The *Xenopus Brachyury* promoter is activated by FGF and low concentrations of activin and suppressed by high concentrations of activin and by paired-type homeodomain proteins

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The mesoderm of *Xenopus laevis* arises through an inductive interaction in which signals from the vegetal hemisphere of the embryo act on overlying equatorial cells. One candidate for an endogenous mesoderm-inducing factor is activin, a member of the TGFβ superfamily. Activin is of particular interest because it induces different mesodermal cell types in a concentration-dependent manner, suggesting that it acts as a morphogen. These concentration-dependent effects are exemplified by the response of *Xbra*, expression of which is induced in ectodermal tissue by low concentrations of activin but not by high concentrations. *Xbra* therefore offers an excellent paradigm for studying the way in which a morphogen gradient is interpreted in vertebrate embryos. In this paper we examine the trancriptional regulation of *Xbra2*, a pseudoallele of *Xbra* that shows an identical response to activin. Our results indicate that 381 bp 5′ of the *Xbra2* transcription start site are sufficient to confer responsiveness both to FGF and, in a concentration-dependent manner, to activin. We present evidence that the suppression of *Xbra* expression at high concentrations of activin is mediated by paired-type homeobox genes such as *goosecoid*, *Mix.1*, and *Xotx2*.

[*Key Words: Brachyury; Xenopus;* mesoderm induction; activin; FGF; thresholds; homeodomain; *goosecoid; Mix.1; Xotx2*]

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The mesoderm of *Xenopus laevis* arises through an inductive interaction in which signals from the vegetal hemisphere of the embryo act on overlying equatorial cells (Sive 1993; Slack 1994). One candidate for an endogenous mesoderm-inducing factor is activin, a member of the TGFβ superfamily (Asashima et al. 1990; Smith et al. 1990; Thomsen et al. 1990; Dyson and Gurdon 1996). Activin is of particular interest because it induces prospective ectodermal tissue to form different mesodermal cell types in a concentration-dependent manner (Green et al. 1992), and recent evidence suggests that it can function in the embryo as a long-range ''morphogen'' (Gurdon et al. 1994, 1995; Jones et al. 1996; but see Reilly and Melton 1996).

The dose-dependent effects of activin are illustrated by the behavior of *Xenopus Brachyury* (*Xbra*). Expression of *Xbra* is induced in prospective ectodermal tissue as an immediate-early response to activin and to the mesoderm-inducing factor FGF-2 (Smith et al. 1991). Activation of *Xbra* in response to activin only occurs, however, in a narrow ''window'' of activin concentrations: Low doses do not induce expression, intermediate concentrations induce high levels, and yet higher concentrations suppress expression (Green et al. 1992; Gurdon et al. 1994, 1995). Because activin may be able to function as a long-range morphogen, these observations suggest a mechanism by which expression of *Xbra* might be restricted to the marginal zone of the *Xenopus* embryo. Thus, if activin (or an activin-like molecule) is produced in the vegetal hemisphere of the embryo and is able to diffuse from here toward the animal pole, a gradient of activin activity might be established. The concentrations of activin in vegetal and animal regions would, respectively, be too high or too low for expression of *Xbra* to occur, but the concentration in the equatorial region might be just right.

Analysis of the response of *Xbra* to different concen-

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trations of activin provides a powerful tool with which to study the interpretation of a morphogen gradient in vertebrate embryos, but analysis of *Xbra* expression is also of interest because *Brachyury* is an important regulatory gene in early vertebrate development. In mouse, chick, zebrafish, and *Xenopus* embryos, *Brachyury* is expressed at the onset of gastrulation throughout the nascent mesoderm, and transcripts persist thereafter in notochord and in posterior mesoderm (Herrmann et al. 1990; Smith et al. 1991; Schulte-Merker et al. 1992; Kispert et al. 1995b). Loss of *Brachyury* function in mouse, zebrafish and *Xenopus* embryos causes loss of posterior mesoderm and impairment of notochord differentiation (Herrmann et al. 1990; Halpern et al. 1993; Schulte-Merker et al. 1994; Conlon et al. 1996). *Brachyury* encodes a nuclear sequence-specific DNA-binding protein that functions as a transcription activator (Schulte-Merker et al. 1992; Kispert and Herrmann 1993; Kispert et al. 1995a; Conlon et al. 1996). Widespread expression of *Xbra* in *Xenopus* embryos causes ectopic mesoderm formation (Cunliffe and Smith 1992), and the character of the mesoderm formed in such experiments depends on the concentration of *Xbra* mRNA, the stage at which expression of *Xbra* begins, and the genes with which it is coexpressed (Cunliffe and Smith 1992, 1994; O'Reilly et al. 1995; Tada et al. 1997). Thus, *Brachyury* acts as a genetic switch specifying mesodermal fate in embryonic cells, and normal development of the embryo must depend on precise spatial and temporal control of *Brachyury* expression.

As a first step toward understanding *Xbra* transcriptional control, we have isolated the promoter region of *Xenopus Brachyury*. The gene we have cloned appears to be a pseudoallele of *Xbra*, and we designate it *Xbra2*. We describe the structure of the 5′-flanking region of *Xbra2* and demonstrate that the promoter confers mesoderm-specific responsiveness to linked reporter genes. A reporter construct containing 381 bp of *Xbra2* 5′-flanking sequence responds to both fibroblast growth factor (FGF) and activin and, like the endogenous gene, is down-regulated by high concentrations of activin. We provide evidence that this down-regulation is attributable to suppression of transcription by homeobox-containing genes such as *goosecoid* (Cho et al. 1991) and *Mix.1* (Rosa 1989), both of which are induced by high concentrations of activin (Green et al. 1992; Gurdon et al. 1994, 1995, 1996), and by *Xotx2* (Pannese et al. 1995). Together, these observations provide the first insights into a "threshold" phenomenon in vertebrate embryos.

Results

Isolation of genomic clones encoding the 5′ end of Xenopus Brachyury

Genomic clones containing the first exon of *Xenopus Brachyury* were obtained as described in Materials and Methods. Clone 3A1 contained ~2.2 kb of DNA 5′ to the probable translation initiation codon, and comparison with *Xbra* cDNA revealed that the exon 1/intron 1 boundary is located at codon 67 (Fig. 1). There were significant differences in nucleotide sequence between 3A1 and the original *Xbra* cDNA (Smith et al. 1991), although

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-1254 AAAAATTACT TTGAAATTCG AAGTTTTTGA ATTCGAAAAT TCACTTCGAC CCTTAGTAAA

-1194 TGGGCCCTGA ATGTTCTAAC AATCTATTTA GAAGTATTTA GACTCTGTAA TGTAATGTTA
        ApaI

-1134 AAGATAGTGA ATTATATCAC TATACAGTTT TATGGCATAA TTACACATAA CGCCATAGTA

-1074 TAAGTTTTGA TGCTAGACTT TAGTAAACCT TGAGAAACAG CTTACATAGC GCAGAAGTGA

-1014 TACTAGGTAT ATATATGTGC ACACCAATCG CACAAGTGGC TGCAGCAATG TATTATCACC

-954  TTCTATGCAC AGTAAATGAA CACTATAGTG CATATTGGTG AAACAAACCA AATAAATATG

-894  ATGACAGCCC AAATAAATCA TTTATATACG GTAGAATCCA CATTTTACAT TTTCAGGGGA

-834  CAAGAAAAAA AATGGTGTAA TATCCGGTAA TATATAAGAT CAGGGACAGA CATTATATAT

-774  TTGATGGGAC CACAAACAAA CAATGTAAAA TGAGGGGAAA ACTATAAAAT AAGGGGTTAT

-714  AAAATTGATA TATTGGTGGG ACCACAAACA AACTATGTAA AATGAGGAAA AAATTTAAAT

-654  TAGGGTATGT ACAATTTGAT ATATTTTACA TTGTAAACTT AAAACTAGGG GATGTAAAAA

-594  TTAAATTTTG AATTGTAAAC TTAAAATTAG GGGATGTACA ATTTTTAAAT TGTAAACTTA

-534  AAATTAGGGG ATGTAAAATT GAGGTTTTAC TTTTATAAAT TTCTAAACTG CCCCTTAAAA

-474  TACACCTTTT TTCTTTAAAT GCAATTAAAA AATTGTACCT ATTATTTTTG TATGTAATAA
                                Ant

-414  TAATCCTTTA AGTATCAGAA CCTTATTTTT ATACATCTGC CATTATACCA TTATGTCATT
        Bcd

-354  TGAACAATCT ATCCAGGCCA CCTAAAATAT AGAATGATAA AGTGACCAGG TGTCAGTTCT

-294  TACTGGATGT AAGTTTATTG AAGGCAGGCT GGGGGGGGGG GGGTGACACA CCACTTCCTC

-234  ATTGCAGCTC TTTGATATAG GTGTAAACAT TTGGGGATTT ACATAAAGTA CCTCTGACTT

-174  GCAATTAAAT TCCCAGGATT ATCATAGAGC TCTCTGGGGT TTCTGTGCTC CAATCAGCAG
        Ant            Bcd         SacI

-114  TTGCCTCACC AACATCAAAG AGAAAACTGA CCAATGAGCT GCCTGGCACC GCCGTTGATG

                                                                    +1
-54   AAGACACCAC AGCTTGTCCA GGCCTATTTA TGGAAGAGTG AAATATCTCT ATCTTAATCA

+7    GTGCCTCTTA TTCCCCTAAT GTCAATTGGA TTTACTACCT GCTGATCAAT CGCACCTTGG
                                                      BclI

+67   GTTTTGTTCC GATTAGTGGA AAAGCTGCTA AAATTTTTCC CCCAGTCTGT GTGTTACGAA

                          M   S   A   T   E   S   C   A   K   N   V   Q   Y   R
+127  GCCTCCCTTT CTGGAGGAAT GAGTGCGACC GAGAGCTGCG CCAAGAACGT GCAGTACCGG

      V   D   H   L   L   S   A   V   E   N   E   L   Q   A   G   S   E   K   G   D
+187  GTAGATCATC TGCTGAGCGC TGTGGAGAAT GAGCTCCAGG CTGGCAGCGA AAAGGGGGAC
                                          SacI
      P   T   E   K   E   L   K   V   S   L   Q   E   R   D   L   W   T   R   F   K
+247  CCCACCGAGA AGGAGCTGAA GGTGAGTCTG CAGGAGCGGG ACCTGTGGAC GAGGTTTAAG

      E   L   T   N   E   M   I   V   T   K   N   G   R
+307  GAGCTCACCA ATGAGATGAT CGTCACTAAG AATGGAAGGT AAAATCCCAG AAACTAATAA
                                              sd

+367  CTTTCTTGTC TAGAGTGCAG GAGTTTCTAC CTAGTGTAGA TTTATATCAT CATGATCATG
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Figure 1. Sequence of exon 1 of *Xbra2* (boldface type) together with 1.2 kb of 5′-flanking sequence. The first 82 bases of intron 1 are also included. Transcription initiation site is indicated by +1 and is also the first nucleotide in boldface type. ATTA sequences are double-underlined, and Antennapedia- and Bicoid-specific sites are marked **Ant** and **Bcd,** respectively. This sequence has been submitted to the EMBL database, accession number AJ001528.

they are 99% identical at the amino acid level over the first 67 amino acids. This suggests that 3A1 is derived from a second *Xenopus Brachyury* gene, which we designate *Xbra2.* Consistent with this suggestion, the spatial and temporal expression patterns of *Xbra2* proved to be indistinguishable from those of *Xbra* and different from other *Xenopus* T-box-containing genes (data not shown; see Lustig et al. 1996; Ryan et al. 1996; Stennard et al. 1996; Zhang and King 1996). Furthermore, *Xbra2* expression, like that of *Xbra,* is induced in animal caps by FGF-2 and activin (data not shown).

DNA (2.1 kb) immediately upstream of the Xbra2 *promoter drives mesoderm-specific gene expression in* Xenopus *embryos*

The *Xbra2* transcription initiation site was defined using primer extension and is located 144 nucleotides upstream of the initiator methionine codon (data not shown). As a first step in the analysis of *Xbra2* regulation, 2.1 kb of *Xbra2* 5′-flanking sequence and ~50 bp of 5′-untranslated region were subcloned into the luciferase reporter plasmid pGL2, thus generating *Xbra2*.pGL2. This construct (or the promoterless vector pGL2Basic) was coinjected with the internal control pSV.β-galactosidase into blastomeres of tier A or tier C of *Xenopus* embryos at the 32-cell stage. These give rise predominantly to the ectoderm or the mesoderm of the embryo, respectively (Dale and Slack 1987). Injected embryos were assayed for luciferase and β-galactosidase activities at gastrula stage 12. The ratio of luciferase to β-galactosidase activity provides a normalized measure of luciferase expression. Figure 2A shows that although injection of the *Xbra2* reporter into tier A results in low luciferase activity, extracts of tier C-injected embryos showed high levels. No significant luciferase activity was observed after injection of the promoterless plasmid pGL2Basic. Measurement of luciferase activity at different stages of development (Fig. 2B) showed that reporter gene activity increased dramatically from the mid-blastula stage to the neurula stage. This suggests that sequences within 2.1 kb of 5′-flanking region confer mesoderm-specific and temporally correct transcriptional activity to *Xbra2.*

To monitor transcripts originating from the *Xbra2* promoter directly, we constructed *Xbra2*.βg, in which 2.1 kb of *Xbra2* 5′-flanking region was placed upstream of the human β-globin gene, and analyzed levels of β-globin mRNA by RNase protection. Figure 2B (inset) shows that injection of this construct into tier A elicits only weak synthesis of correctly processed transcript, whereas injection into tier C causes strong activation.

The ability of sequences within 2.1 kb of *Xbra2* 5′-flanking region to drive mesoderm-specific expression was then examined using the construct *Xbra2*.GFP, in which green fluorescent protein (GFP) (Zernicka-Goetz et al. 1996) was used as a reporter. *Xenopus* embryos at the four-cell stage were injected in each blastomere with a total of 100 pg of reporter DNA, and GFP activity was visualized by direct observation (not shown) followed by in situ hybridization using a GFP probe. Mosaic GFP

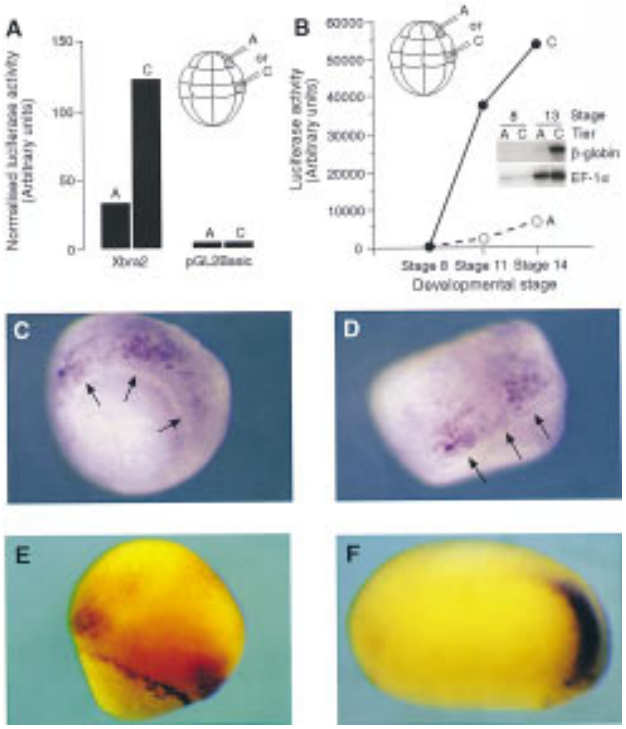


Figure 2. Mesoderm-specific expression of reporter gene constructs containing 2.1 kb of *Xbra2* 5′-flanking sequence. (*A*) *Xbra2*.pGL2 or the promoterless plasmid pGL2Basic was coinjected with pSV.β-galactosidase into a single blastomere of either tier A (prospective ectoderm) or tier C (prospective mesoderm) of 32-cell stage embryos (see *inset*). Embryos were collected for luciferase and β-galactosidase activity assays at stage 12.5. (*B*) Luciferase activity in extracts of embryos at stage 8, 11, or 14 injected with *Xbra2*.pGL2 into a single blastomere of either tier A or tier C at the 32-cell stage. (*Inset*) RNase protection assay for expression of *Xbra2*.βg in embryos injected into a single blastomere of tier A or tier C and collected for assay at stage 8 or stage 13. EF-1α was used as a loading control. This experiment has been performed four times, with similar results each time. (*C,D*) *Xbra2*.GFP was injected equatorially into all four blastomeres of four-cell stage embryos, and distribution of GFP transcripts at stage 10.5 was determined by whole-mount in situ hybridization. Arrows point to the blastopore lip. (*E,F*) *Xbra2*.GFP was used to generate transgenic embryos that were analyzed as in *C* and *D* at gastrula (*E*) or tailbud (*F;* anterior to the *left*) stage.

expression was seen in prospective mesodermal cells at stage 10.5 (Fig. 2C,D). This expression pattern was specific to *Xbra2* flanking sequence, because the promoter of the ubiquitously expressed and constitutively active EF-1α directed widespread mosaic expression (not shown).

To overcome problems associated with mosaic inheritance of injected DNA and to test the ability of the *Xbra2* promoter to drive mesoderm-specific expression when integrated into chromatin, we generated transgenic embryos carrying *Xbra2*.GFP (Fig. 2E,F). At the gastrula stage, most transgenic embryos showed expression of GFP mRNA in a ring around the blastopore, as is seen

with the endogenous gene. However, the reporter gene differed from the endogenous gene because the ring was discontinuous, with a gap on the dorsal side (Fig. 2E). This observation is consistent with experiments using the mouse *Brachyury* promoter, where there are separate elements for primitive streak and notochord expression (Clements et al. 1996). At the tailbud stage, GFP mRNA was normally found in the posterior mesoderm, coincident with endogenous *Xbra* expression (Fig. 2F); there is no expression of *Xbra* RNA in the notochord at this stage (Green et al. 1997). Together, these results show that 2.1 kb of *Xbra2* 5′ sequence is sufficient to direct expression to a large subset of sites where the endogenous gene is expressed; sequences located elsewhere are required for expression in the presumptive notochord.

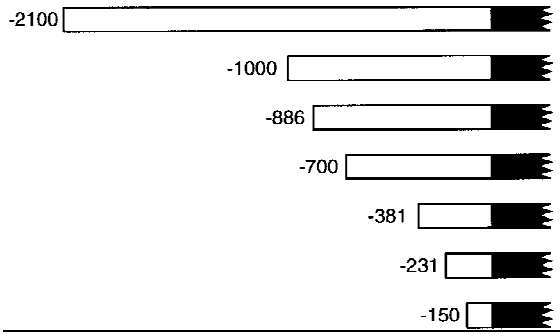
Sequence within 381 bp of the transcription start site is sufficient to elicit responses to FGF and activin

We then tested the ability of FGF, of members of the MAP kinase pathway, and of activin to activate *Xbra2*.pGL2. The reporter construct was coinjected with a reference plasmid into *Xenopus* embryos at the one-cell stage. Animal caps were dissected at blastula stages and were incubated in medium containing FGF or activin or were left untreated. Some embryos were also injected with RNA encoding v-Ras or a constitutively active form of MEK1, MEK1$^{S217E/S221E}$ (Umbhauer et al. 1995). Reporter gene activities were analyzed in animal cap extracts at gastrula stage 12.5. FGF caused a strong induction of luciferase activity in animal caps injected with *Xbra2*.pGL2 (Figs. 3 and 4A,B). In animal caps expressing v-*ras* or MEK1$^{S217E/S221E}$, the *Xbra2*.pGL2 construct was activated in the absence of FGF (Fig. 4A). Neither FGF nor v-Ras caused activation of the promoterless vector pGL2Basic (data not shown). These results suggest that elements mediating FGF-dependent gene activity are located within 2.1 kb of upstream flanking sequence.

Activin treatment of animal caps also caused expression of the *Xbra2*.pGL2 reporter construct. These experiments gave variable results, however, and it seemed possible that this was attributable to cells within the animal caps experiencing different concentrations of activin; expression of the endogenous *Xbra* gene is very sensitive to activin concentration (Green et al. 1992; Gurdon et al. 1994, 1995). In an effort to obtain a more uniform distribution of activin, activin RNA was coinjected with the *Xbra2*.pGL2 reporter construct and a reference plasmid into the animal hemispheres of *Xenopus* embryos at the one-cell stage (see Gurdon et al. 1994). When low levels of RNA were injected, strong activation of reporter gene activity was observed (Figs. 3 and 4B).

To identify elements in the *Xbra2* promoter responsible for mediating the response to FGF and activin, a deletion series of *Xbra2*.pGL2 was generated, and constructs were tested for their ability to respond to inducing factors. The degree of induction varied from experiment to experiment, perhaps owing, among other factors, to mosaic expression of the reporter construct (see Discussion). Nevertheless, removal of 5′ sequences down to −381 bp did not prevent induction by FGF and activin, whereas 231 bp was unable to elicit a significant response (Fig. 3).

Examination of Figure 3 suggests that levels of reporter gene activation in response to FGF and activin are higher in constructs containing 2.1 and 1.0 kb of flanking sequence than in those containing only 381 bp. However, the variation in levels of induction observed in different experiments made it difficult to study this issue in a quantitative manner, and the variation also complicated the precise localization of activin or FGF response elements.



	Fold activation					
	FGF			Activin		
	Range	Mean	n	Range	Mean	n
-2100	1.0 - 13.7	4.2	15	3.3 - 43.1	17.5	4
-1000	1.0 - 13.0	6.1	4	4.8 - 9.0	6.9	2
-886	0.5 - 37.0	9.0	15	2.0 - 14.5	9.2	6
-700	1.9 - 6.0	3.3	4	3.4 - 4.4	3.9	2
-381	0.2 - 11.0	3.2	12	1.2 - 7.1	4.0	4
-231	0.7 - 2.0	1.3	9	1.1	1.1	2
-150	1.0 - 3.3	1.7	4	1.0 - 1.3	1.2	2

Figure 3. FGF and activin activate expression of a reporter gene construct containing 2.1 kb of *Xbra2* 5′-flanking sequence. Sequences upstream of −381 in the *Xbra2* 5′-flanking sequence are dispensable for response of *Xbra2* to FGF (25 ng/ml) and activin. Embryos were injected with the indicated constructs and reference plasmid and, in some cases, with 1 pg of activin mRNA, as described in Materials and Methods. Normalized firefly luciferase activity from stage 12.5 caps is expressed as fold activation over untreated caps. (n) Number of experiments.

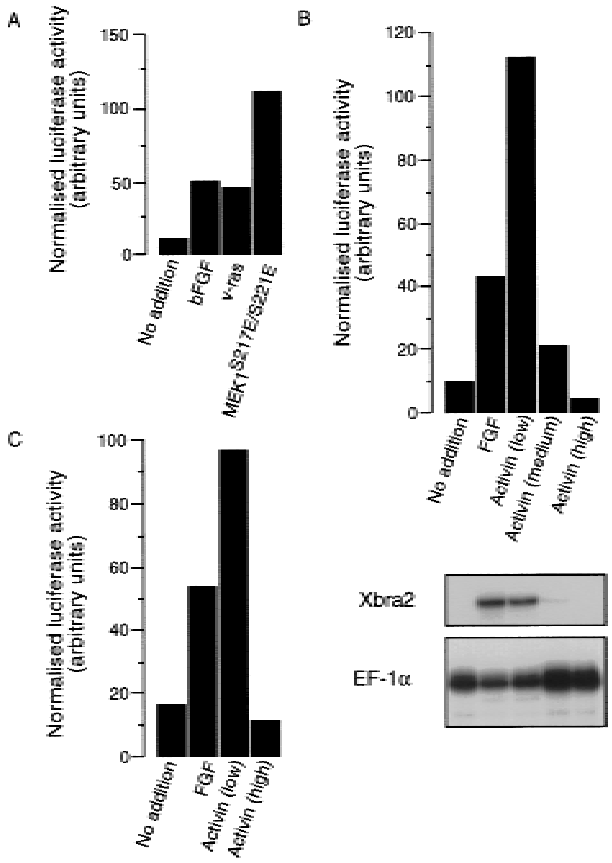


Figure 4. Activation by FGF and components of the MAP kinase pathway of a reporter gene construct containing 2.1 kb of *Xbra2* 5′-flanking sequence. Activation of the same reporter in response to activin, and of a reporter containing 381 bp of flanking sequence, is dose dependent. (*A*) Normalized luciferase activity in animal caps injected with *Xbra2*.pGL2. One group of caps was also injected with v-Ras mRNA, a second with $MEK1^{S217E/S221E}$, and a third was treated with FGF-2. Analysis was performed at control stage 12.5. This experiment has been performed 3 times with $MEK1^{S217E/S221E}$ alone, 10 times with v-Ras alone, and once with $MEK1^{S217E/S221E}$ and v-Ras in the same experiment. Similar results were obtained each time. (*B*) Normalized luciferase activity in animal caps treated with FGF-2 or injected with different amounts of activin mRNA (in this experiment "low" represents 1 pg, "medium" represents 5 pg, and "high" represents 50 pg). Analysis was at control stage 12.5. Half of each group of caps was assayed for luciferase and β-galactosidase activities, and the other half was assayed for expression of endogenous *Xbra2* by RNase protection. Note that high levels of activin suppress expression of both endogenous *Xbra2* and the reporter construct. This experiment has been performed out three times, with similar results each time. (*C*) A similar experiment to that in *B* using −381*Xbra2*.pGL2 (in this experiment "low" represents 1 pg and "high" represents 100 pg activin RNA).

The 5′-flanking region of the Xbra2 *gene confers dose-dependent responsiveness to activin*

The observation that efficient induction of *Xbra2*.pGL2 is elicited only by low concentrations of activin was reminiscent of the response of the endogenous gene, and this inspired us to examine the phenomenon in detail. Therefore, different amounts of activin RNA were coinjected with the *Xbra2*.pGL2 reporter construct and a reference plasmid into *Xenopus* embryos, animal caps were dissected at stage 8, and levels of reporter and of endogenous *Xbra2* expression were determined at stage 12.5. Injection of low levels of activin mRNA (1 pg in Fig. 4B) strongly activated *Xbra2*.pGL2 in animal cap explants, whereas injection of intermediate levels (5 pg) gave much lower activation, and a high concentration (50 pg) reduced luciferase activity to below baseline levels. This dose-response profile mirrored endogenous *Xbra2* expression (Fig. 4B). These results show that 2.1 kb of *Xbra2* 5′-flanking sequence contains elements mediating a dose-dependent response to activin. Further experiments revealed that similar results could be obtained with only 381 bp of *Xbra2* flanking sequence (Fig. 4C).

Suppression of Brachyury *expression by homeobox-containing genes*

High levels of activin cause *Xbra2*.pGL2 activity to fall to a level below that observed in the absence of activin (Fig. 4B,C), suggesting that *Xbra* transcription is suppressed actively. Previous work, in which the concentration-dependent induction of *Xbra* and other genes was studied at different times after activin treatment, identifies two candidate repressors as goosecoid and Mix.1 (Green et al. 1994; see also Artinger et al. 1997). When assayed shortly after activin treatment, levels of *Xbra* change little as the concentration of activin is raised, whereas expression of both *goosecoid* and *Mix.1* increases dramatically. These genes might therefore be responsible for the subsequent down-regulation of *Xbra* that occurs at high levels of activin. Additional circumstantial evidence suggesting that goosecoid and Mix.1 may regulate *Xbra* expression includes the observations that *goosecoid* functions as a transcriptional repressor (Goriely et al. 1996; Smith and Jaynes 1996) and that under certain circumstances the two proteins can bind DNA cooperatively (Wilson et al. 1993). *Goosecoid* and *Mix.1* both encode paired class homeodomains (Rosa 1989; Blumberg et al. 1991), and another member of this family that is also expressed in the early *Xenopus* embryo is *Xotx2* (Blitz and Cho 1995; Pannese et al. 1995).

To examine whether goosecoid, Mix.1, or Xotx2 might cause down-regulation of *Xbra*, *Xenopus* embryos were injected at the four-cell stage with RNA encoding these proteins, and expression of *Xbra* and *Xbra2* was examined by in situ hybridization at the early gastrula stage. All three gene products caused down-regulation of *Xbra* expression (see Fig. 5A,B for examples of down-regulation with *goosecoid* and *Xotx2*), whereas injection of RNA encoding a mutant version of *Xotx2*, in which the lysine at position 9 of the recognition helix of the homeodomain is replaced by glutamic acid (*Xotx2* K → E), had no effect (Fig. 5C).

Suppression of *Xbra2* expression by goosecoid, Mix.1, and Xotx2 was also revealed in experiments in which

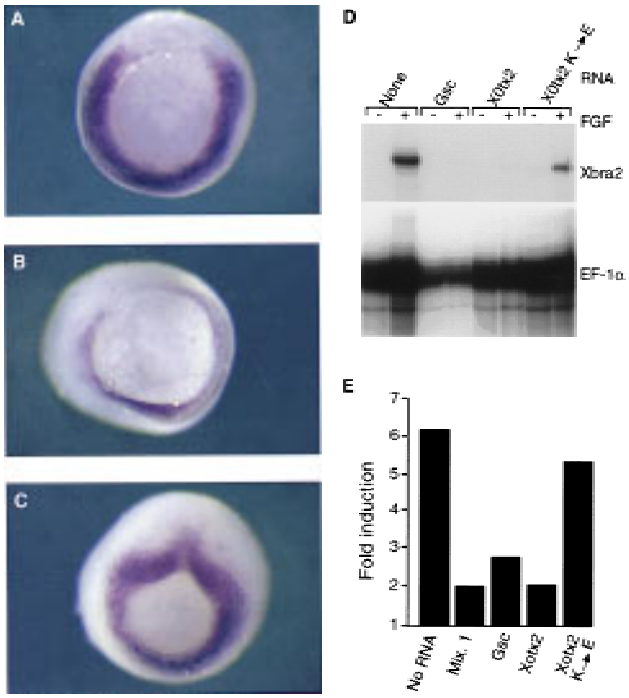


Figure 5. Suppression of *Xbra* expression in gastrula-stage embryos by paired-type homeodomain proteins. Embryos at the four-cell stage were injected equatorially with 500 pg of *goosecoid* (*A*), *Xotx2* (*B*), or *Xotx2* K → E (*C*) RNA in one blastomere, and the expression pattern of *Xbra* was analyzed by whole-mount in situ hybridization at the early gastrula stage. Note lack of *Xbra* expression in part of the equatorial region of embryos in *A* and *B*. All embryos shown are from the same experiment; that in *C* is at a slightly later stage than those in *A* and *B*. (*D*) Suppression of FGF-induced *Xbra2* mRNA in animal caps by *goosecoid, Mix.1,* and *Xotx2* but not by *Xotx2* K → E. Embryos at the one-cell stage were injected with 1 ng of the indicated RNAs, and animal caps were cut at stage 8 and incubated in FGF until stage 12. *Xbra2* and EF-1α transcripts were detected by RNase protection. Note that FGF-induced expression of *Xbra2* is strongly inhibited by *goosecoid, Mix.1,* and *Xotx2* but not by *Xotx2* K → E. (*E*) Suppression of FGF-induced *Xbra2*.pGL2 luciferase activity in animal caps by *goosecoid, Mix.1,* and *Xotx2* but not by *Xotx2* K → E. Embryos were coinjected with 1 ng of the indicated RNAs, 30 pg of *Xbra2*.pGL2, and 10 pg of pRL–SV reference plasmid. Animal caps were excised at stage 8 and incubated with FGF until stage 12.5, and firefly and Renilla luciferase activities were determined. Activity of *Xbra2*.pGL2 is presented as fold activation over uninduced levels. This experiment was repeated twice for all four RNAs and four additional times with *goosecoid,* with similar results.

animal pole explants derived from injected embryos were treated with FGF. Expression of *Xbra2* in response to FGF was strongly down-regulated by all three proteins but not by the control construct *Xotx2* K → E (Fig. 5D); similar results were seen when activin was used instead of FGF (not shown). The effects of goosecoid, Mix.1, and Xotx2 were specific, because activation of *Pintallavis* (Ruiz i Altaba and Jessell 1992) was not affected (not shown).

We then studied the ability of goosecoid, Mix.1, and Xotx2 to suppress FGF-induced activity of the *Xbra2*.pGL2 reporter in animal pole explants. The presence of all three gene products caused a general reduction in *Xbra2*.pGL2 reporter activity in such caps (not shown), and as illustrated in Figure 5E, overexpression of goosecoid, Mix.1, and Xotx2, but not of Xotx2 K → E, caused a reduction in FGF-induced reporter activity, suggesting that these transcription factors modulate *Xbra2* expression by suppressing its transcriptional activity. Similar results were seen when animal pole explants were treated with activin (not shown).

Goosecoid, Xotx2, and Mix.1 bind to sequences within the 5′-flanking region of Xbra2

The suppression of *Xbra2* transcription by goosecoid, Mix.1, and Xotx2 may occur indirectly through activation of downstream repressors or directly, by binding to sites in the *Xbra2* regulatory region. Most homeodomain-containing proteins bind to sequences containing a TAAT core (Treisman et al. 1992), and there are four such motifs within the 381 bp of *Xbra2* flanking sequence that confers suppression of reporter gene expression in response to high levels of activin (Fig. 1). One of these motifs, GGATTA, matches the consensus binding site of the *Drosophila* morphogen Bicoid, to which goosecoid also binds (Blumberg et al. 1991; Wilson et al. 1993). Recognition of the GG dinucleotide 5′ of the ATTA motif is specified by lysine at position 50 of the homeodomain, a residue also present in Otx2. Another site present in the *Xbra2* 5′-flanking region, CAATTAA, conforms to the consensus for the Antennapedia class of homeobox proteins, which have glutamine at position 50 of the homeodomain (Desplan et al. 1988; Hanes and Brent 1991): Mix.1 belongs to this class.

Goosecoid, Xotx2, and Mix.1 proteins were prepared by in vitro translation and tested for the ability to bind to the Bicoid and Antennapedia sites in the *Xbra2* 5′-flanking region (nucleotides −174 to −152). Both goosecoid and Xotx2 bind the −174/−152 probe specifically (Fig. 6). As expected, no specific binding was observed with Xotx2 K → E (Fig. 6). Mix.1 also forms a complex with the −174/−152 probe, but this is rather weak, perhaps reflecting a lower affinity for the site; Wilson et al. (1993) have also observed that Mix.1 binds only weakly to a single site.

*Goosecoid acts on the −381*Xbra2 *promoter through Antennapedia and Bicoid sites*

To investigate whether the Antennapedia and Bicoid binding sites identified in the −174/−152 region are involved in suppression of *Xbra2* expression, we have mutated them in the background of the −381*Xbra2* construct, the shortest promoter fragment that is inducible by FGF and low concentrations of activin. This construct was chosen over larger ones because it has only two sites predicted to bind paired-type homeodomain proteins. To further simplify analysis, we have concentrated on

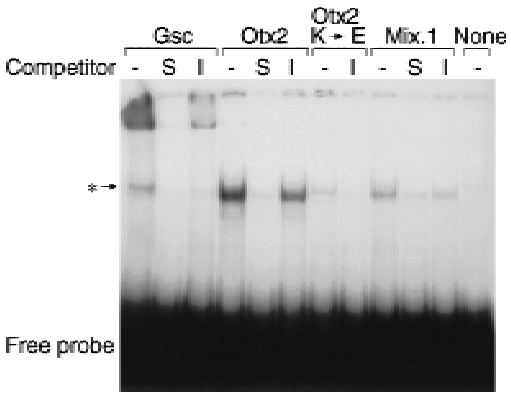


Figure 6. *goosecoid, Xotx2,* and *Mix.1* bind to the −174/−152 region of the *Xbra2* promoter. *goosecoid, Xotx2,* and *Mix.1* were translated in a coupled transcription–translation system; the DNA template used in each reaction is indicated above the brackets. In some cases, binding reactions were preincubated with a 200-fold molar excess of specific competitor (unlabeled −174/−152 oligonucleotide, S) or with a 200-fold molar excess of irrelevant competitor (unlabeled −43/−19 oligonucleotide, I). Complexes were resolved on a 4% polyacrylamide gel. The asterisk (*) indicates the position of a nonspecific DNA-binding complex; this complex varied between lanes and between different experiments.

goosecoid as a representative paired-type homeodomain protein, and to compare directly the activities of wild-type and mutant constructs, we have coinjected them into the same embryos, with the wild-type construct driving expression of Renilla luciferase and the mutant construct driving firefly luciferase.

Figure 7A shows that activin-induced reporter activity is suppressed by goosecoid in the wild-type reporter construct but not in the mutant construct in which the Antennapedia and Bicoid binding sites are destroyed (Fig. 7C). Induction of the mutant construct by low levels of activin in these experiments appears to be weaker than induction of the wild-type construct (Fig. 7A).

To investigate whether goosecoid is also able to suppress *Xbra2* promoter activity in a heterologous system, we have used transient transfections of NIH-3T3 mouse embryo fibroblasts. The activity of −381*Xbra2*.luciferase reporter is very low in these cells and could not be used reliably as a baseline for studies of suppression. We therefore tested the Antennapedia and Bicoid sites by cloning the −381/−150 region of *Xbra2* upstream of the SV40 minimal promoter. Figure 7B shows that goosecoid suppresses activity of the wild-type −381/−150.SV40 reporter but not that of the mutant version. Additional experiments indicate that mutation of the Bicoid site alone is sufficient to abolish the effect of goosecoid, as expected from its binding site preference (not shown).

Discussion

In this paper we demonstrate that sequences within 381 bp of the *Xbra2* transcription initiation site confer FGF- and activin-mediated gene activation in animal cap explants. The response of reporter constructs to activin, like the response of the endogenous gene, is concentration dependent, thus offering an opportunity to study a threshold phenomenon in a vertebrate embryo. Our results suggest that the down-regulation of *Xbra2* expression in response to high concentrations of activin is attributable to suppression by homeodomain-containing proteins such as goosecoid, Xotx2, and Mix.1.

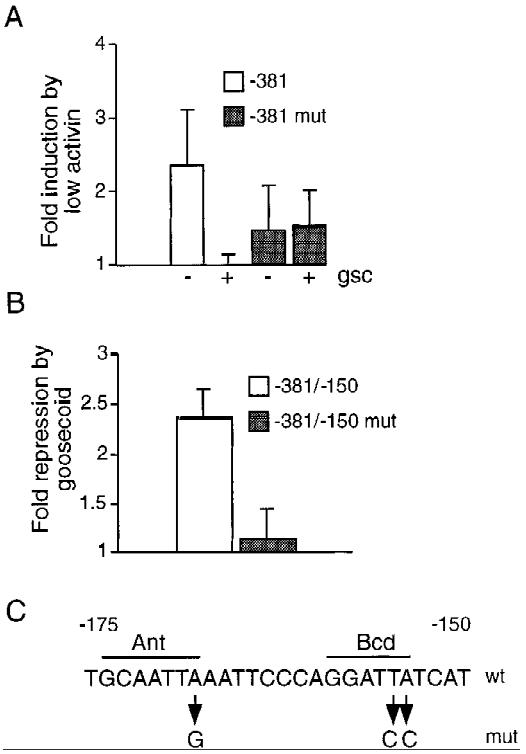


Figure 7. Suppression of −381*Xbra2*.luciferase activity by goosecoid requires Antennapedia and Bicoid homeodomain-binding sites. (*A*) Embryos were injected with 15 pg mutated −381*Xbra2*.firefly luciferase (see *C*), 15 pg −381*Xbra2*.Renilla luciferase, and 30 pg pEF-1α.β-galactosidase; in addition to this combination of DNAs, some embryos were injected with 1 pg of activin mRNA or with a mixture of 1 pg of activin mRNA and 1 ng of *goosecoid* mRNA. Animal caps were excised at the blastula stage and incubated until stage 12.5, when firefly and Renilla luciferase and β-galactosidase activities were determined. The graph shows fold induction by activin in the presence or absence of goosecoid, as obtained with the wild-type and mutated reporters. Normalized luciferase activities from four independent experiments were used in this figure. Bars show S.D.S. (*B*) NIH-3T3 cells were cotransfected in duplicate with a combination of CMV overexpression vector (either empty or *goosecoid* expressing), wild-type or mutated *Xbra2*/SV40 reporter, and reference plasmid. Luciferase activities were determined, and the results are expressed as the fold repression of reporter activity by goosecoid for each construct. Data from four experiments was used for this graph. Bars show S.D.S. (*C*) Sequence of the wild-type and the mutant Antennapedia and Bicoid sites.

Expression of Xbra2 *and sequence of the* Xbra2 *promoter*

The existence of two *Brachyury* genes, *Xbra* and *Xbra2*, is consistent with the pseudotetraploid nature of the *Xenopus laevis* genome (Kobel and Du Pasquier 1986). The regulation of *Xbra2* is similar to that of *Xbra* (Smith et al. 1991), suggesting that they play similar roles in mesodermal differentiation.

Inspection of the sequence of the *Xbra2* promoter region reveals, in addition to potential binding sites for homeodomain-containing proteins, the presence of three sites similar to the half-palindrome binding site for Xbra protein (AGGTGT or the complementary ACACCT) at positions −473 to −468, −307 to −302, and −217 to −211 (Kispert and Herrmann 1993). Such sequences would normally be predicted to occur only once every 2048 bp; so the clustering of three within 262 bp may be of significance. Because *Xbra* can activate its own transcription (Rao 1994), it will be of interest to determine whether Xbra can bind to these sequences in vitro and whether mutations in them impair autoregulation. Recent experiments suggest, however, that *Xbra* activates its own transcription indirectly (Tada et al. 1997).

The *Xbra2* promoter sequence bears no homology to the regulatory region of its mouse homolog, *Brachyury* (*T*), even though the mouse sequence is sufficient to direct expression in the primitive streak (Clements et al. 1996). It is possible that homologous sequences are located at nonconserved sites or that different mechanisms regulate the expression of these orthologs. Because regulatory elements are conserved frequently between orthologs (Nonchev et al. 1996), it will be of interest to understand this apparent difference in the mechanism of regulation between *Xbra* and *T*, as it may shed light on the evolution of regulatory pathways. There is also little similarity between the *Xbra2* promoter sequence and that of *Ciona intestinalis Brachyury* (Corbo et al. 1997), although we note that a putative *Suppressor of hairless* site (TTCCCAGG), which may play a role in regulation of *Ciona intestinalis Brachyury* expression (Corbo et al. 1997), is present between nucleotides −165 and −158.

It is also noteworthy that the activin-responsive regions of the *Xenopus* genes *goosecoid* (Watabe et al. 1995), *Mix.2* (Huang et al. 1995; Vize 1996), and *XFD-1′* (Kaufmann et al. 1996) are dissimilar and that none shows significant homology to the *Xbra2* promoter. These results suggest that there are multiple activin-responsive transcription factors, rather than a single dedicated factor that is shared by all activin-inducible immediate-early genes.

Xbra2 *promoter sequences up to −2.1 kb respond to endogenous mesoderm-inducing signals*

Xbra2 5′-flanking sequence (2.1 kb) drives mesoderm-specific expression of reporter constructs in *Xenopus* embryos. Lack of reporter gene expression in the dorsal marginal zone (Fig. 2E) indicates, as in the mouse embryo (Clements et al. 1996), that additional *Xbra* promoter elements are required for notochord expression.

Sequences within −381 bp of the Xbra2 *transcription start site respond to both FGF and activin*

Experiments using animal cap tissue revealed that 2.1 kb of *Xbra2* 5′-flanking sequence is sufficient to confer responsiveness to FGF, to components of the FGF signal transduction pathway, and to activin. Our attempts to map precisely FGF- and activin-response elements were thwarted, however, by variability from experiment to experiment. This variability is likely to be owing to several factors, including mosaic expression of the reporter construct (Vize et al. 1991), heterogeneity of different embryo batches, and perhaps the absence of some elements required for full activity of the *Xbra2* promoter. It may also be that distinct elements are required for activation and for subsequent maintenance of *Xbra2* expression (not shown; see also Clements et al. 1996). We are currently testing the effects of additional *Xbra2* genomic sequences; preliminary results show that adding 2 kb of *Xbra2* sequence downstream of +54 has no effect on the inducibility of the −2.1 kb promoter by activin. Nevertheless, our data (Fig. 3) allow us to conclude that elements that respond to FGF and activin reside between −381 bp and −231 bp of the *Xbra2* transcription start site. The response of the −381-bp construct is weaker than that of the larger −1.0- or −2.1-kb reporter constructs (Fig. 3), suggesting that elements between −1000 and −381 are required for enhancement of the transcriptional response.

Dose-dependent effects of activin on activation of the Xbra2 *promoter sequences*

Although identification of activin- and FGF-responsive elements in the *Xbra2* promoter has proved troublesome, we observe consistently that whereas low concentrations of activin elevate expression of a reporter gene driven by the *Xbra2* promoter, high concentrations cause a strong down-regulation. In this respect, the *Xbra2* reporter construct resembles the endogenous *Xbra* gene (Green et al. 1992, 1994; Gurdon et al. 1994, 1995; Jones et al. 1996), and the behavior of the reporter construct offers an opportunity to investigate the mechanism by which *Xbra* expression is activated only in a narrow window of activin concentrations. This phenomenon may be important in ensuring that *Xbra* and *Xbra2* are expressed in the right regions of the embryo. For example, levels of activin, or an activin-related molecule such as Vg1 (Weeks and Melton 1987), might be too high in the vegetal hemisphere of the late blastula/early gastrula to allow expression of *Xbra* and too low in the animal hemisphere; only in the marginal zone might the concentration be appropriate (Gurdon et al. 1994, 1995; Jones et al. 1996).

Suppression of Xbra2 *expression by goosecoid, Mix.1, and Xotx2*

High concentrations of activin cause the expression of *Xbra2* reporter constructs to fall below baseline levels,

implying active suppression of *Xbra2* expression. This might occur through the induction, by high concentrations of activin, of gene products that suppress expression of *Xbra2*. As discussed in Results, two candidate *Xbra2* repressors are *goosecoid* and *Mix.1*, both of which are induced by high levels of activin (Green et al. 1992, 1994; Gurdon et al. 1994, 1995, 1996). *goosecoid* and *Mix.1* both encode paired class homeodomains (Rosa 1989; Blumberg et al. 1991), and another member of this family is *Xotx2*, which is also expressed in the marginal zone of the early *Xenopus* embryo and may be a target of *goosecoid* regulation (Blitz and Cho 1995; Pannese et al. 1995). Our experiments show that overexpression of all three homeobox-containing genes leads to suppression of *Xbra2*, both in whole embryos and in the animal cap mesoderm induction assay. This suppression can occur at the level of transcriptional regulation, because a similar effect is observed using *Xbra2* reporter constructs. Suppression of *Xbra* expression by *goosecoid* has also been reported by Artinger et al. (1997).

In our attempts to understand the roles of homeobox-containing genes in the suppression of *Xbra* expression, we have focused on goosecoid and on a −174/−152 promoter fragment that contains Antennapedia and Bicoid binding sites to which goosecoid, Mix.1, and Xotx2 bind (Fig. 6; see also Artinger et al. 1997). Mutating these sites in a −381*Xbra2* reporter construct abolishes suppression of activin-induced activity by goosecoid (Fig. 7A). Similarly, the integrity of these sites is necessary for repression by goosecoid of the activity of a heterologous promoter fusion carrying a −381/−150 fragment in NIH-3T3 cells (Fig. 7B). Together, these data argue that goosecoid, and perhaps Mix.1 and Xotx2 as well, down-regulate *Xbra* expression through direct transcriptional repression.

Might these genes repress *Xbra* expression during normal *Xenopus* development? The case is strong for *goosecoid*, which appears to be transiently coexpressed with *Xbra* both in intact embryos (Artinger et al. 1997; also cf. Gurdon et al. 1994; Vodicka and Gerhart 1995; Ryan et al. 1996) and in activin-treated animal caps (Gurdon et al. 1994, 1995, 1996). Furthermore, Goriely et al. (1996) suggest that goosecoid functions as a repressor, and our own results (Fig. 7B) and those of Artinger et al. (1997) are consistent with this. Of the other two genes, *Xotx2* is expressed maternally and is strongly up-regulated at the onset of zygotic transcription; its gastrula-stage domain of expression is very similar to that of *goosecoid*, and *goosecoid* may regulate expression of *Xotx2* (Blitz and Cho 1995). The mammalian ortholog of *Xotx2* is able to transactivate from a *bicoid* target sequence, but in certain contexts some transcription activators are also able to function as repressors (Roberts and Green 1995). Finally, *Mix.1* (Rosa 1989) and its pseudoallele *Mix.2* (Vize 1996) are both expressed in the endoderm and mesoderm of the gastrula-stage embryo (see The *Xenopus* molecular marker resource; URL http://vize222.zo.utexas.edu/) and overlap with the expression domain of *Xbra*. The *Mix* genes may particularly be involved in ensuring that *Xbra* is not expressed in the vegetal hemisphere of the embryo. Like Otx2, Mix.1 has been suggested to function as a transcriptional activator (Mead et al. 1996), but, particularly in the presence of goosecoid, with which it can bind DNA cooperatively (Wilson et al. 1993), it may also act as a repressor.

Conclusion

Overall, our results are consistent with the simplest model imaginable to explain the response of *Xbra* to different concentrations of activin. At low doses, activin induces expression of *Xbra*. At higher concentrations, it also activates expression of genes such as *goosecoid* and *Mix.1*. These homeodomain-containing proteins then repress transcription of *Xbra* to give the observed off–on–off pattern that is observed as the concentration of activin is increased.

Materials and methods

Construction and screening of a size-selected phage λ genomic library

Attempts to isolate genomic clones containing the first exon of *Xbra* by screening conventional genomic libraries were unsuccessful. We therefore made a library from size-selected restriction fragments known to contain 5′ exons. These were identified by hybridizing a fragment containing the first 350 bp of *Xbra* cDNA to restriction digests of genomic DNA. *Bam*HI fragments ranging from 4 kb to 6 kb were then isolated from an agarose gel and cloned into *Bam*HI-digested Lambda ZapExpress vector (Stratagene). The resulting library was screened at high stringency using the first 350 bp of *Xbra* cDNA, and two identical clones, 3A1 and 3B1, were isolated.

Primer extension analysis

An oligonucleotide primer complementary to nucleotides +110 to +129 in *Xbra2* was end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. After purification, the labeled primer was mixed with 50 μg total RNA in 0.4 M KCl and incubated at 70°C for 5 min followed by 50°C for 1 hr. Nucleic acids were precipitated with ethanol, resuspended in water, and reverse-transcribed with Superscript II reverse transcriptase (Life Sciences). RNase A was added to 0.1 mg/ml, and after 10 min at room temperature cDNA was analyzed by denaturing PAGE.

Plasmid constructs

Luciferase-based vectors A *Spe*I–*Bcl*I fragment containing 53 bp of exon 1 and 2.1 kb of 5′-flanking sequence was isolated from 3A1 and subcloned into the promoterless luciferase vector pGL2Basic (Promega) to generate *Xbra2*.pGL2. One-kilobase and 150-bp deletion constructs were made using *Pst*I and *Sac*I, respectively.

A finer deletion series of the *Xbra2* flanking sequence was generated using the polymerase chain reaction, using the same 3′ primer (nucleotides +34 to +48) in all cases and various 5′ primers. A −381/+54 fragment was transferred into the Renilla luciferase vector pRLNull (Promega) to generate the plasmid used in Figure 7A. Antennapedia and Bicoid sites in −381.*Xbra2* were mutated using the Chameleon kit (Stratagene) and oligonucleotide 5′-GACTTGCAATTGATTCCCAGGATCCTCCATAGAGCT-3′. Fragments (−381/−150) from the wild-type and

mutant −381.*Xbra2* plasmids were removed with *Sac*I and cloned into pGL3Promoter (Promega).

Human β-globin-based vector The luciferase gene of *Xbra2*.pGL2 was removed by digestion with *Hin*dIII and *Sal*I and replaced with the β-globin gene isolated from p128.β/C (Mohun et al. 1989).

GFP-based vector A *Hin*dIII–*Bam*HI cDNA fragment encoding GFP (Zernicka-Goetz et al. 1996) was used to replace the luciferase gene in a pGL3 Promoter (Promega) construct carrying 2.1 kb of the *Xbra2* promoter.

Vectors used for normalization pSV.β-galactosidase contains the SV40 early promoter and enhancer driving *lacZ* (Promega). pEF-1α.β-galactosidase contains the EF-1α promoter derived from the plasmid pXEX (Johnson and Krieg 1994). This drives higher levels of *lacZ* than pSV.β-galactosidase. pRL–SV (Renilla luciferase under the control of the SV40 promoter and enhancer) and pRL–CMV were from Promega.

Xenopus *embryo culture, microinjection, and transgenics*

Xenopus laevis embryos were obtained according to Smith and Slack (1983) and staged according to Nieuwkoop and Faber (1967). Animal caps were dissected at stage 8, and they were cultured in 75% NAM (Slack 1984). Where FGF-2 (25 ng/ml) was included in the medium, bovine serum albumin was added to 0.1%. Embryos were injected with RNA as described (Smith 1993).

Xbra2 reporter constructs were injected in circular or linearized form; reference plasmids were injected in circular form. Embryos were microinjected at the one- or two-cell stage with 10–30 pg of *Xbra2* reporter DNA together with 80 pg of pSV.β-galactosidase or 80 pg of pEF-1α-β-galactosidase, or 10 pg of pRL-SV.

Transgenic embryos were generated as described by Amaya and Kroll (1997) using *Mlu*I-digested *Xbra2*.GFP.

RNase protection assays

RNase protections were performed as described (Smith 1993). *Xbra2*–β-globin fusion transcripts from *Xbra2*.βg were detected using a probe made from plasmid *Xbra2*.βgcDNA; details are available on request. To detect transcripts of the endogenous *Xbra2* gene, a 370-bp *Sac*I fragment comprising nucleotides −148 to +222 of *Xbra2* was subcloned into pBluescript to generate pSac370.9. This plasmid was linearized with *Bam*HI and transcribed with T7 polymerase. Loading control probe EF-1α has been described previously (Cunliffe and Smith 1992).

In vitro transcription

In vitro transcription using SP6 or T7 RNA polymerase was as described by Smith (1993). v-Ras cDNA was the gift of Dr. M. Whitman (Harvard Medical School, Boston, MA). Other clones include MEK1$^{S217E/S221E}$ (Umbhauer et al. 1995), *Xenopus* activin B (Thomsen et al. 1990), *Xotx2* and *Xotx2* K → E (Pannese et al. 1995), and *goosecoid* (Cho et al. 1991). *Mix.1* cDNA (gift of F. Rosa, Ecole Norwale Supérieure, Paris, France) was cloned into pSP64TXB (a modified form of pSP64T prepared by Dr. M. Tada); the resulting plasmid was linearized with *Eco*RI and transcribed with SP6 polymerase. Amounts of RNAs used in injections were v-Ras, 100 pg; MEK1$^{S217E/S221E}$, 1 ng; activin B, 1–100 pg (see legends to Figs. 3 and 4); *goosecoid, Mix.1, Xotx2,* and *Xotx2* K → E, 0.5 ng per blastomere.

Whole-mount in situ hybridization

Whole-mount in situ hybridization to albino *Xenopus* embryos was according to Harland (1991) except that the substrate used for the chromogenic reaction was Boehringer Mannheim Purple AP Substrate. *Xbra2* transcripts were detected using the plasmid pSac370.9ΔEM; details are available on request. A probe specific for *Xbra* was made by digesting pXT1 (Smith et al. 1991) with *Stu*I and transcribing with T7 RNA polymerase. A probe specific for GFP was made by cloning a blunt-ended *Hin*dIII–*Bam*HI GFP fragment (Zernicka-Goetz et al. 1996) into *Stu*I-digested pCS2+ (Rupp et al. 1994).

Luciferase and β-galactosidase assays

When firefly luciferase assays were performed using β-galactosidase for normalization, embryos (~5) or animal caps (~20) were frozen on dry ice and then lysed in 5–10 volumes of lysis buffer (Promega). The lysate was clarified by brief centrifugation; 40% was assayed for luciferase activity using the Promega Luciferase Assay System, and the remaining 60% was assayed for β-galactosidase activity (Sambrook et al. 1989).

In experiments where Renilla luciferase was used for normalization, dual-luciferase assays were performed according to the manufacturer's recommendations (Promega). Twelve to 20 animal caps per sample were lysed in 30–50 µl of lysis buffer; 0.2 to 0.5-animal cap equivalents were used in measurements of luciferase activity.

Electrophoretic mobility-shift assay

Proteins for use in binding reactions were translated in the TNT coupled transcription–translation system (Promega). All four proteins translated with similar efficiency, as assessed by incorporation of [^{35}S]methionine and analysis by SDS-PAGE. One microliter of translation reaction was used in each binding reaction, which included 12 mM HEPES at pH 7.6, 60 mM KCl, 0.5 mM dithiothreitol (DTT), 3 mM spermidine, 0.5 mM PMSF, 12% glycerol, 3 µg of pBluescript KS as a nonspecific DNA competitor, and ~0.4 ng (5×10^4–7×10^4 cpm) of ^{32}P-labeled probe. Probe was made by annealing oligonucleotides 5′-*TCGA*GCAATTAAATTCCCAGGATTATC and 5′-*TCGA*GATAATCCTGGGAATTTAATTGC and labeling them with Klenow enzyme in the presence of [^{32}P]dCTP, dATP, dTTP, and dGTP (Sambrook et al. 1989). Nucleotides shown in italics are not derived from *Xbra2* sequences. Binding reactions were preincubated for 20–25 min at room temperature, after which probe was added. After a further 10 min at room temperature, samples were analyzed on a 4% polyacrylamide gel.

Cell culture and transfections

NIH-3T3 mouse fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM) (Sigma) supplemented with 10% newborn calf serum, 2 mM L-glutamine, 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin. Calcium-phosphate transfections were performed as described (Sambrook et al. 1989) in six-well plates. A total of 5 µg of DNA per well was used: 4 µg of pCDNA3 or pCDNA3–*goosecoid,* 0.5 µg of *Xbra2* reporter plasmid, and 0.5 µg of reference plasmid (either pRL–CMV or pRL–SV40). Cells were analyzed for luciferase activities 3 days after transfection as described above. Each sample was transfected in duplicate. *goosecoid* cDNA (Blumberg et al. 1991) was cloned into the pcDNA3 vector (containing the CMV promoter/enhancer; Invitrogen) using *Eco*RI and *Xho*I.

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