# Goosecoid and Mix.1 repress Brachyury expression and are required for head formation in Xenopus

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## SUMMARY

The *Xenopus* homologue of *Brachyury*, *Xbra*, is expressed in the presumptive mesoderm of the early gastrula. Induction of *Xbra* in animal pole tissue by activin occurs only in a narrow window of activin concentrations; if the level of inducer is too high, or too low, the gene is not expressed. Previously, we have suggested that the suppression of *Xbra* by high concentrations of activin is due to the action of genes such as *goosecoid* and *Mix.1*. Here, we examine the roles played by *goosecoid* and *Mix.1* during normal development, first in the control of *Xbra* expression and then in the formation of the mesendoderm. Consistent with the model outlined above, inhibition of the function of either gene product leads to transient ectopic expression of *Xbra*. Such embryos later develop dorsoanterior defects and, in the case of interference with *Mix.1*, additional defects in heart and gut formation. Goosecoid, a transcriptional repressor, appears to act directly on transcription of *Xbra*. In contrast, Mix.1, which functions as a transcriptional activator, may act on *Xbra* indirectly, in part through activation of *goosecoid*.

Key words: *Xenopus*, Mesoderm, *Brachyury*, *goosecoid*, *Mix.1*

## INTRODUCTION

The body plan of the *Xenopus* embryo is specified through the asymmetric distribution of maternal determinants followed by a series of inductive interactions (Harland and Gerhart, 1997). The first such interaction is mesoderm induction, in which signals from the vegetal hemisphere of the embryo act on overlying equatorial cells and cause them to become mesoderm rather than ectoderm (Harland and Gerhart, 1997). The best candidates for endogenous mesoderm-inducing factors include members of the TGF-β superfamily, including activin and Vg1 (Harland and Gerhart, 1997; Slack, 1994). Of these factors, the most intensively studied is activin, which is capable of inducing different endodermal and mesodermal cell types in a concentration-dependent manner. Thus, low concentrations of activin induce ventral mesoderm and high concentrations activate genes normally expressed in anterior endodermal tissues (Green et al., 1992; Gurdon et al., 1996).

In an effort to understand mesoderm induction and the concentration-dependent effects of activin, we have studied the regulation of *Xenopus Brachyury* (*Xbra*). At the early gastrula stage, *Xbra* is expressed throughout the marginal zone of the embryo and, as gastrulation proceeds, transcripts are lost from newly involuted mesoderm but persist in the notochord (Smith et al., 1991). Expression of *Xbra* is induced in explants of animal pole tissue by activin, but stable activation occurs only in a narrow window of activin concentrations (Gurdon et al., 1996); if levels are too low, or too high, the gene is not expressed. This phenomenon may

underlie the restriction of *Xbra* expression to the marginal zone of the embryo. Levels of activin, or an activin-like molecule, may be too high in the vegetal hemisphere, and too low in the animal hemisphere, for expression of *Xbra* to occur, but levels in the equatorial region may be just right. The concentration-dependent response of *Xbra* to activin may therefore represent a useful model for the problem of germ layer specification during early development.

Previous work has suggested that the downregulation of *Xbra* expression at high concentrations of activin is due to repression of transcription by the homeobox-containing genes *goosecoid* and *Mix.1* (Latinkic et al., 1997). Both genes are induced by high concentrations of activin (Gurdon et al., 1996), and overexpression of either causes downregulation of *Xbra*, both in the embryo and in explants of animal pole tissue (Artinger et al., 1997; Latinkic et al., 1997). The effects of *goosecoid* and *Mix.1* are likely to occur at the level of transcription, because they can also repress *Xbra* reporter constructs (Latinkic et al., 1997).

Here we examine the roles played by *goosecoid* and *Mix.1* in normal development, first in the control of *Xbra* expression and then in the development of the mesendoderm. Consistent with the model outlined above, inhibition of the function of either gene product leads to transient ectopic expression of *Xbra*. Such embryos later develop dorsoanterior defects, suggesting that the activities of *goosecoid* and *Mix.1* are both required for normal head development. As well as having reduced heads, embryos in which *Mix.1* function is inhibited have additional defects in heart and gut formation, suggesting

that *Mix.1* has a broader role in the development of dorsoanterior endoderm.

Our data are consistent with the idea that Goosecoid, a transcriptional repressor, acts directly on transcription of *Xbra*. In contrast, Mix.1 functions as a transcriptional activator, and probably acts on *Xbra* indirectly, in part through activation of *goosecoid*. Coexpression of *Mix.1* and *goosecoid* in animal cap explants leads to the synergistic induction of the endodermal marker *XSox17α*, another gene induced by high concentrations of activin. Together, these observations suggest that *Mix.1* and *goosecoid* act together to promote dorsoanterior endodermal differentiation and to suppress expression of mesodermal genes like *Xbra*.

## MATERIALS AND METHODS

### Plasmid constructs

All recombinant DNA manipulations were performed by standard techniques (Sambrook et al., 1989). Full construction details and maps of all constructs are available on request.

A *goosecoid* cDNA (Blumberg et al., 1991) was cloned as a *Hin*dIII-*Eco*RI fragment in its reverse orientation into pcDNA3 (Invitrogen) to create pCMV-*csg*. GscVP16 was constructed by adding two copies of the VP16 minimal transcriptional activation domain (amino acids 413-454; gift of Dr J. Brickman) to a pcDNA3-*gsc* construct.

A *Mix.1* cDNA (Rosa, 1989) was cloned into pcDNA3 as a *Bam*HI-*Apa*I fragment to create the antisense construct pCMV-*1.xiM*, or as a *Hin*dIII-*Bam*HI fragment into a derivative of pcDNA3 containing two HA tags (B. V. L., unpublished) to create a wild-type overexpression construct. *Mix.1-En^R* and *Mix.1HD-En^R* were constructed by using PCR to fuse the *Mix.1* coding sequence with a double-haemagluttinin (HA)-tagged Engrailed repressor domain (Conlon et al., 1996, and M. Tada, personal communication). Junctions created by cloning were verified by sequencing.

### Reporter constructs

P3 (top strand: 5′-agctTGAG/TCTCTAATTGAATTACTGTACA; bottom strand: 5′-agctTGTACAGTAATTCAATTAGACTCA) or P3C (top strand: 5′-gatcCTGAGTCTAATCCGATTACTGTACG; bottom strand: 5′-gatcCGTACAGTAATCGGATTAGACTCAG) oligonucleotides were annealed and cloned into the *Hin*dIII or *Bgl*II sites, respectively, of pGL3Promoter (Promega), which contains the SV40 minimal promoter. Clones were isolated that contained two head-to-tail inserts of each oligonucleotide. (P3)$_6$/luc was obtained by cloning 6 copies of the P3 site into a reporter containing the E4 minimal promoter (kind gift of M. Tada). A *goosecoid* promoter fragment (Watabe et al., 1995) was obtained by genomic PCR and cloned into pGL3Basic to create −300gsc/luc (gift of Niall Armes and Masa Tada). −207gsc/luc and −190gsc/luc were also created by PCR. The nucleotide co-ordinates designate the most 5′ base pairs of the *goosecoid* promoter retained in the construct and for both constructs the following 3′ primer was used: 5′-GACCTCGAGCTCTCCCATCTGTGCCTCTTC-3′. PCR products were digested with *Mlu*I and *Xho*I and cloned into the same sites of pGL3Basic.

### *Xenopus* embryos and microinjection

Fertilisation, culture and microinjection of *Xenopus* embryos were as described (Latinkic et al., 1997). They were staged according to Nieuwkoop and Faber (1975).

### RNAase protection assays

RNAase protection assays were carried out as described (Smith, 1993), except that rapid aqueous hybridisation was used (Mironov et

al., 1995). Probes included *Xbra* (Smith et al., 1991), *goosecoid* (Armes and Smith, 1997; Cho et al., 1991), *EF-1α* (Sargent and Bennett, 1990), *ornithine decarboxylase* (*ODC*) (Isaacs et al., 1992), *chordin* (Howell and Hill, 1997) and *XSox17α* (Hudson et al., 1997).

### Whole-mount in situ hybridisation and immunocytochemistry

Whole-mount in situ hybridisation was carried out essentially as described (Harland, 1991). Probes included *Xbra* (Smith et al., 1991), *goosecoid* (Cho et al., 1991) and *XMLC2* (Chambers et al., 1994). Whole-mount staining with monoclonal antibodies MZ15 (Smith and Watt, 1985) and 12/101 (Kintner and Brockes, 1984) was performed as described (Smith, 1993). Injected cells were labelled by coinjecting nuclear *lacZ* RNA followed by X-Gal staining, or by co-injecting biotinylated dextran (Molecular Probes) and detecting with ExtrAvidin-Alkaline Phosphatase (Sigma), using Fast Red as a substrate (Boehringer Mannheim).

### In vitro transcription

In vitro transcription using SP6 or T7 RNA polymerase was as described (Smith, 1993).

### Luciferase and β-galactosidase assays

Dual-luciferase assays on NIH3T3 and animal cap extracts were carried out according to the manufacturer's recommendations (Promega), essentially as described (Latinkic et al., 1997). In experiments where β-galactosidase was used as a reference, enzymatic assays were performed as described (Sambrook et al., 1989).

### Cell culture and transfections

NIH3T3 mouse embryo fibroblasts were cultured in Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 10% newborn calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin (Sigma). Calcium phosphate transfections were performed as described (Sambrook et al., 1989) in 6-well plates. 5 μg DNA per well was used. Unless indicated otherwise, this comprised 4 μg of pcDNA3 or the indicated derivative, 0.5 μg of reporter plasmid, and 0.5 μg of pRL-TK (Promega) or EF-1α/*lacZ* as a reference plasmid. Cells were analysed 3 days after transfection for luciferase activity as described above. Each sample was transfected in duplicate.

### Immunofluorescence

Indirect immunofluorescence was performed with anti-HA mouse monoclonal antibodies and secondary anti-mouse-FITC antibody. Bright-field and fluorescent images were electronically overlaid.

### Electrophoretic mobility shift assays

Proteins for use in binding reactions were translated in the TNT coupled transcription-translation system, according to the manufacturer's recommendations (Promega). Electrophoretic mobility shift assays were performed as described (Latinkic et al., 1997). In experiments where the identity of complexes was tested by the addition of anti-HA antibodies (1 mg/ml; Boehringer Mannheim), 1 μl of antibody was added after addition of probe, and samples were incubated for an additional 15-20 minutes on ice. The probe derived from the *Xbra2* promoter, and the non-specific competitor, were as described (Latinkic et al., 1997). The sequence of the DE and PE is shown in Fig. 4B; annealed oligonucleotides had 5′-GATC single-stranded overhangs.

## RESULTS

### Interference with goosecoid function causes ectopic expression of *Xbra*

Misexpression of *goosecoid* in *Xenopus* embryos or in activin-

or FGF-treated animal caps suppresses transcription of *Xbra* (Artinger et al., 1997; Latinkic et al., 1997). Since goosecoid can bind to the *Xbra* promoter (Artinger et al., 1997; Latinkic et al., 1997), and can repress *Xbra* reporter constructs in a heterologous system in a sequence-specific manner (Latinkic et al., 1997), it is likely that this repression occurs in a direct fashion.

To investigate whether goosecoid regulates *Xbra* expression during normal development, we inhibited the function of the gene in two different ways. In the first, we interfered with the ability of goosecoid to repress transcription (Mailhos et al., 1998) by adding to it the VP16 transcription activation domain (Fig. 1A; see Materials and Methods). The resulting gscVP16 construct differs from that recently described by Ferreiro et al. (1998) because it includes the entire coding region of goosecoid (in an effort to increase specificity) and because we use two copies of a minimal VP16 activation domain, which in our hands is less toxic than the entire activation domain. GscVP16, like wild-type goosecoid (Latinkic et al., 1997), binds to nucleotides −172 to −154 of the *Xbra2* promoter (not shown).

The ability of gscVP16 to interfere with the function of wild-type goosecoid was tested in NIH3T3 cell transient transfection assays using a luciferase reporter construct (pP3C-SV40/luc) in which two P3C sites (see Materials and Methods), to which goosecoid (Wilson et al., 1993) and gscVP16 (data not shown) bind, are positioned upstream of the SV40 minimal promoter. Fig. 1B shows that gscVP16 does not activate pP3C-SV40/luc, but does interfere with the ability of wild-type goosecoid to repress it, even at a ratio of 1:3. Transfection of different quantities of gscVP16 (0.1-4.0 µg) suggests that the inability

of gscVP16 to activate transcription is unlikely to be due to squelching effects (not shown). Rather, it is likely that the VP16 domain, positioned at the C terminus of the protein, interferes with the N-terminally located repression domain (Mailhos et al., 1998). The apparent lack of transcription activation by gscVP16 is an advantage in our studies, because its effects should be restricted to preventing goosecoid-mediated repression; it will not exceed this remit by inappropriate activation of goosecoid targets.

Dorsal, but not ventral, injection of both *gscVP16* RNA and pCMV-*gscVP16*, in which *gscVP16* expression is driven by the CMV promoter, leads to ectopic activation of *Xbra* (Fig. 2B,C). Embryos allowed to develop to tadpole stages showed a range of anterior deficiencies, including cyclopia and loss of head. Notochord and somite formation, revealed using monoclonal antibodies MZ15 and 12/101, respectively, were essentially normal (Fig. 2E-H).

These results suggest that *goosecoid* represses expression of *Xbra* during normal development. To confirm this conclusion, we used an antisense approach in which a plasmid directing expression of antisense *goosecoid* RNA under the control of the CMV promoter (pCMV-*csg*) was injected into *Xenopus* embryos at the 4-cell stage. Expression of *Xbra* was then analysed at gastrula stages by whole-mount in situ hybridisation. Previous work has shown that antisense *goosecoid* constructs causes anterior defects in *Xenopus* embryos, probably by interfering with translation of goosecoid protein (Steinbeisser et al., 1995).

Fig. 2A shows that embryos injected with pCMV-*csg* display patches of ectopic *Xbra* expression in anterior regions, suggesting that the reduction of goosecoid activity in these territories causes activation of *Xbra*. No such patches were observed in control embryos in which empty vector was injected (Fig. 2D). Embryos injected with pCMV-*csg*, and with antisense *goosecoid* RNA, lacked anterior structures (Fig. 2I,J, and data not shown).

Together, these observations show that interference with *goosecoid* function leads to ectopic activation of *Xbra*, suggesting that *goosecoid* is involved in repression of *Xbra* in the dorsoanterior mesendoderm of *Xenopus* embryos. This result is consistent with previous work indicating that goosecoid represses transcription of *Xbra* directly (Artinger et al., 1997; Latinkic et al., 1997). In addition, both approaches indicate that *goosecoid*-like activity is required for normal development of dorsoanterior mesendoderm.

## Mix.1 is a transcriptional activator

We next tested the role of *Mix.1* in restriction of *Xbra* expression. Like *goosecoid*, *Mix.1* can suppress expression of *Xbra* (Latinkic et al., 1997) and, like goosecoid, Mix.1 contains a paired-type homeodomain.

Although it suppresses expression of *Xbra* in embryos and animal cap assays, Mix.1 has been stated to act as a transcriptional activator (Lemaire et al., 1998; Mead et al., 1996). Our own experiments demonstrate that Mix.1 causes activation of a reporter gene containing six copies of the P3 binding site (two palindromic core sequences TAAT, separated by three nucleotides) placed upstream of a TATA box (see Fig. 6F), as well as activation of a reporter construct containing two copies of P3 placed upstream of the SV40 promoter (Fig. 3A). Mix.1 can also transactivate the *Xbra* promoter construct
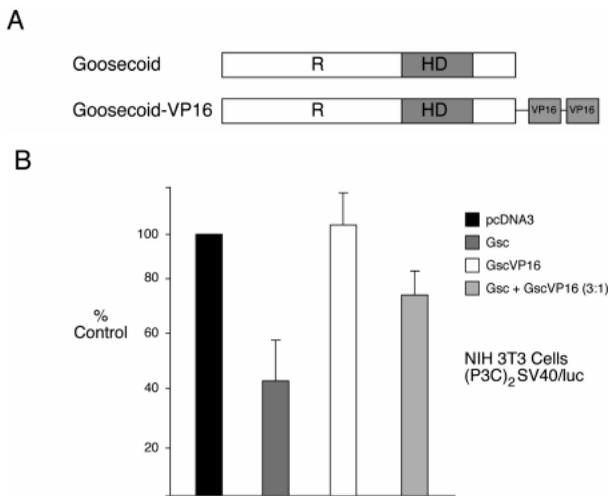


**Fig. 1.** Creation and characterisation of an interfering goosecoid construct. (A) Goosecoid contains a homeodomain (HD) and an N-terminal repression domain (R). Goosecoid-VP16 comprises the entire Goosecoid amino acid sequence with the addition at the C terminus of two minimal VP16 transcription activation domains (amino acids 413-454). (B) Goosecoid-VP16 does not activate transcription but does interfere with the ability of Goosecoid to repress transcription. NIH3T3 cells were transfected with 500 ng (P3C)₂SV40/luc, 200 ng RL/TK as a reference plasmid and a total of 4 µg of pcDNA3-based overexpression plasmids. Standard errors are indicated and are based on three experiments.
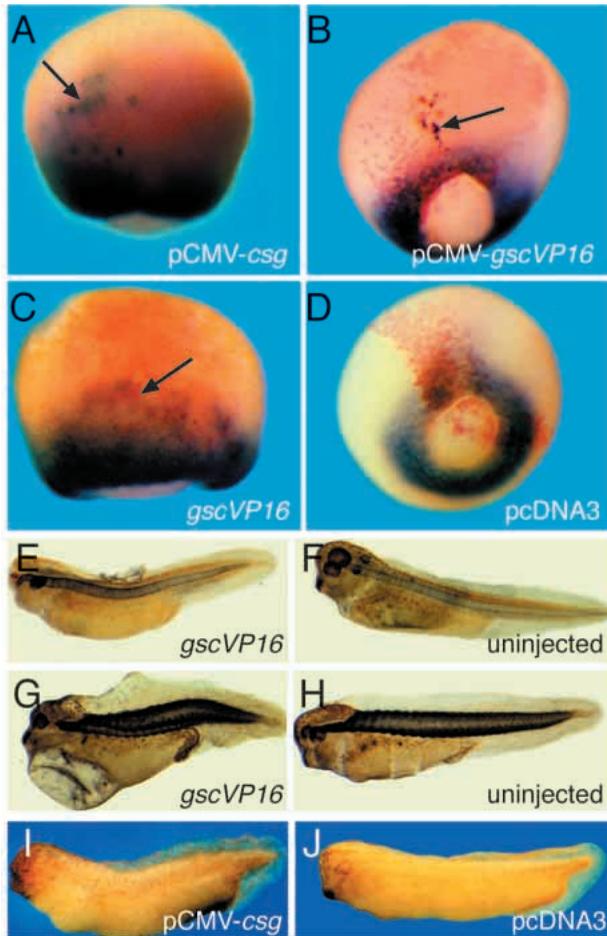
**Fig. 2.** Interference with *goosecoid*-like activity causes ectopic expression of *Xbra* and results in anterior truncations.
(A-D) Embryos were injected dorsally with 100 pg of the indicated DNAs (A,B,D) or RNA (C) together with biotinylated dextran as a cell lineage marker (pink-red staining). They were fixed at mid-gastrula stages and expression of *Xbra* was analysed by in situ hybridisation. Both *gscVP16* and antisense *goosecoid* cause ectopic expression of *Xbra* in a cell-autonomous fashion (arrows). In a representative experiment (carried out three times), this was observed in 40% of cases following injection of pCMV-*csg* (*n*=20; A), 22% of cases following injection of pCMV-*gscVP16* (*n*=22; B) and 83% of cases following injection of *gscVP16* RNA (*n*=23; C). pcDNA3 had no effect on *Xbra* expression (D). (E-H) Dorsal injection of RNA encoding gscVP16 (E,G) causes anterior deficiencies, including cyclopia in 83% of cases. Such embryos contain both notochord (E; MZ15 staining) and muscle (G; 12/101 staining). (I,J) Antisense goosecoid constructs also cause anterior truncations. Embryos were injected in two dorsal blastomeres at the four-cell stage with 100 pg of pCMV-*csg* (I) or pcDNA3 (J). 42% of embryos injected with pCMV-*csg* develop anterior deficiencies, ranging from mild cyclopia to complete loss of head. Similar results were obtained with antisense *goosecoid* RNA.

−381*Xbra2*/luc; this requires the homeodomain binding sites within the −381 promoter, arguing that the effect is specific (Fig. 3A).

Thus, when tested in a simple heterologous system, Mix.1 behaves as a transcriptional activator; in contrast, when expressed in embryos, or in animal caps, *Mix.1* suppresses expression of *Xbra* (Latinkic et al., 1997). How does Mix.1
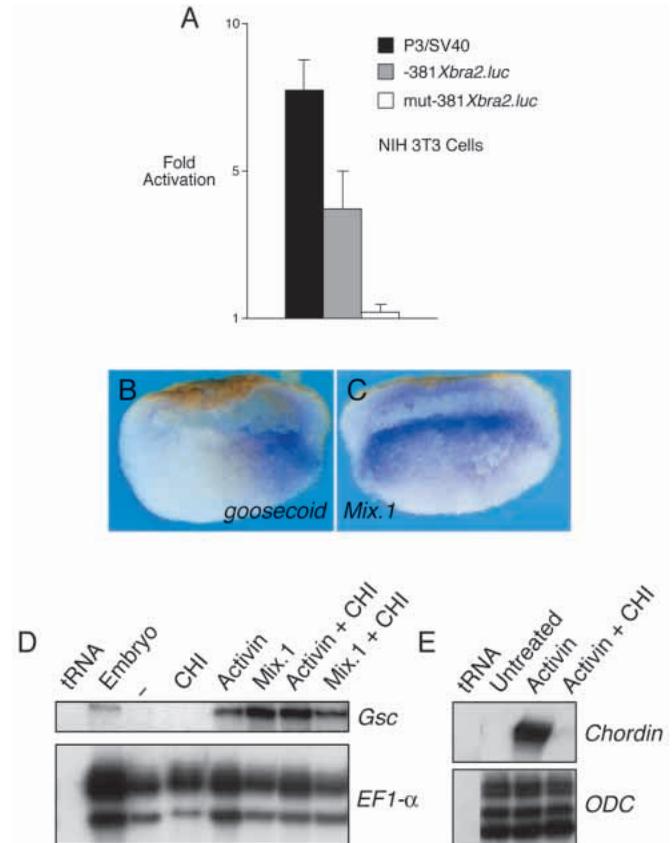


**Fig. 3.** *Mix.1* is a transcription activator and one of its potential targets is *goosecoid*. (A) pCMV-*Mix.1* was cotransfected with the indicated reporter constructs and into NIH3T3 cells. Normalised luciferase activities were used to calculate fold activation, using activity in cells transfected with empty vector as baseline. The reporter mut −381.*Xbra2.luc*, in which both Paired-type homeodomain binding sites of the −381.*Xbra2* promoter are mutated (Latinkic et al., 1997), is not activated by *Mix.1*. (B,C) *goosecoid* and *Mix.1* are transiently co-expressed. *Xenopus* embryos were fixed at stage 10 and cut into left and right halves, which were processed separately for in situ hybridisation. There is extensive overlap of the expression domains of *Mix.1* and *goosecoid* on the dorsoanterior side (right in B and left in C). (D) Like activin, *Mix.1* activates expression of *goosecoid* in animal caps in the presence of cycloheximide. *Mix.1* RNA (200 pg) was injected into fertilised eggs, and animal caps were dissected at mid-blastula stage 8. Cycloheximide (10 μg/ml) was added to the indicated samples. Uninjected animal caps were treated with 8 U/ml activin as indicated. All caps were frozen at early gastrula stage 10.5 and processed by RNAase protection. (E) The cycloheximide treatment regime is sufficient to block induction of chordin by activin.

cause suppression of *Xbra* in the embryo and what is the biological significance of this effect?

## Mix.1 activates *goosecoid* expression

One way in which Mix.1 might suppress expression of *Xbra* is by potentiating the action of a repressor. Another is that it acts indirectly, through the activation of a transcriptional repressor and a third possibility is a combination of the two models in which *Mix.1* induces a repressor whose activity it potentiates. One potential target gene of Mix.1 is *goosecoid*. Inspection of published data suggests that the two genes are transiently co-
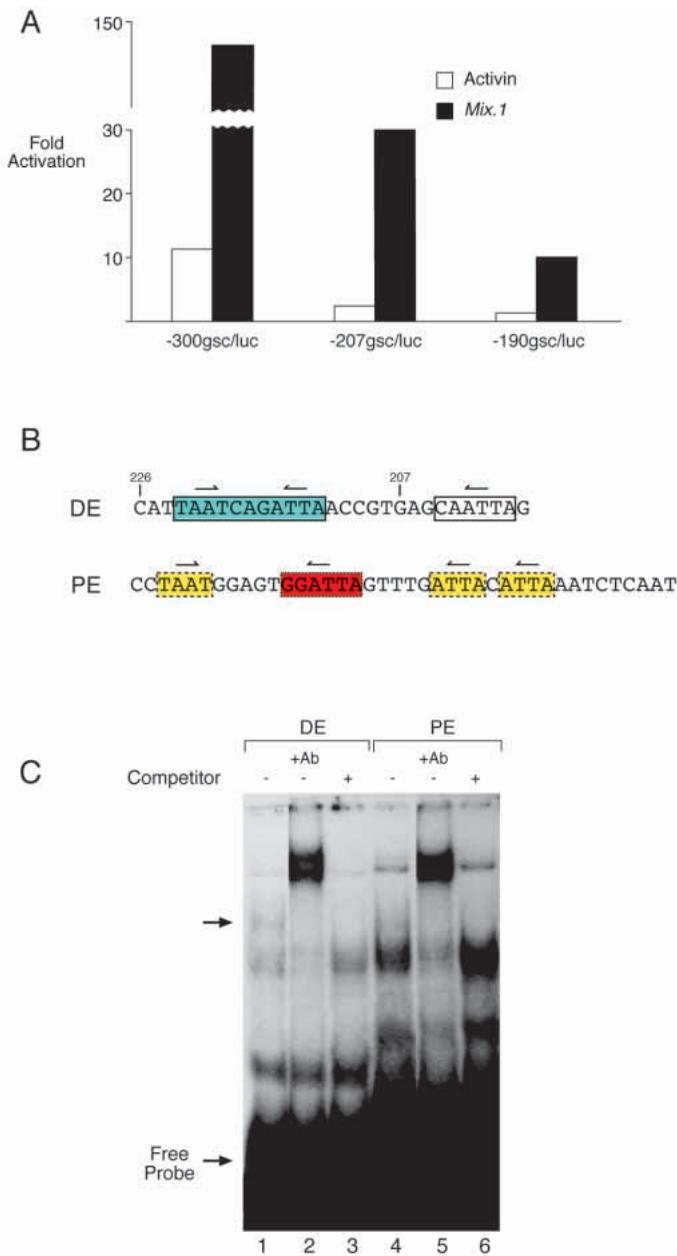
**Fig. 4.** Mix.1 activates *goosecoid* reporter constructs and binds to the distal and proximal elements (DE and PE) of the goosecoid promoter. (A) The indicated reporter constructs were injected into the animal poles of *Xenopus* embryos together with *Mix.1* or activin mRNA. Animal caps were excised at stage 8 and cultured for 3 hours at room temperature, after which time luciferase activities were determined. This experiment has been carried out three times, and an additional two times using the –300gsc/luc construct. Usually, the levels of activation of –300gsc/luc by activin and Mix.1 were comparable, and of the order of 30- to 50-fold. (B) The sequence of the DE includes three core TAAT sites, two of which are in the preferred palindromic orientation (blue box). The PE includes three core TAAT sites (yellow) and a Bicoid-type (K50) site (red). (C) Mix.1-HA binds both to the DE and to the PE. Binding is specific (arrow, lanes 1 and 4), and the complex is abolished by addition of anti-HA antibody, which results in the formation of a more stable lower-mobility complex (lanes 2 and 5).

confirmed by demonstrating that cycloheximide also inhibits induction of *chordin*, which is known to be induced indirectly by activin (Howell and Hill, 1997; Sasai et al., 1994) (Fig. 3E).

To investigate whether the induction of *goosecoid* by Mix.1 occurs directly, we asked whether Mix.1 can activate a -300 base pair *goosecoid* reporter construct (Watabe et al., 1995), both in NIH3T3 cells (not shown) and in animal caps (Fig. 4A). In each system, over-expression of Mix.1 leads to activation of reporter gene activity. Progressive 5′ deletions of the *goosecoid* promoter caused a gradual reduction in Mix.1 responsiveness, suggesting that multiple elements are involved (Fig. 4A).

Inspection of the *goosecoid* promoter sequence (Fig. 4B) reveals two clusters of putative Mix.1 binding sites (Wilson et al., 1993) within the distal and proximal elements, which confer responsiveness to activin and Wnt signalling respectively (Watabe et al., 1995); these regions also appear to be necessary for the response to Mix.1. The distal element (DE) contains a P3 site, deletion of which causes the greatest loss of activity, and one core TAAT site. The proximal element (PE) includes two inverted repeats of the core binding site separated by 7 base pairs. As expected, Mix.1 binds with higher affinity to the DE than to the PE (Fig. 4C).

These results suggest that one mechanism by which *Mix.1* suppresses expression of *Xbra* is through activation of *goosecoid*. However, it is still possible, as suggested above, that Mix.1 potentiates the repressor action of goosecoid. This question was investigated by measuring *goosecoid* and *Xbra* reporter gene activity in NIH3T3 cells in the presence of Mix.1, goosecoid and a combination of the two proteins. At a ratio of 1:1, goosecoid inhibited Mix.1-induced activation of both reporter constructs, but no evidence for potentiation of repression was obtained in this heterologous system (data not shown). It remains possible, however, that *Mix.1* does enhance the activity of a repressor such as *goosecoid* in vivo.

### Inhibition of Mix.1-like function causes transient ectopic expression of *Xbra*

The above results are consistent with the suggestion that Mix.1 regulates expression of *Xbra* indirectly, through the activation of repressor molecules such as goosecoid. We next asked whether *Mix.1* regulates expression of *Xbra* during normal development. To this end, we first made a construct (pCMV-*1.xiM*) in which the entire *Mix.1* cDNA is driven in the antisense orientation by the CMV promoter. This is essentially

expressed in the organiser and in vegetal tissue during normal development (Medina et al., 1997; Vodicka and Gerhart, 1995), and both are induced in animal caps by high concentrations of activin (Gurdon et al., 1996). To compare directly the expression patterns of *goosecoid* and *Mix.1*, we dissected *Xenopus* embryos at the late blastula and early gastrula stages into left and right halves, which were then processed separately for whole-mount in situ hybridisation. Our results show that the two genes are expressed in overlapping domains on the dorsal side, and that *goosecoid* is expressed in deep dorsoanterior endoderm (Fig. 3B,C).

We next asked whether Mix.1 can induce expression of *goosecoid*. Fig. 3D shows that misexpression of *Mix.1* in *Xenopus* animal caps is sufficient to induce expression of *goosecoid*, and that this induction can occur in the presence of cycloheximide, suggesting that it reflects direct transcriptional activation. The efficacy of cycloheximide treatment was
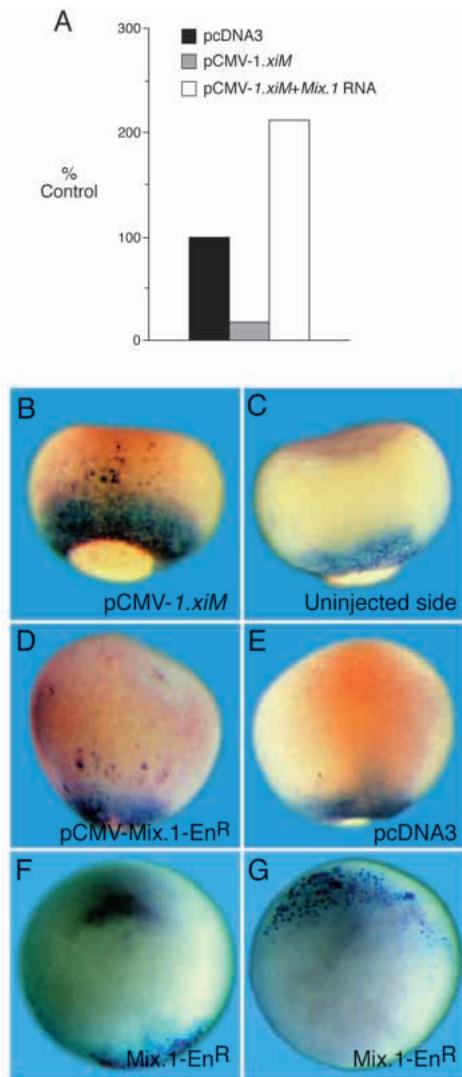
**Fig. 5.** (A) pCMV-*1.xiM* inhibits expression of (P3)$_6$/luciferase in vegetal pole tissue. Inhibition is reversed by injection of Mix.1 RNA. Embryos received vegetal injections of (P3)$_6$/luc (100 pg) together with 100 pg pcDNA3 or pCMV-*1.xiM*. Expression of the reporter construct was inhibited by pCMV-*1.xiM*, presumably through inhibition of endogenous *Mix.1* activity. Injection of 10 pg *Mix.1* RNA reversed inhibition. This experiment was carried out twice. (B-G) Transient activation of *Xbra* by antisense *Mix.1* and *Mix.1-En$^R$* and downregulation of goosecoid by *Mix.1-En$^R$*. pCMV-*1.xiM* (B; uninjected side shown in C), pCMV- *Mix.1-En$^R$* (D) or pcDNA3 (E) (100 pg) was injected in the two dorsal blastomeres of *Xenopus* embryos at the 4-cell stage together with biotinylated dextran as a lineage marker (showing as pink-red staining). Embryos were fixed at mid-late gastrula stages, and *Xbra* transcripts were revealed by in situ hybridisation. In a representative experiment, ectopic expression of *Xbra* occurred in a cell-autonomous manner in 75% of embryos injected with pCMV-*1.xiM* (*n*=12), 73% of embryos injected with pCMV- *Mix.1-En$^R$* (*n*=11) and in none of the embryos injected with pcDNA3 (*n*=6). Similar results were obtained with RNA injections (not shown; experiment carried out three times). (F,G) Interfering with *Mix.1*-like activity in early embryos causes downregulation of *goosecoid*. Embryos were injected at the 4-cell stage with RNA encoding Mix.1-En$^R$, together with β-galactosidase RNA as a lineage marker. Dorsal injections (G) cause dramatic downregulation of *goosecoid* expression, whereas ventral injections (F) have no effect.

the same strategy as used above for *goosecoid*. The efficacy of the construct was tested by injecting pCMV-*1.xiM* into the vegetal hemisphere of *Xenopus* embryos together with the (P3)$_6$/luc reporter construct. pCMV-*1.xiM* proved significantly to inhibit (P3)$_6$/luciferase activity, presumably due to interference with endogenous *Mix.1* function and this interference was reversed by injection of RNA encoding wild-type *Mix.1* (Fig. 5A).

At the mid/late-gastrula stage, 75% of embryos injected with pCMV-*1.xiM* displayed ectopic patches of *Xbra* expression on the injected side (Fig. 5B,C), whereas all embryos injected with empty vector showed a normal *Xbra* expression pattern (Fig. 5E).

To test the specificity of the results obtained with pCMV-*1.xiM*, we devised a second approach in which the activation function of Mix.1 was compromised by fusing it to the Engrailed repressor domain (Conlon et al., 1996). Two constructs were made (Fig. 6A): one included only the homeodomain of Mix.1 and sequences N terminal to it (*Mix.1HD-En$^R$*), while the other included the entire open reading frame of Mix.1 (*Mix.1-En$^R$*). Like the wild-type protein, both fusions bind the P3 oligonucleotide (Fig. 6B) and both are nuclear proteins (Fig. 6C-E). When tested in NIH3T3 cells on the P3 reporter, both Mix.1HD-En$^R$ and Mix.1-En$^R$ behave as transcriptional repressors and inhibit activation by wild-type Mix.1 (Fig. 6F). Complete inhibition of Mix.1 activity was achieved with a 1:2 ratio of Mix.1HD-En$^R$ or Mix.1-En$^R$ to Mix.1, and partial inhibition was achieved even with a ratio of 1:10, arguing that our interfering reagents act as active repressors (data not shown). Mix.1-En$^R$ also prevents activation of *Xbra* and *goosecoid* reporter gene constructs in NIH3T3 cells (data not shown), and it inhibits *Mix.1* function in animal cap assays (Fig. 6G). We note that in NIH3T3 cells our repressor fusions inhibited activation not only by Mix.1 but also by the highly related paired-type homeobox protein Bix.1 (Tada et al., 1998) (data not shown).

Having established that Mix.1-Engrailed repressor fusions act as transcriptional repressors and inhibit the function of *Mix.1* in vitro, we tested their effects on *Xbra* and *goosecoid* expression during normal development. Embryos were injected at the 4-cell stage with pCMV-*Mix.1-En$^R$* or *Mix.1-En$^R$* RNA and analysed for *goosecoid* or *Xbra* expression by in situ hybridisation at mid to late gastrula stages. Injection of both constructs leads to an upregulation of *Xbra* (Fig. 5D and data not shown) and a suppression of *goosecoid* in a cell-autonomous fashion (Fig. 5F,G). The upregulation of *Xbra* was transient, and undetectable by stage 13. These observations are consistent with the suggestion that *Mix.1* regulates expression of *Xbra* during normal development, and that this regulation occurs through activation of *goosecoid* and perhaps other transcriptional repressors.

We have also asked whether proteins such as *Mix.1* and *goosecoid* participate in the repression of *Xbra* mediated by high doses of activin by using cycloheximide to block their translation. This treatment resulted in expression of *Xbra* even at high activin concentrations (not shown).

## Interference with Mix.1 function causes deficiencies in anterior structures and in endodermal differentiation

Use of an antisense *goosecoid* construct and *gscVP16* confirms

that goosecoid activity is required for anterior patterning during *Xenopus* development (Fig. 2E-J). What is the role of *Mix.1*? Embryos injected dorsally at the 4-cell stage with pCMV-*1.xiM*, or RNA encoding antisense *Mix.1*, develop with dorsoanterior deformities, ranging from mild cyclopia to complete loss of head (Fig. 7). Most of these embryos also have abnormal gut morphology and defective heart formation, as judged by in situ hybridisation using the heart-specific marker *XMLC2* (Fig. 7A-C). However, notochord was present in all specimens, indicating that these embryos are posteriorised rather than ventralised (Fig. 7F-H).

Embryos injected on their dorsal sides with RNA encoding Mix.1HD-En$^R$ or Mix.1-En$^R$ displayed similar but distinct phenotypes, with both constructs causing a reduction in dorsoanterior structures. We concentrate here on results obtained with *Mix.1-En$^R$*, since it is likely to be more specific (see Discussion). Certainly, the phenotypes of embryos injected with RNA encoding Mix.1-En$^R$ are indistinguishable from those obtained with antisense *Mix.1*.

Injection of *Mix.1-En$^R$* RNA causes a reduction in anterior structures, varying from a slight decrease in head size and cement gland to complete loss of head. Intermediate phenotypes include cyclopia, together with a greatly reduced cement gland. There are in addition defects in

posterior endoderm, including a reduction of gut size and inhibition of normal gut coiling (Fig. 7I-K), a phenotype also observed with injection of antisense *Mix.1* RNA. Injection of *1.xiM* RNA into the vegetal pole region of the embryo often results in deformities in the gut region, while injections in the dorsoequatorial region usually cause a combined head and gut phenotype. As with antisense *Mix.1*, injection of *Mix.1-En$^R$* RNA frequently interferes with heart formation, as revealed by in situ hybridisation using a probe specific for *XMLC2*.
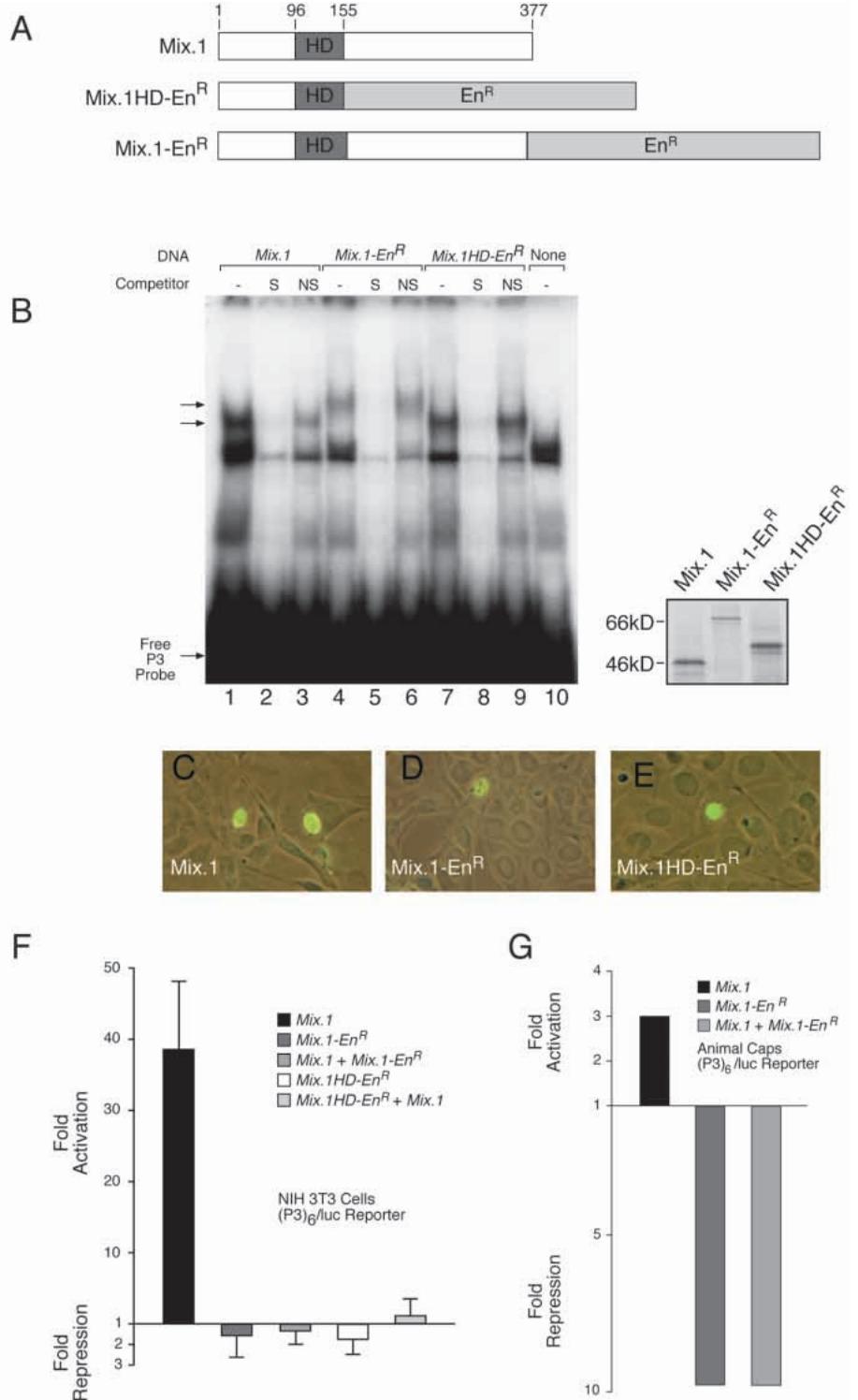


**Fig. 6.** Mix.1HD-En$^R$ and Mix.1-En$^R$ bind DNA and behave as transcriptional repressors that interfere with the activity of wild-type Mix.1 in NIH3T3 cells and in animal caps. (A) The engrailed repressor domain was added to the Mix.1 homeodomain (creating Mix.1HD-En$^R$) or to the full-length *Mix.1* open reading frame (creating Mix.1-En$^R$). (B) Mix.1, Mix.1-En$^R$ and Mix.1HD-En$^R$ bind the palindromic P3 site. Arrows indicate specific complexes; upper arrow at left refers to Mix.1-En$^R$ and the lower arrow refers to Mix.1 and Mix.1HD-En$^R$. S, specific competitor (unlabelled probe); NS, non-specific competitor. Inset shows polyacrylamide gel electrophoresis of translated proteins. (C-E) Mix.1 (C), Mix.1HD-En$^R$ (D) and Mix.1-En$^R$ (E) are nuclear proteins. (F) Mix.1HD-En$^R$ and Mix.1-En$^R$ behave as transcriptional repressors that interfere with the ability of Mix.1 to activate (P3)$_6$/luc in NIH3T3 cells. These experiments were carried out four times. (G) Mix.1HD-En$^R$ and Mix.1-En$^R$ interfere with the ability of Mix.1 to activate (P3)$_6$/luc in animal caps. Embryos received injections of 100 pg interfering *Mix.1* constructs, 10 pg CMV-*Mix.1*, 20 pg of (P3)$_6$/luc and 10 pg of RL/TK reference plasmid). This experiment was performed twice, with similar results each time.
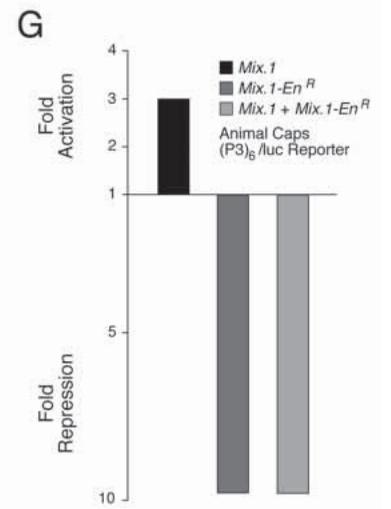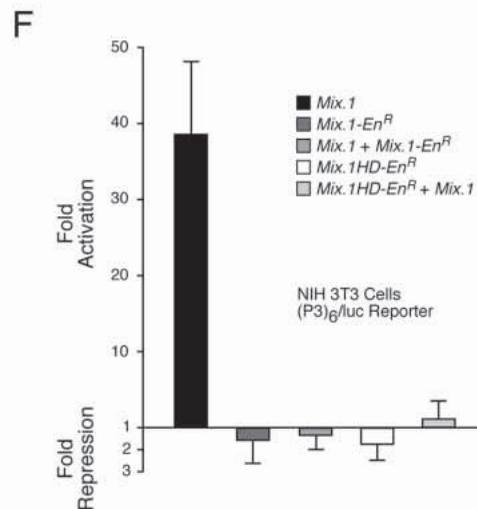
**Fig. 7.** Interference with *Mix.1* function causes defects in head, heart and gut development. (A-C) Examples of embryos injected with the indicated expression constructs were analysed by in situ hybridisation using a probe specific for the heart marker *XMLC2*. In a typical experiment, 63% of embryos injected with pCMV-*Mix.1-En$^R$* lack a heart (A; *n*=27), as do 50% of those injected with pCMV-*1.xiM*, (B; *n*=36). All embryos injected with empty vector pcDNA3 show XMLC2 staining (C; *n*=12). Note reduction in head development in A and B. (D) Embryos injected with 100 pg RNA encoding Mix.1-En$^R$ RNA or antisense Mix.1 RNA (not shown) have dorsoanterior defects, but form notochord (not shown) and skeletal muscle, as revealed by staining with monoclonal antibody 12/101 (D). (E) Uninjected embryo is the same as that shown in Fig. 2H because the experiment was carried out at the same time. (F-H) Embryos injected with the indicated expression constructs develop defects in gut and in anterior structures but do form notochord, as revealed by



staining with monoclonal antibody MZ15 (93%; *n*=29). (I-K) Embryos injected with 100 pg antisense *Mix.1* RNA or RNA encoding Mix.1-En$^R$ (not shown) show reduced gut size and abnormal gut coiling. Note that vegetal injections tend to result in gut defects and largely normal head development (I), whereas dorsal injections affect both head and gut development (J). Overall, dorsoanterior defects were observed in 48% of *Mix.1-En$^R$*-injected embryos (*n*=186) and in 57% of *1.xiM*-injected embryos (*n*=81).
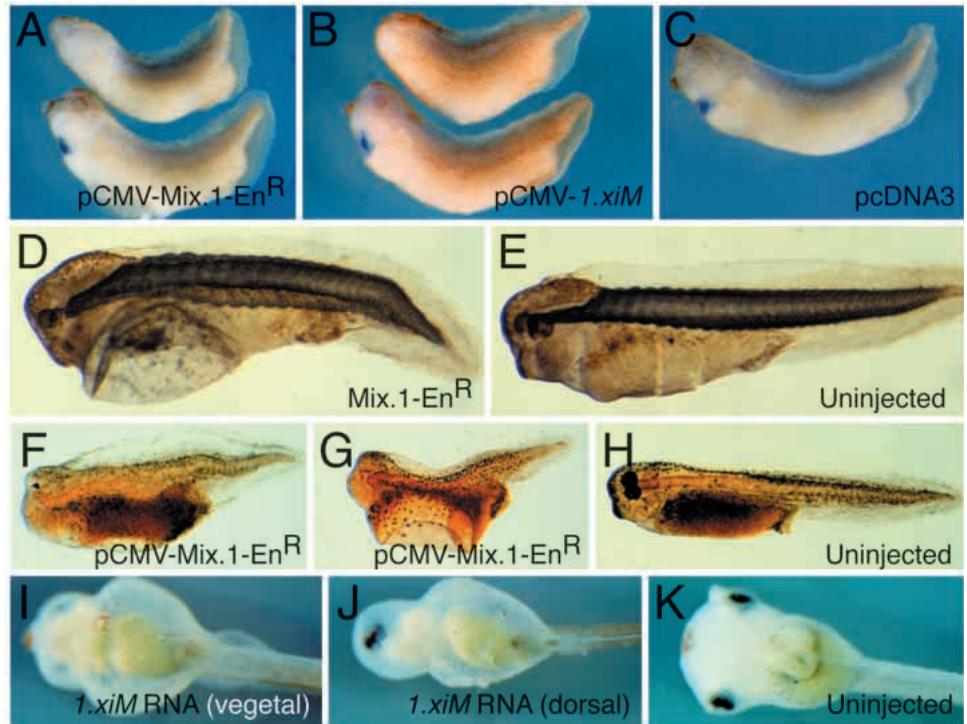
Since Mix.1-En$^R$ interferes with the formation of dorsoanterior endodermal tissues, we next asked whether wild-type *Mix.1* is able to induce early endodermal markers in animal cap explants. While *Mix.1* alone induces only very weak but reproducible expression of *XSox17α* in animal caps, this effect is greatly increased, in a synergistic manner, by co-injection of *goosecoid* RNA (Fig. 8). We also observe that *Mix.1-En$^R$* causes a downregulation of *XSox17α* expression in whole embryos (not shown).

## Mix.1-En$^R$ and M11

The results presented in this paper, like those of Lemaire et al. (1998) suggest that *Mix.1* plays a role in development of the endoderm, a conclusion that contrasts with previous work indicating that the gene is required for differentiation of ventral mesoderm (Mead et al., 1996). The interfering *Mix.1* construct used by Mead and colleagues (designated M11) introduces a proline between helices two and three of the homeodomain, a mutation that is thought to interfere with DNA binding (Mead et al., 1996). We compared the effects of Mix.1-En$^R$ and M11 by injecting RNA encoding the two proteins into *Xenopus* embryos and dissecting animal caps at the mid-blastula stage. Animal pole explants injected with RNA encoding M11 form cement glands (Fig. 9; see Lemaire et al., 1998) and express the neural marker N-CAM (not shown), whereas those injected with *Mix.1-En$^R$* are indistinguishable from uninjected controls (Fig. 9).

The induction of cement gland and N-CAM expression by M11 suggests that the effects of this reagent are not limited to *Mix.1*, because *Mix.1* is not expressed, to detectable levels, in animal pole tissue (Rosa, 1989). This is discussed below.

## DISCUSSION

This work addresses the roles of *goosecoid* and *Mix.1* in the control of *Xbra* expression and in germ layer specification in *Xenopus*. Our approach has been to impair the functions of *goosecoid* and *Mix.1*. In doing so, in an effort to ensure specificity, we have used two different approaches (antisense RNA as well as dominant-interfering constructs) and have considered our results significant only if the two methods give similar results. In order to achieve maximum specificity, our dominant-interfering constructs contain the entire open reading frames of *goosecoid* or *Mix.1* rather than just the homeodomains. The homeodomain binds particular DNA sequences, but this is not sufficient to account for specificity of action in vivo, which is further refined by protein-protein interactions (Mann and Affolter, 1998). This consideration is particularly important in the case of Mix.1, which has recently been shown to be the founder member of a subfamily of at least six homeodomain-containing proteins with overlapping expression patterns and activities (Ecochard et al., 1998; Henry and Melton, 1998; Rosa, 1989; Tada et al., 1998; Vize, 1996). The dominant-interfering constructs were tested by characterising their DNA binding and transcriptional activities both in *Xenopus* and in a heterologous system and on a variety of promoters. These experiments have provided the first direct evidence that Mix.1 acts as a transcription activator and that Mix.1-En$^R$ functions as an active repressor.

Consistent with previous work demonstrating that *goosecoid* and *Mix.1* suppress expression of *Xbra* (Artinger et al., 1997; Latinkic et al., 1997), we find that inhibition of the function of
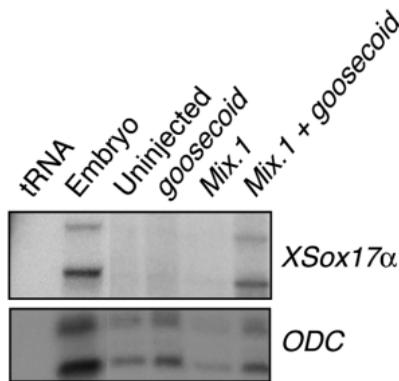
**Fig. 8.** *Mix.1* and *goosecoid* act synergistically to induce expression of *XSox17α* in animal caps. *Xenopus* embryos were injected with the indicated combinations of RNAs (200 pg *goosecoid*, 200 pg *Mix.1* or 100 pg *goosecoid* together with 100 pg *Mix.1*), animal pole regions were dissected at mid-blastula stage 8, and expression of *XSox17α* was analysed by RNAase protection at the equivalent of stage 10.5. This experiment has been carried out three times.

these genes during normal development leads to ectopic expression of *Xbra*. This effect was transient, indicating that simple de-repression is not sufficient to cause stable activation of *Xbra*; rather, continued expression of the gene must require region-specific activation signals. The transient activation of *Xbra* was not able to induce ectopic tail formation, as is seen following the more stable activation of *Xbra* during gastrula stages that is obtained using hormone-inducible constructs (Tada et al., 1997). We note that activation of *Xbra* was not observed in a recent study which also investigated the consequences of inhibiting *Mix.1* function in early development (Lemaire et al., 1998). This discrepancy may arise from the transient nature of ectopic *Xbra* activation, which makes it difficult to detect. Consistent with our observations, however, fusion of the *Mix.1*-like gene *Mixer* with the engrailed repressor domain proved to cause 'higher and less concentrated' expression of *Xbra* (Henry and Melton, 1998). Together, our results suggest that both *goosecoid* and *Mix.1* play a role in the regulation of *Xbra* expression during normal development.

## Comparing the functions of *goosecoid* and *Mix.1*

Our study focuses on the roles of *goosecoid* and *Mix.1* in regulating expression of *Xbra*, but the results also address the roles of the two genes in the development of the whole embryo. Ours is not the first attempt to study the functions of these two homeobox-containing genes. For *goosecoid*, as mentioned in Results, Steinbeisser et al. (1995) have already used an antisense approach, and Ferreiro et al. (1998) have employed two 'antimorphic' constructs. The first of these 'antimorphs' uses an approach similar to our *gscVP16*, but the construct differs in that it lacks the N-terminal 113 amino acids of goosecoid and uses the entire VP16 activation domain. The second construct, and the main focus of the study, is a Myc-tagged version of *goosecoid* (MT*gsc*) which, surprisingly, acts as a

powerful transcription activator (Ferreiro et al., 1998). The effects of MT*gsc* may, therefore, go beyond preventing goosecoid-mediated repression by inappropriately activating the expression of *goosecoid* target genes.

The phenotypes obtained in the three studies are broadly similar in that all display loss of head, but they differ in significant details. In particular, embryos obtained following expression of MT*gsc* lack a notochord and are described by Ferreiro et al. (1998) as ventralised. By contrast, notochord formation is normal in the embryos obtained in our study and in that of Steinbeisser et al. (1995) and are best described as posteriorised. These results show that *goosecoid* function is required in dorsoanterior mesendoderm and not in dorsal mesoderm.

The function of *Mix.1* has been addressed by Mead et al. (1996), using the M11 construct in which a proline is inserted between helices 2 and 3 of the homeodomain, and more recently by Lemaire et al. (1998), who fuse the engrailed repressor construct to the Mix.1 homeodomain. These papers differ quite dramatically in their conclusions, with Mead et al. (1996) suggesting that *Mix.1* is required for ventral mesoderm formation and Lemaire et al. (1998) arguing that it is needed for head development and endoderm formation. Our own data using an antisense approach and an engrailed repressor construct that includes the entire *Mix.1* open reading frame agree with Lemaire and colleagues. Like these authors, we found that M11 induces cement gland formation in animal caps. Therefore, it may be interfering with the functions of other homeobox-containing genes such as *Xvent-1*, *Xvent-2* (Gawantka et al., 1995; Ladher et al., 1996) and *msx1* (Suzuki et al., 1997). Our results do differ in one respect, however, from those of Lemaire et al. (1998), because we see no expansion of mesodermal tissues following interference with *Mix.1* function.

We note that, although interference with *Mix.1* function affects anterior development, posterior and ventral structures appear normal. Thus, although *Mix.1* is expressed in ventral regions of the vegetal pole, it appears not to be required there.

The effects of *Mix.1* on *Xbra* and on dorsoanterior development may be mediated, at least in part, through its ability to amplify or maintain expression of *goosecoid* in anterior endodermal tissue. Thus, the two genes are transiently co-expressed in dorsoanterior endoderm at the early gastrula stage (Fig. 3B,C), *Mix.1* can induce expression of *goosecoid* in animal caps (Fig. 3D) through direct binding to the *goosecoid* promoter (Fig. 4), and interference with *Mix.1* function causes downregulation of *goosecoid* expression (Fig. 5F,G). These results support the proposed indirect mode of



**Fig. 9.** *M11*, but not *Mix.1-En^R*, causes cement gland formation in *Xenopus* animal caps. Animal caps were derived from uninjected embryos (A), or embryos injected with *M11* RNA (B) or RNA encoding Mix.1-En^R (C). Caps were cultured to the equivalent of stage 33 and photographed. Only caps injected with M11 RNA formed cement glands (C). This experiment has been carried out three times.

action of *Mix.1*. The effects of pCMV-*Mix.1-En^R* on *Xbra* expression provide further support for this model: if Mix.1 were acting directly on Xbra transcription, then Mix.1-En^R, an active repressor, would have been expected to downregulate *Xbra*, not activate it.

The phenotypes resulting from interference with *Mix.1* and *goosecoid* function may be due to interference with normal gastrulation movements. Overexpression of wild-type *goosecoid*, for example, is known to cause inappropriate anterior migration of mesodermal cells (Niehrs et al., 1993). Furthermore, *Mix.1* has recently been shown to cause adhesion of animal pole cells and *goosecoid* acts synergistically with *Mix.1* to promote this effect (Wacker et al., 1998). This synergism is reminiscent of the effects of the two genes in inducing *Xsox17α* (Fig. 8) and is consistent with their ability to form heterodimers on the P3C site (Wilson et al., 1993).

The function of *Mix.1* appears not to be restricted to the prospective head because embryos injected with interfering *Mix.1* constructs also have defects in heart and gut development (Fig. 7). Unless Mix.1 protein is unusually long-lived, it is likely that *Mix.1* is involved in the earliest steps of endoderm formation, because the gene is not expressed after the end of gastrulation (Henry and Melton, 1998; Rosa, 1989). The effects of interference with *Mix.1* function on heart development may be indirect; heart development requires an inductive signal from the endoderm (Nascone and Mercola, 1995) and downregulation of *Mix.1* function may affect this process.

Even though *Mix.1* appears to be required for proper formation of the gut, simple misexpression of the gene is not sufficient to specify endoderm in animal pole tissue (this work and Lemaire et al., 1998). Rather, in combination with *Siamois*, *Mix.1* induces expression of *cerberus* (Lemaire et al., 1998), which is expressed in anterior endoderm (Bouwmeester et al., 1996), whereas in combination with *goosecoid* it induces *XSox17α* (Hudson et al., 1997), a general endoderm marker (Fig. 8).

Together, our results provide evidence that *Mix.1* and *goosecoid* promote endodermal differentiation while suppressing mesoderm, and are required for dorsoanterior development of the *Xenopus* embryo.

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