of the embryo, extending anteriorly towards the animal pole approximately as far as the hypoblast has involuted (Fig. 6E,F). During gastrula stages, both the hypoblast and epiblast are stained (Fig. 6C,E), indicating that there is no germ layer restricted activity of the distal element. Extensive analysis of the morphology of stained pregastrula embryos (Schmitz and Campos-Ortega, 1994), as well as of the staining patterns in early neurula embryos confirmed that the activity of the distal element is exclusively dorsally localized (data not shown).

To determine whether the distal element responds to activin in vivo, EVSa2TLac was coinjected with various amounts of synthetic activin βB mRNA. Injected embryos were grown until controls reached the shield stage, fixed and stained. Coinjection with 500 fg activin mRNA clearly induced distal element activity to a larger domain (Fig. 6G). Although the domain was extended in many cases, activity remained mainly dorsal. Coinjection with

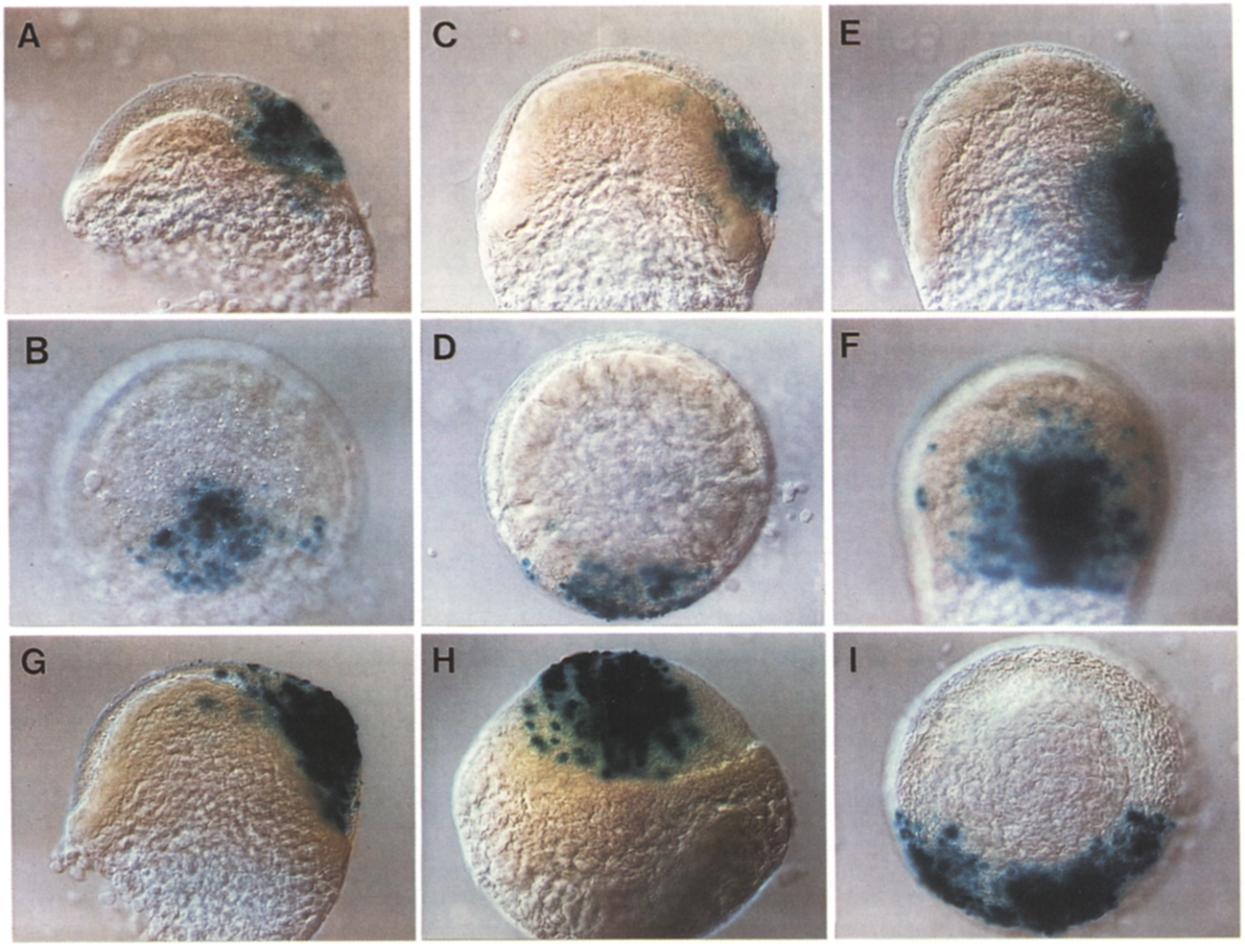


Fig. 6. Spatial activity of the distal element in blastula and gastrula embryos. Embryos of the 2–4 cell stage were injected with 10 pg of EVSa2TLac construct, allowed to develop until various stages were reached, fixed and stained for β-galactosidase activity. Typically 80% of the injected embryos showed staining. Dorsal is to the right in lateral views. (A) and (B) Lateral and animal pole views, respectively, of a 4.3 hpf embryo showing distal element activity before gastrulation in a confined region at the future dorsal side of the embryo. (C) and (D) Lateral and animal pole views, respectively, of a 6 hpf embryo showing localized distal element activity at the dorsal side of the embryo in the shield region. (E) and (F) Lateral and dorsal views, respectively, of an 8 hpf embryo, showing distal element activity at the dorsal side. (G) Lateral view of a 6 hpf embryo, coinjected with 10 pg EVSa2TLac and 500 fg of *Xenopus* activin βB mRNA. Distal element activity is enhanced and spatially extended, but still confined to the dorsal region of the embryo. (H) Lateral view of a 6 hpf embryo, coinjected with 10 pg EVSa2TLac and 500 pg of *Xenopus* activin βB mRNA. The embryo shows no signs of epiboly or gastrulation. The activity of the distal element is strongly induced. (I) Animal view of a 6 hpf embryo injected with 10 pg of EVSaTLacZ and subsequently treated with 0.3 M LiCl at the 32–64 cell stage. The region of distal element activity has extended along the blastoderm margin. The embryos shown in this figure represent typical patterns as obtained in at least 80 injected embryos from at least two independent injection experiments.

500 pg activin mRNA severely disturbs morphogenesis, apparently resulting in a developmental arrest at the blastula stage. In these embryos distal element activity is detected in a large, intensely stained patch in the center of the embryo, but no correct spatial coordinates could be identified, since the embryos were abnormal (Fig. 6H). Coinjection of EVSa2TLac with 500 pg of a control mRNA did not influence embryonic development, the pattern, or the intensity of distal element activity (not shown).

Finally, activity of the distal element was determined in embryos dorsalized with lithium chloride. In about 50% of the dorsalized embryos, a pattern was detected as shown in Fig. 6I. The domain of activity is radially extended, spanning a 120° region along the marginal zone, similar to findings on *gsc* expression in lithium treated embryos (Stachel et al., 1993). The percentage of embryos with abnormal distal element activity is in agreement with the percentage of severe phenotypes obtained in lithium treated embryos (Joore, unpublished results).

These in vivo experiments suggest that the distal element is exclusively active in a localized region at the dorsal side of the embryo, the region where endogenous *gsc* transcripts are expressed (Stachel et al., 1993; Schulte-

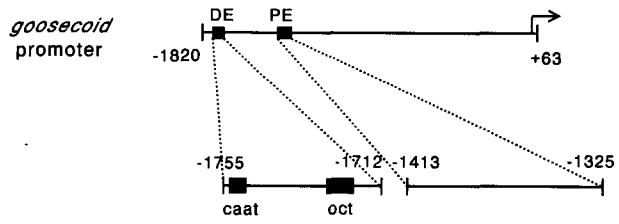
Merker et al., 1994). The activity is detected before the involution movements start, in a domain which marks the future dorsal side of the embryo and which will contribute to the shield and dorsal parts of the embryonic axis, suggesting that regulation of this element is involved in the earliest steps of *gsc* induction. Experiments in dorsalized embryos further substantiate the congruency between the spatio-temporal activity of the distal element and *gsc* expression. Finally, coinjection experiments with activin mRNA demonstrate that the distal element is induced by activin in intact embryos, strongly supporting our observations in dispersed blastula cells.

3. Discussion

We have analyzed the induction of activity of zebrafish *goosecoid* (*gsc*) promoter constructs in response to activin A and bFGF in dispersed zebrafish blastula cells. Two enhancer elements were identified that mediate the responses to growth factors: a distal element of 43 bp, which responds to activin independently of *de novo* protein synthesis and a proximal element of 88 bp, which only responds to the combination of activin and bFGF. Both elements are important for activin induction of the *gsc* promoter and specifically bind nuclear proteins present in blastula stage embryos. The proximal element contains sequences that respond to *Xwnt1* in intact embryos (Fig. 7). The distal, activin responsive, element activates transcription in a defined dorsal domain of the embryo and coinjection of activin βB mRNA enhances the activity in vivo. Finally, in embryos dorsalized with lithium chloride, the domain of distal element activity is radially expanded.

3.1. Spatial activity of the gsc promoter resembles gsc mRNA expression

The spatial activity of a 1.8 kb *gsc* promoter fragment is confined to the dorsal midline region of midgastrula embryos, in agreement with the expression pattern of *gsc* mRNA. In some embryos, activity was restricted to the hypoblast region, but in the majority of the embryos examined, the promoter was active in the epiblast as well. At the midgastrula stage (75% epiboly), Thisse et al. (1994) identified a domain with *gsc* expression in ectodermal cells overlying the posterior region of *gsc* expression in the mesoderm, which may explain the activity of the *gsc* promoter in the epiblast. Alternatively, in microinjection experiments, multiple promoter copies are introduced in embryonic cells, which, in combination with the sensitive β-galactosidase assay, may result in detection of ectopic promoter activity. This problem may be, at least partly, overcome by stably introducing a single copy of a *gsc* promoter LacZ construct in the zebrafish genome. We are currently in the process of generating such stable transgenic zebrafish lines.



	Distal element 43 bp	Proximal element 88 bp
induction by activin	yes	no
synergism between activin + bFGF	no	yes
important for induction by activin	yes	yes
induction by wnt1	not done	yes

Fig. 7. Summary of the inductions by activin, bFGF and *Xwnt1* on the distal and proximal elements in the *gsc* promoter. Numbers represent basepair positions relative to the transcription start site, as indicated by the arrow.

3.2. Analysis of promoter constructs in dispersed blastula cells

We have applied a novel method for studying growth factor regulated promoter activity in embryonic cells, utilizing dissociated blastula cells from embryos injected with promoter-reporter constructs. Our method serves several purposes. Firstly, promoter regulation is analyzed in cells where the promoter is regulated in vivo, which are the cells most likely to express all essential components of the relevant growth factor signal transduction pathways, as well as the appropriate transcription factors. Secondly, in dispersions, all cells are exposed to defined and equal concentrations of growth factors (Green et al., 1990; Green and Smith, 1992). This method eliminates most of the experimental variation, typical for assessment of promoter activity in whole embryos upon microinjection, since activity is determined in a mixture of cells derived from many injected embryos, thus eliminating the effects of mosaiscism (Joore, 1995). Although it has been shown that dissociation of embryonic cells may alter their developmental potential, and that reaggregation is necessary to establish sharp threshold responses to activin (Wilson and Melton, 1994; Green et al., 1994; Symes et al., 1994), these effects are relatively slow effects, and probably do not interfere with rapid growth factor responses. Furthermore, it has been reported in dissociated *Xenopus* animal cap cells, that activin induced expression of *gsc* remains constant, while, for instance, *Xbra* expression declines if the cells are not reaggregated (Cornell and Kimelman, 1994, personal communication therein). It is important to note that we were able to extend our findings to intact embryos, showing that dispersed blastula cells serve as a valid model to study promoter inductions by mesoderm inducing factors.

3.3. Two cis-acting elements cooperatively regulate induction of the goosecoid promoter in response to mesoderm inducing factors

The distal element in the *gsc* promoter (Fig. 7) is to our knowledge the first enhancer element described to mediate the induction of a developmental regulatory gene by activin. This element mediates an immediate early response to activin, which is in agreement with the activin induction of *gsc* transcripts in the absence of de novo protein synthesis in *Xenopus* animal caps (Cho et al., 1991). Our experiments indicate that outside this region other activin responsive regions reside on the *gsc* promoter, which may add up to a full induction of *gsc* transcription. It has been shown that *gsc* responds with an optimum to relatively high concentrations of activin in *Xenopus* animal caps (Gurdon et al., 1994), thus other activin concentrations than employed in our experiments may induce distal element activity to a greater extent. Furthermore, other TGFβ-like growth factors such as Vg1 or *nodal*, may be more potent inducers of distal element activity than activin. Vg1 is a potent inducer of *gsc* expression in *Xenopus* animal caps (Thomson and Melton, 1993) and *nodal* mRNA injections into zebrafish zygotes result in an ectopic shield with induced *gsc* and *Lim1* expression in that region (Toyama et al., 1995). Although Vg1 may still satisfy the criteria for acting as an endogenous mesoderm inducer, *nodal* may rather be involved in the induction and/or maintenance of the primitive streak in the mouse and equivalent structures in other vertebrates (Zhou et al., 1993; Conlon et al., 1994). Finally, since *gsc* responds to high activin concentrations (Green et al., 1992; Gurdon et al., 1994), activin response elements regulating *gsc* may have evolved to become relatively inefficient transcriptional inducers.

The proximal element responds to costimulation with activin and bFGF only (Fig. 7). Several lines of evidence suggest interactions between activin and bFGF signaling pathways. First, inhibition of activin signaling by injection of a dominant negative activin receptor in *Xenopus* embryos completely blocks mesoderm induction, suggesting that also bFGF mediated induction is inhibited (Hemmati-Brivanlou and Melton, 1992). Second, Green et al. (1992) showed in dissociated and reaggregated *Xenopus* blastula cells that bFGF lowers the concentration of activin needed for muscle actin induction. Third, inhibition of bFGF signaling by dominant negative FGF receptors reduces the activin mediated induction of mesodermal markers in *Xenopus* animal caps (LaBonne and Whitman, 1994; Cornell and Kimelman, 1994). Effects on *gsc* induction by activin were observed by LaBonne and Whitman (1994), who used 8 ng of dominant negative FGF receptor mRNA, whereas Cornell and Kimelman (1994) who used 1 ng, did not, suggesting that only a strong inhibition of FGF signaling affects *gsc* induction by activin. Our data indicate that specific transcriptional mechanisms exist that respond to the combined action of activin and bFGF, which may mediate the crosstalk between these factors on the *gsc* promoter.

Interestingly, a deletion in the proximal element, which does not respond to activin alone, has an approximately equal impact on the activin induction of the *gsc* promoter, compared to a deletion of the distal element (Fig. 7). This suggests a cooperative action of these enhancer elements on activin induction of the *gsc* promoter, by increasing the inductive capacity on the distal element. This is further substantiated by the observation that a construct encompassing both the distal and the proximal element coupled to a heterologous promoter is induced by activin to approximately the same extent as the full length promoter.

3.4. Wnt1 responsive sequences are located in the proximal element

A 49 bp region derived from the proximal element of the *gsc* promoter is activated by overexpression of *Xenopus wnt1* (Fig. 7). Overexpression of *Xwnt1* by means of RNA injection induces the formation of a complete secondary body axis in *Xenopus* (Sokol et al., 1991), suggesting that *gsc* expression is induced in these embryos. More directly, ectopic *gsc* expression is induced by injection of *Xwnt8* mRNA in *Xenopus* embryos (Steinbeisser et al., 1993), as well as in zebrafish embryos injected with zebrafish *wnt8* paralogs (Kelly et al., 1995). In these experiments *wnt* protein was expressed before midblastula transition (MBT), using microinjected mRNA, whereas we observed effects with DNA constructs overexpressing wnt protein only after MBT. Nevertheless, our results strongly suggest that the zebrafish *gsc* promoter is regulated by wnt-like factors in vivo, in agreement with a role for members of the wnt family in mesodermal patterning and axis formation.

3.5. Spatial activity of the distal activin responsive element is confined to the dorsal domain in the embryo

In vivo experiments show that the activity of the distal element is strictly confined to the dorsal domain in the embryo. The spatial activity of the distal element strongly resembles endogenous *gsc* expression, although some significant differences exist. First, *gsc* mRNA expression is confined to the hypoblast region during early gastrulation (Stachel et al., 1993; Schulte-Merker et al., 1994), whereas activity of the distal element is not germ layer restricted. The activity of the 1.8 kb *gsc* promoter is not germ layer restricted either, which may indicate that specific regulatory elements are not contained in this promoter fragment. Second, the region of distal element activity at 75% epiboly is wider than the region of *gsc* ex-

pression at the same stage, as revealed by in situ hybridization experiments (Stachel et al., 1993; Schulte-Merker et al., 1994). These differences suggest that the distal element may not be the only determinant of the spatial regulation of *gsc* expression. We cannot exclude in these experiments that other regulatory elements, included in the EVSa2TLac construct outside the region defined as distal element, influence the observed spatial patterns. However, binding of nuclear proteins to the promoter fragment used in the in vivo analysis is restricted to the region of the distal element, suggesting that only these sequences are important for transactivation. Furthermore, we do not know whether the distal element is exclusively regulated by activin or activin-like signals in the embryo. Importantly, the induction of activity of the distal element in embryos coinjected with activin βB mRNA shows that the distal element exhibits a clearly detectable response to activin, thereby substantiating its relevance for *gsc* promoter induction in vivo. We show that low amounts of activin βB mRNA extended the region of distal element activity at the dorsal side of the embryo. High amounts of activin βB mRNA severely disturbed embryogenesis, but the results suggest ectopic induction of distal element activity. We believe that low amounts of activin mRNA are below threshold levels to induce distal element activity in ventral regions of the embryo. Finally, the pattern of activity of the distal element in dorsalized embryos suggests that lithium exerts its effects by activation of activin signaling pathways, or alternatively by repressing the action of antagonists of activin signaling pathways, such as bone morphogenetic proteins (reviewed in Harland, 1994).

4. Materials and methods

4.1. Materials

Recombinant bovine activin A (a kind gift of Dr. P. de Waele, Innogenetics, Belgium) was stored lyophilized at −80°C and was used as a 10 μg/ml solution in 40% (w/v) acetonitrile, 0.1% (w/v) trifluoroacetic acid. Basic FGF (bFGF, Boehringer) was stored at −20°C as 10–50 μg/ml solutions in phosphate buffered saline. Cycloheximide (Sigma) was stored at −20°C as a 10 mg/ml solution in distilled water.

4.2. Fish and embryos

Zebrafish were kept at 27.5°C. Embryos were obtained by natural matings and cultured in embryo medium (Westerfield, 1994) at 28.5 or at 33°C. Staging of embryos was done according to The Zebrafish Book (Westerfield, 1994), with stage indications in hours post fertilization (hpf), referring to equivalent stages of embryos grown at 28.5°C.

4.3. Cloning of goosecoid regulatory sequences

Approximately 5×10^5 plaques of a zebrafish gastrula cDNA-library (gift of Dr. D. Grunwald) were screened with a [α-^{32}P]dCTP labeled random primed probe derived from a 900 bp BamHI/SspI fragment from the *Xenopus laevis goosecoid* B cDNA (gift of Dr. E. De Robertis). Hybridization was carried out overnight at 42°C in 50% formamide, 5× SSC, 5× Denhardt's solution, 20 mM phosphate buffer (pH 6.8), 10 mM EDTA (pH 8.0), 0.1% SDS and 0.1 mg/ml sonicated salmon sperm DNA. Following hybridization, the filters were rinsed 10 min in 3× SSC containing 0.1% SDS at room temperature, three times 20 min in 3× SSC, 0.1% SDS at 55°C and three times 20 min in 1× SSC, 0.1% SDS at 55°C. Positive phages were isolated in a single round of purification and plasmids were excised by the protocol recommended by the manufacturer (Stratagene). Following characterization of the positive clones by partial sequence analysis, a full length cDNA clone (pGSC211) was sequenced to completion. The open reading frame of clone pGSC211 encodes a hypothetical protein of 240 amino acids, identical to the published sequence (Stachel et al., 1993). To clone *gsc* promoter sequences, approximately 6×10^5 plaques of a zebrafish genomic library (gift of Dr. Anders Fjose) were screened with the full-length zebrafish *gsc* cDNA probe (pGSC211). Hybridization was carried out as described above for the cDNA-screening. After two rounds of screening, two positive clones were obtained and Southern blot analysis revealed that lambda phage 7.2 contained *gsc* upstream sequences, whereas the other positive phage only contained coding and downstream sequences. A 4 kb EcoRI/EcoRI fragment derived from clone 7.2 was subcloned (pGCEEsk) and partial sequence analysis revealed an overlap with 5′ cDNA sequences. A 1.8 kb EcoRV-EcoRI promoter fragment was sequenced to completion.

4.4. Plasmid construction

Plasmid GCEEsk containing a 4 kb *gsc* promoter EcoRI fragment was cut with EcoRV, with StyI/EcoRV (filled in) and with HincII, subsequently circularized to obtain GCEVEsk, GCSEsk and GCHEsk, respectively. The insert of GCEEsk was released with BamHI/HindIII with the HindIII site filled in. The vector ptkLacZ (a gift from Dr. M. Schartl) was cut with XbaI/BglII with the XbaI site filled in, which removes the tk portion. The promoter fragment was cloned 5′-3′ with respect to the LacZ coding sequences into this vector to obtain EVE-LacZ. Inserts of GCEVEsk, GCSEsk and GCHEsk were released with BamHI/XhoI and cloned into pLUC (a gift from Dr. M. Schartl) opened with SalI/BamHI to obtain EVELuc, SELuc and HELuc, respectively. GCEVEsk was cut with EcoRV/StyI and the blunt ended 495 bp

EcoRV-StyI fragment was cloned 5′-3′ into a blunt ended ptkLuc vector (a gift from Dr. M. Schartl) opened with SalI to obtain EVStkLuc. GCEVEsk was cut with EcoRV/HincII and with HincII/StyI, the latter filled in and the 236 bp EcoRV-HincII and the 259 bp HincII-StyI fragments were cloned into a blunt ended SalI digested ptkLuc vector and into SmaI linearized pBluescript SK–, to obtain EVHtkLuc, HStkLuc, EVHsk and HSsk. Inserts in EVHtkLuc and HStkLuc are in the 3′-5′ direction. The insert of EVHsk was isolated with HindIII/BamHI and cut with SauIIIA. The BamHI-SauIIIA fragment was cloned into BamHI linearized ptkLuc, giving EVSatkLuc and EVSa2tkLuc, with 1 and 2 copies of the insert in 5′-3′ orientation, respectively. The SauIIIA-HindII fragment was filled in and cloned in 5′-3′ orientation into SalI linearized and filled in ptkLuc. The insert of HSsk was isolated with KpnI/BamHI, cut with HinfI, filled in and recut with XhoI. The HinfI-HinfI fragment was cloned 5′-3′ into BamHI linearized blunt ended ptkLuc, the HinfI-XhoI fragment was cloned into HindIII/SalI linearized ptkLuc with the HindIII site filled in, to obtain HfHftkLuc and HfStkLuc, respectively. For the unilateral deletion constructs, the insert of GCEVEsk was isolated with EcoRV/EcoRI, filled in and cloned 5′-3′ into blunt-ended BamHI linearized pLuc, giving EVELucII. EVELucII was linearized with PstI/SalI and unilateral deletions were made starting at the SalI site into the 5′ end of the promoter using an exonuclease III/mung bean nuclease deletion kit according to the manufacturer's recommendations (Stratagene). The clones were sequenced and −1776Luc, −1755Luc, −1748Luc and −1745Luc were selected. To clone EVEΔ27Luc, a PCR product was amplified from GCEVEsk using a reverse primer in the promoter insert starting at −1354 with a StyI site attached (5′-ACTCCTAGGTTGTTCAAATGAGGGACTAA) and a forward primer in the Bluescript polylinker (5′-TGACCATGATTACGCCAAGC). The PCR product was cut with XhoI/StyI and cloned with the 1388 bp StyI-BamHI fragment from GCEVEsk into the SalI/BamHI linearized pLuc vector. To clone PE1tkLuc, the following synthetic oligonucleotides were annealed and ligated 5′-3′ into the BamHI linearized ptkLuc vector:

PE1 S: 5′-gatcCATCATGTAATTTAGTCCCTCATTTGAACAATAAATAAATAAATAATAT

PE1 A:5′-gatcATATTATTTATTTATTTATTGTTCAAATGAGGGACTAAATTACATGATG

Lower case nucleotides were attached for cloning purposes, the uppercase nucleotides of PE1S represent basepairs −1384 to −1335 of the *gsc* promoter. In order to construct EVSa2TLac, an SphI/BamHI fragment containing 5 Gal4 sites and an adenovirus E1b TATA box from a Gal4E1bCAT reporter (Gill et al., 1990) was cloned into the SphI/BglII linearized tkLacZ vector (a gift from Dr. M. Schartl), which removes the tk portion, to obtain GTLac. The BamHI-SauIIIA fragment described above used to clone EVSatkLuc was cloned into BamHI linearized pBluescript SK–, as a dimer with both 5′ ends at the SmaI site (EVSa2sk). The insert was isolated with XbaI/SmaI and cloned into the SphI digested, blunt ended, XbaI digested GTLac, which removes the Gal4 sites from the vector, to obtain EVSa2TLac. Constructs were checked by restriction fragment analysis and sequencing. For microinjection experiments cesium chloride density gradient purified or Qiagen Tip 100 purified DNA was used.

4.5. DNA microinjection

Microinjection needles were pulled on a Sutter pipette puller model P-80/PC using 1 mm borosilicate glass capillaries (GC100TF Clark Electromedical Instruments) and broken under a microscope to obtain approx. 5 μm tips. Supercoiled EVELacZ plasmid DNA at a concentration of 6 μg/ml in 10 mM HEPES pH 7.0, 50 mM KCl, 0.1 mM EDTA (injection buffer) was injected in 0.5 nl volumes at the interface between the yolk and the blastomere of one cell stage embryos, using a Narishige microinjector. For all other experiments in whole embryos, supercoiled plasmid DNA at a concentration of 20–40 μg/ml in injection buffer was injected in a volume of 0.5 nl into the blastomeres of 2–4 cell stage embryos. For dissociated blastula experiments 0.5 nl of a 60 μg/ml (unless otherwise indicated) solution of supercoiled plasmid DNA in injection buffer was injected at the interface between the yolk and the blastomeres of 2–4 cell stage embryos.

PE1tkLuc was injected into the cytoplasm of 2–4 cell stage embryos (10 pg/embryo), together with 10 pg of SV40 promoter driven expression constructs, expressing *Xwnt1* (pSG5-Xwnt1) (Noordermeer et al., 1989), *Xwnt5c* (pSG5-Xwnt5c) (J.G. Koster, B.J. Stegeman and O.D. Destrée, unpublished data), or the empty expression vector (pSG5; Green et al., 1988). Embryos were grown until 7 hpf (60–70% epiboly) at 28.5°C and luciferase activity was determined in pools of 10 embryos.

4.6. Preparation of dissociated blastula cells and growth factor treatment

Injected 4 hpf embryos (typically 20–80) were transferred to 500 μl phosphate buffered saline without calcium and magnesium (PBS0), triturated and dissociated by pulling gently five times through a 20-g injection needle. Chorion debris was removed by filtering through a nylon mesh, which was washed with 500 μl PBS0. Cells were pelletted for 4 min at 400 × *g*, the supernatant was removed and the cells were resuspended at 2.5 embryo equivalents per 10 μl in Leibovitz L15 medium (pH 7.3) Subsequently 10 μl aliquots were dispensed in eppendorf tubes with frequent mixing of the cell suspension. Activin A and bFGF were diluted to 80 ng/ml in Leibovitz L15 medium and 5 μl was added to the cells. Samples were adjusted to a final volume of 20 μl with medium. Cells

were incubated for 2 h at 26°C and luciferase activity was determined. For cycloheximide experiments, embryos were injected with 60 pg DNA per embryo. Blastula cells were preincubated for 30 min at room temperature with 10 μg/ml cycloheximide and subsequently incubated with growth factors for 2 h at 26°C.

4.7. Luciferase assays

Embryos injected with luciferase reporter constructs were transferred to Eppendorf vessels (10 embryos/ sample) and lysed in 200 μl lysis buffer (1% Triton X-100, 25 mM glycylglycine, 15 mM magnesium sulfate, 4 mM EGTA, 1 mM DTT, pH 7.6) using an Eppendorf micropestle. Samples were left on ice for 10 min followed by a 5 min centrifugation (10 000 × *g*) at 4°C to pellet debris. Dissociated blastula cells were harvested in 150 μl lysis buffer. Luciferase assays were performed as described (Brasier et al., 1989) on 75 μl lysate.

4.8. RNase protection probe and lysate RNase protection assays

A luciferase RNase protection probe construct LucPPsk was made by cloning a 338 bp ClaI-XbaI fragment from pLuc, representing the 3′ end of luciferase coding sequences, into pBluescript SK– linearized with ClaI/XbaI. An 138 bp antisense RNA probe labeled with [α-^{32}P]UTP was synthesized with T3 RNA polymerase (Gibco) from RsaI digested LucPPsk. Cold sense RNA (338 bp) was synthesized with T7 RNA polymerase (Stratagene) from XbaI linearized LucPPsk. For lysate RNase protections (Gillespie et al., 1992; Haines and Gillespie, 1992), dissociated blastula cells derived from the equivalent of 10 injected embryos were pelleted and resuspended in 20 μl 5 M guanidine thiocyanate/0.1 M EDTA (GSCN/EDTA). Five microliters of GSCN/EDTA containing 1 million cpm of labeled RNA probe were added and hybridization was carried out overnight at 37°C. RNase digestion and electrophoresis were carried out as described (Gillespie et al., 1992; Haines and Gillespie, 1992) with modifications. Briefly, following hybridization, 500 μl of RNase solution (0.4 M NaCl, 0.35 mg/ml salmon sperm DNA, 10 mM Tris–Cl, pH 7.5) containing 20 μg/ml RNase A (Boehringer) and 650 U/ml RNase T1 (Gibco) were added and the mixture was incubated at 37°C for 30 min. The samples were deproteinated for 30 min at 37°C with 400 μg/ml proteinase K (Boehringer) and 0.4% SDS, and subsequently precipitated using ethanol, supplemented with 1% (v/v) DEPC. RNA duplexes were analyzed on a native 5% polyacrylamide (PAA, 29:1) gel in 0.5× TBE. Gels were fixed in 10% methanol/10% acetic acid, dried and autoradiographed for 2 days at −80°C with intensifying screens. The results were quantified on a PhosphorImager using ImageQuant software (Molecular Dynamics).

4.9. Gel retardation assays

Nuclear extracts were isolated from dissociated blastula cells derived from 4 hpf embryos. Embryos were dechorionated with 0.5 mg/ml pronase (Boehringer) in embryo medium (Westerfield, 1994) and washed at least five times in embryo medium. Embryos were transferred to PBS0 and dissociated by gentle pipetting through a yellow tip. Cells were collected by centrifugation and washed with PBS0. Nuclear extracts were prepared as described by Andrews and Faller (1991). Ten micrograms of extract was incubated with 20 000 cpm of probe in a total volume of 20 μl containing 10 mM Tris–Cl (pH 7.5), 1 mM EDTA, 2 mM magnesium chloride, 5% glycerol, 1 mM DTT, 1 mM PMSF, 0.01% NP40, 2 μg poly-(dIdC) and 10 μg/ml linearized pBluescript SK– DNA for 30 min at room temperature. Competitor DNA was added before addition of the labeled probe. Samples were analyzed on a 5% PAA gel (29:1) in 0.5× TBE. Gels were fixed in 10% methanol/10% acetic acid, dried and autoradiographed. The EcoRV-SauIIIA fragment (−1820 to −1712) was gel purified from a SauIIIA digest on the insert of EVHsk released with BamHI/XhoI, and the HinfI-StyI fragment (−1413 to −1325) was gel purified from a HinfI digest on the insert of HfSsk released with BamHI/XhoI. Fragments were end-labeled by Klenow fill-in in the presence of [α-^{32}P]dATP and [α-^{32}P]dCTP (3000 Ci/mmol) and purified from PAA gels. The following sets of synthetic oligonucleotides were annealed and used as cold competitors:

ARR1 S: 5′-tcgaGCCAATGTCACCGAGCAGCGTC
A: 5′-tcgaGACGCTGCTCGGTGACATTGGC

ARR2 S: 5′-tcgaGCCAATGTCACCGAGCAGCGTC-TTCCTTCATG
A: 5′-tcgaCATGAAGGAAGACGCTGCTCGG-TGACATTGGC

ARR3 S: 5′-tcgaGCCAATGTCACCGAGCAGCGTC-TTCCTTCATGCAAATTCCAT
A: 5′-tcgaATGGAATTTGCATGAAGGAAGA-CGCTGCTCGGTGACATTGGC

PE2 S: 5′-gatcCGAACAATAAATAAATAAATAA-TA
A: 5′-gatcTATTATTTATTTATTTATTGTTCG

Ad4oct S: 5′-CGAATATGCAAATAAGGC (Verrijzer et al., 1990)
A: 5′-GCCTTATTTGCATATTCG

Nucleotides in lower case are attached for cloning or fill-in purposes. A 219 bp SmaI/BamHI fragment from the human RARβ promoter (a gift from G. Folkers) was used as aspecific competitor in gel retardation experiments.

4.10. LiCl treatment, RNA coinjection, β-galactosidase staining, microscopy

For lithium chloride treatment, injected embryos were

grown to the 32–64 cell stage, transferred for 10 min to a 0.3 M lithium chloride solution in embryo medium, washed extensively and incubated further in embryo medium at 28.5 or 33°C.

Capped synthetic activin βB mRNA was prepared as described using SP6 RNA polymerase (Thomson et al., 1990). Capped synthetic TGFβ receptor II mRNA was a gift of Dr. C. de Vries and was used as a control RNA in microinjection experiments. RNA was added to injection plasmids immediately before microinjection to prevent degradation.

Embryos injected with LacZ constructs were fixed at the appropriate stage for 45 min at 4°C in 2.5% (v/v) formaldehyde, 0.8% (v/v) glutaraldehyde and 0.02% (v/v) NP40 in phosphate buffered saline (PBS), followed by two washes in PBS at 4°C. Following manual dechorionation using forceps, embryos were stained overnight at 28.5°C in a solution containing 4 mM $K_4Fe(CN)_6$, 4 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$ and 0.4 mg/ml X-gal (Boehringer) in PBS. Subsequently, the embryos were rinsed in PBS, transferred through a graded series of methanol and cleared in a mixture of two-thirds benzoyl benzoate and one-third benzoyl alcohol (Murray's). Results were examined using a Zeiss Axiovert microscope equipped with differential interference contrast (DIC) and recorded with a Sony optical memory disc recorder.

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