Development 125, 2371-2380 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV2293

A role for the vegetally expressed *Xenopus* gene *Mix.1* in endoderm formation and in the restriction of mesoderm to the marginal zone

Patrick Lemaire*,‡, Sébastien Darras*, Danielle Caillol* and Laurent Kodjabachian†

Laboratoire de Génétique et Physiologie du Développement, Institut de Biologie du Développement de Marseille, CNRS-INSERM-Université de la Méditérannée-AP de Marseille, Campus de Luminy, Case 907, F-13288 Marseille Cedex 9, France

*These three authors contributed equally to this work

†Present address: NIH, NICHHD, Blg 6B, Rm 4B/420, 9000 Rockville Pike, Bethesda, MD, 20892-2790, USA

‡Author for correspondence (E-mail: lemaire@lgpd.univ-mrs.fr)

Accepted 20 April; published on WWW 3 June 1998

SUMMARY

We have studied the role of the activin immediate-early response gene *Mix.1* in mesoderm and endoderm formation. In early gastrulae, *Mix.1* is expressed throughout the vegetal hemisphere, including marginal-zone cells expressing the trunk mesodermal marker *Xbra*. During gastrulation, the expression domains of *Xbra* and *Mix.1* become progressively exclusive as a result of the establishment of a negative regulatory loop between these two genes.

This mutual repression is important for the specification of the embryonic body plan as ectopic expression of *Mix.1* in the *Xbra* domain suppresses mesoderm differentiation. The same effect was obtained by overexpressing VP16Mix.1, a fusion protein comprising the strong activator domain of viral VP16 and the homeodomain of Mix.1, suggesting that Mix.1 acts as a transcriptional activator.

***Mix.1* also has a role in endoderm formation. It cooperates with the dorsal vegetal homeobox gene *Siamois* to activate the endodermal markers *edd*, *Xlhbox8* and *cerberus* in animal caps. Conversely, vegetal overexpression of *enRMix.1*, an antimorphic *Mix.1* mutant, leads to a loss of endoderm differentiation. Finally, by targeting *enRMix.1* expression to the anterior endoderm, we could test the role of this tissue during embryogenesis and show that it is required for head formation.**

Key words: *Mix.1*, *Xenopus*, Endoderm, Mesoderm, *Siamois*, Organiser, Head

INTRODUCTION

In early *Xenopus* gastrulae, the three germ layers are arranged along the animal-vegetal axis. The presumptive endoderm is located in the vegetal-most cells, the presumptive mesoderm is found around the equator, while the ectoderm is derived from animal cells. Mesoderm is induced in the equatorial region of the embryo by FGF or activin-like signals emitted by vegetal cells (reviewed in Slack, 1994). In addition to their role in mesoderm induction, these two signalling pathways, which are also active in vegetal cells, may be involved in endoderm formation (Labonne and Whitman, 1997; Watabe et al., 1995). Indeed, treatment of ectodermal cells with activin leads to the formation of endoderm as well as mesoderm, while inhibition of activin or FGF signalling in vegetal cells leads to the loss of anterior endodermal markers (Henry et al., 1996). How a cell decides to adopt a mesodermal or endodermal fate in response to activin-like or FGF factors is still a major issue which will greatly benefit from a better understanding of the role and regulation of genes expressed in presumptive endoderm or mesoderm around the onset of gastrulation.

Xbra, the homologue of the mouse *Brachyury* (*T*) gene, encodes a T-box transcription factor expressed in a ring of equatorial cells fated to form trunk and tail mesoderm (reviewed in Papaioannou and Silver, 1998). Loss of function of *brachyury* leads to a severe reduction in posterior mesoderm derivatives (Papaioannou and Silver, 1998). Other transcription factors mark a more restricted subset of *Xenopus* mesodermal cells. For example, *goosecoid* marks head mesoderm, while *msx.1*, *Xpo* and *Xvent-1* mark ventrolateral mesoderm (reviewed in Stennard et al., 1997).

Fewer early genes have shown to be involved in endoderm formation. VegT/Antipodean/Xombi/Brat (referred to here as VegT) encodes a T-box transcription factor whose transcripts are first detected maternally at the vegetal pole of *Xenopus* eggs and which is later expressed zygotically in the vegetal and equatorial regions of early gastrulae (reviewed in Stennard et al., 1997). Overexpression of this gene in ectoderm leads to both mesoderm and endoderm differentiation, and *VegT* function is required for mesoderm formation (Stennard et al., 1997). In contrast to VegT, Sox17α and Sox17β, two members of the HMG-box family of transcription factors, are specifically expressed in the presumptive endoderm from the mid-gastrula stage onwards. Overexpression of *Sox17* leads to the conversion of ectoderm into endoderm but not mesoderm, while overexpression of a dominant-negative mutant converts endoderm into mesoderm (Hudson et al., 1997).

Mix.1, and its close relative *mix.2*, are zygotically

expressed in the vegetal hemisphere of late blastulae and early gastrulae and code for proteins containing a paired-like homeodomain (Rosa, 1989; Vize, 1996). The results presented here indicate that *Mix.1*, whose expression becomes progressively restricted to the endoderm, is a potent suppressor of mesoderm formation and an important cofactor in endoderm differentiation.

MATERIALS AND METHODS

Construction of expression constructs for *Mix.1*, *enRMix.1*, *VP16Mix.1* and *VP16Siamois*

The pBluescript RN3 expression vector (Lemaire et al., 1995) was used for all constructs. pBSRN3Mix.1 was obtained by cloning the *Mix.1* 2A7 cDNA (Rosa, 1989) into the *Eco*RI site of pBluescript RN3 in sense orientation. pBSRN3VP16Mix.1 was constructed as follows: the region encoding aminoacids 413-490 from VP16 (GenBank entry HEHSV165), preceded by a methionine and flanked by *Eco*RI and *Bam*HI cloning sites, was PCR-amplified with the oligonucleotides VP16-F (5′-cgg aat tca act ttg gcc ATG GCC CCC CCG ACC GAT GTC AGC-3′; *Eco*RI site underlined; *VP16* sequences in capitals) and VP16-R (5′-gga tcc cag agc aga ttt ctc tgg CCC ACC GTA CTC GTC AAT-3′, *Bam*HI site underlined, the *VP16* sequences in capitals). The region encoding amino acids 90-163 of Mix.1 followed by a stop codon and flanked by *Bam*HI and *Not*I cloning sites was amplified with the oligonucleotides Mix.1EV-F (5′-cca gag aaa tct gct ctg gga tCC TCT TTG GTC CCA GCA T-3′, *Bam*HI site underlined, *Mix.1* sequences in capitals) and Mix.1EV-R (5′-ata aga atg cgg ccg cTA AAG AAT GGG CTT GGT GGC TT-3′; *Not*I site underlined, *Mix.1* sequences in capitals). The two amplified fragments were mixed and reamplified with the two oligos VP16-F and Mix.1EV-R. Because of the partially complementary sequences of VP16-R and Mix.1EV-F, a 519 bp fragment was amplified, coding for a fusion protein between the activator domain of VP16 and the homeodomain of Mix.1 flanked by six aminoacids on either side. This fragment was cloned into the *Eco*RI-*Not*I sites of pBluescriptRN3 to generate pBSRN3VP16Mix.1. pBSRN3VP16Sia was generated in the same way except that the homeodomain of Siamois flanked by 6 aminoacids on either side was PCR-amplified with the two oligonucleotides SiaEV-F (5′-cca gag aaa tct gct ctg gga tCC TCT CCA GCC ACC AGT A-3′, *Bam*HI site underlined; Sia sequences in capitals) and SiaEV-R (5′-ata aga atg cgg ccg cTA CTG GGG AGA GTG GAA AGT GG-3′, *Not*I site underlined; Sia sequences in capitals) as indicated in Darras et al. (1997). To construct pBSRN3 enRMix.1, the VP16 sequences (*Eco*RI-*Bam*HI fragment) of pBSRN3 VP16Mix.1 were replaced by an *Eco*RI-*Bam*HI fragment of PBSRN3 enRSia (Darras et al., 1997), containing aminoacids 1-298 from *Drosophila* Engrailed.

Embryo injections and treatments

Embryos were in vitro fertilised, dejellied, cultivated and injected with synthetic capped mRNA as in Darras et al. (1997). Synthetic *Siamois* mRNA was prepared from pBSRN3 XSia-ORF as indicated in Darras et al. (1997). *Mix.1* mRNA was synthesized using T3 RNA polymerase from pBSRN3Mix.1, linearised with *Sfi*I. *Mix.2* RNA was synthesized using SP6 RNA polymerase from a *Bam*HI-linearised pSp64T construct. VP16Mix.1, VP16Sia, enRSia and EnRMix.1 were synthesised using T3 RNA polymerase from *Sfi*I linearised pBSRN3VP16Mix.1, pBSRN3VP16Sia, pBSRN3enRMix.1 and pBSRN3enRSia (Darras et al., 1997), respectively. Animal cap explants were cut at stage 8.5-9 and cultured as in Darras et al. (1997). We found that the quality of the caps affected their response to *Mix.1*. *Mix.1*-injected animal caps derived from very good embryo batches never expressed Xlhbox8 or IFABP (Fig. 6), while experiments carried out with embryos of lesser quality (thick animal caps) occasionally led to the activation of these markers (not shown).

In situ hybridisations and immunostaining

In situ hybridisations were carried out as described (whole mount: Gawantka et al., 1995; sectioned embryos: Darras et al., 1997). *Xbra*, *edd*, *cerberus* and *α-T4 globin* antisense probes were synthesized as in Darras et al. (1997), Sasai et al. (1996), Bouwmeester et al. (1996) and Walmsley et al. (1994), respectively. The *Mix.1* antisense probe was synthesized with T3 RNA polymerase from a pBluescript SK+-based *Mix.1* plasmid (Rosa, 1989) after linearisation with *Hin*dIII. Immunostainings were performed as indicated in Darras et al. (1997).

RT-PCR assays

RT-PCR assays were performed as in Darras et al. (1997) with the following additional primers: *edd* (Forward primer: 5′-CTCGCTCT-GGACAAAACTC-3′; Reverse primer: 5′-GAGGTTGCTGATGGG-AATG-3′); *XlHbox8* (Forward primer 5′-CCTACAGCAACCCCTT-GGTA-3′; Reverse primer: 5′-GGGCTCTTGTGTAGGCTGTC-3′); *IFABP* (Forward primer: 5′-GCCTTTGATGGAACTTGGAA-3′; reverse primer: 5′-CTGTAGGAACCAGGCACCAT-3′), EF1α (Forward primer 5′-AGAATGGACAAACCCGTGAG-3′; Reverse primer 5′-GTGGCAGAATGCAGTCAAGA-3′).

RESULTS

Expression domains of *Mix.1* and *Xbra* during gastrulation

We first compared the expression domains of *Xbra* and *Mix.1* during gastrulation by in situ hybridisation. At stage 10, *Mix.1* is expressed throughout the vegetal hemisphere and largely overlaps with the expression domain of *Xbra* in the marginal zone (Fig. 1A, top panels). The stronger intensity of the *Mix.1* staining in the marginal zone does not reflect a specific enrichment of *Mix.1* transcripts in the cells of this region, as the relative abundance of *FGFR1* (ubiquitous) and *Mix.1* transcripts was similar in dissected marginal and vegetal explants (Fig. 1B).

By stage 10.5, expression of *Mix.1* persists in vegetal cells but *Mix.1* transcripts are absent from the dorsal domain of *Xbra* expression, which corresponds to the presumptive notochord (Fig. 1A bottom panels). *Mix.1* expression, however, is maintained in the deeper, more anterior territories of the organiser (Fig. 1A, arrow). Progressive exclusion of the expression patterns of *Xbra* and *Mix.1* is also apparent on the ventral side. By stage 11, the exclusion of the expression domains of the two genes has become nearly complete (not shown).

A negative regulatory loop between *Xbra* and *Mix.1*

The gradual exclusion of the domains of expression of *Xbra* and *Mix.1* during gastrulation suggested that these two genes may negatively regulate each other.

To test this idea, expression of *Mix.1* was analysed in early gastrulae overexpressing *Xbra* and the lineage tracer NLS-β-galactosidase (Lemaire et al., 1995) in the marginal zone. As shown on Fig. 2A,B, *Mix.1* was downregulated at the site of *Xbra* injection. This repression did not reflect a toxic effect or a general transcriptional repression by *Xbra*, as no repression of the homeobox gene *Xcad3* was observed at the site of injection (not shown). Unlike Rao (1994) and consistent with our results, we find that injection of the same concentration of *Xbra* mRNA in ectoderm led to muscle differentiation without activation of *Mix.1* (not shown). Finally, repression of *Mix.1* is

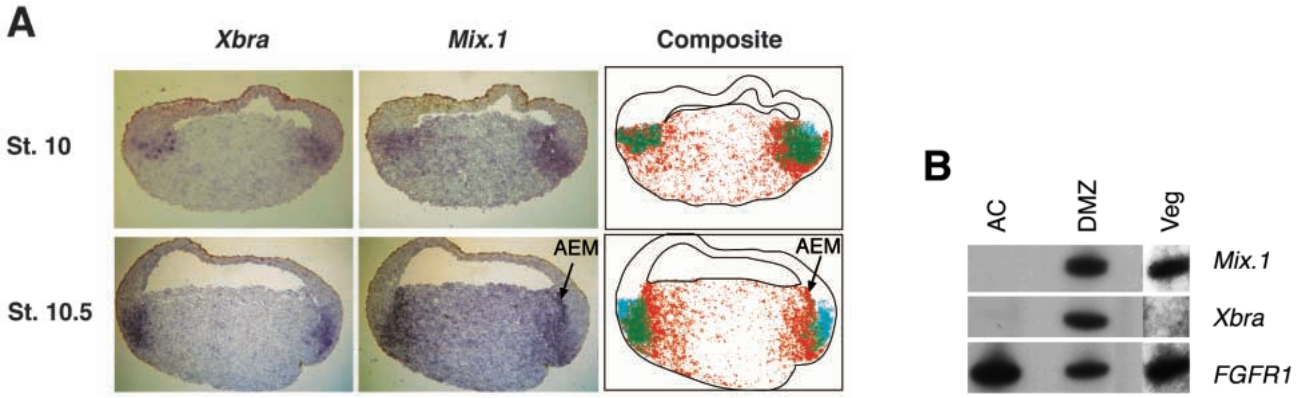

Fig. 1. Expression patterns of *Mix.1* and *Xbra* during gastrulation. (A) In situ hybridisation on sagittal sections through early (stage 10) and mid (stage 10.5) gastrulae using *Xbra* (right) or *Mix.1* (middle) antisense probes. (Right) Computer-generated representations of the expression domains: red, *Mix.1* alone; blue, *Xbra* alone; green, coexpression of *Xbra* and *Mix.1*. Dorsal is to the right. AEM, anterior endomesoderm. (B) RT-PCR analysis of the relative amounts of *Mix.1* transcripts in animal, dorsal marginal and vegetal explants of stage 10 embryos. Because of the low amount of mRNA present in vegetal cells, the left lane was scanned from a longer exposure of the gel. AC, Animal cap; DMZ, Dorsal Marginal Zone explant; Veg, vegetal pole explant. The ubiquitously expressed *FGFR1* gene is used as a loading control.

not a common property of all T-box genes, as ectopic expression of another member of this family, the vegetally expressed *VegT* gene, led to a strong activation of *Mix.1* in the marginal zone (Fig. 2C).

Overexpression of *Mix.1* in the marginal zone led to a complete downregulation of *Xbra* at the early gastrula stage. This repression was obtained when as little as 50 pg of *Mix.1* mRNA were injected in a dorsal, lateral or ventral position (Fig. 2E and not shown) and was restricted to the cells expressing *Mix.1* or their closest neighbours (Fig. 2G). This repression was not a general property of all homeobox genes as equatorial overexpression of the Bmp4-target homeobox gene *msx1* did not lead to a strong repression of *Xbra* (Fig. 2H). Likewise, overexpression of *Bmp4* had no effect on *Xbra* expression (Fig. 2F). Hence suppression of *Xbra* expression is specific to a class of homeodomain proteins to which Mix.1 belongs.

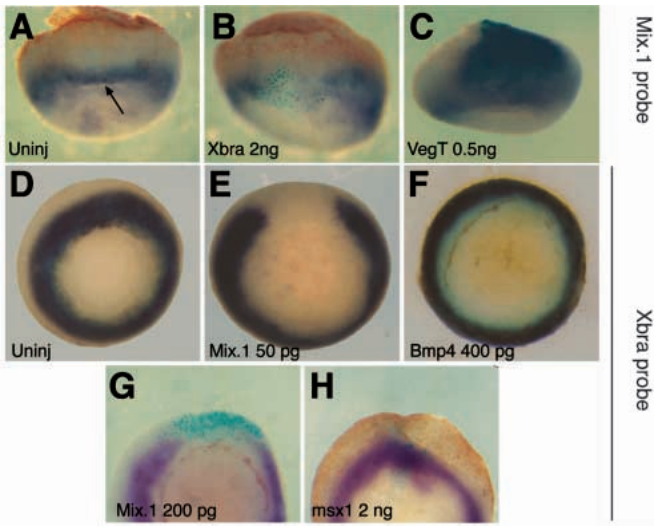

Fig. 2. *Mix.1* and *Xbra* negatively regulate each other. Whole-mount in situ hybridisation with antisense *Mix.1* or *Xbra* probes on stage 10.5 uninjected embryos or sibling embryos injected marginally at the 4-cell stage with NLS-β-galactosidase mRNA and the indicated amount of *Mix.1*, *msx.1*, *VegT*, *Xbra* or *Bmp4* mRNA. (A-C) Dorsal-lateral view. In B, note the absence of the dorsal blastopore lip indicated by an arrow in A; (D-H) vegetal view, dorsal is to the top. The progeny of injected cells is marked by nuclear X-gal staining.

Ectopic expression of *Mix.1* suppresses mesoderm formation

Downregulation of *Xbra* by *Mix.1* suggested that this latter gene may behave as a general suppressor of mesoderm formation. We therefore tested at the mid gastrula stage the effect of *Mix.1* overexpression on: (i) *Xpo* and *Xvent1*, expressed in ventrolateral mesoderm, (ii) *chordin* expressed in dorsal mesoderm (Stennard et al., 1997). Dorsal equatorial injection of *Mix.1* mRNA led to a repression of *chordin* without activation of *Xpo* or *Xvent1* (Fig. 3C,D,G). Conversely, ventral expression of *Mix.1* repressed *Xpo* and *Xvent1* (Fig. 3B,D,E) without activating *chordin* (not shown). This repression of mesodermal markers did not reflect a general transcriptional repression or a toxicity of the injected mRNA as the expression of two endodermal markers was either unaffected or enhanced in *Mix.1*-injected cells (see below).

By the early tadpole stage, embryos injected with *Mix.1* mRNA in a ventral-equatorial position showed relatively normal axial structures, though some defects in the tail and proctodeum were observed (Fig. 4A, arrow, Mead et al., 1996). To test for the presence of ventral mesoderm in these injected embryos, expression of the blood marker α-T4 globin was assayed by whole-mount in situ hybridisation. While normal embryos displayed the classical V-shaped globin expression domain (Fig. 4B), this domain was greatly reduced in *Mix.1*-injected embryos (Fig. 4C,D). RT-PCR analysis of the expression of α-T4 globin in control or Mix.1-injected isolated ventral marginal zone explants supported these results (not shown). We conclude that *Mix.1* overexpression in the ventral marginal zone suppresses terminal differentiation of ventral mesoderm. Similar results were obtained with *Mix.2* (not shown).

Injection of *Mix.1* or *Mix.2* mRNA in the equatorial region

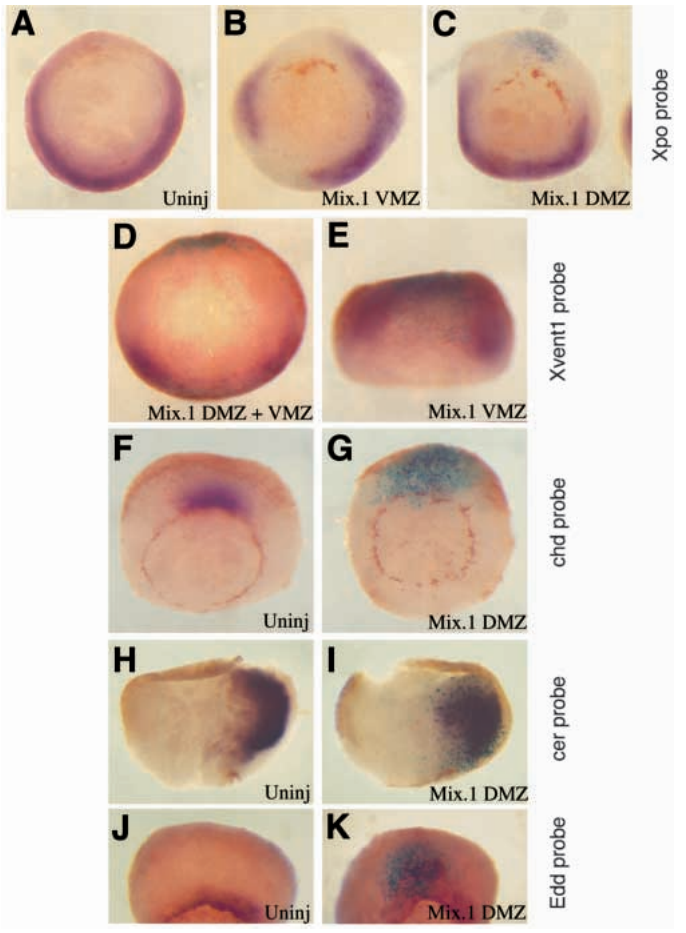

Fig. 3. Effect of the overexpression of *Mix.1* on early mesodermal and endodermal markers. Embryos injected at the 4-cell stage in the ventral marginal zone (VMZ) or dorsal marginal zone (DMZ), with *NLS-β-Gal* and *Mix.1* (50-100 pg) mRNA, were fixed at stage 11 and processed for whole-mount in situ hybridisation with the indicated probes. The position of the progeny of the indicated cells is marked by nuclear X-Gal staining. (A-D,F,G) Vegetal view of stained embryos; (E,J,K) lateral view; (H,I) lateral view of embryos bissected along the sagittal plane prior to the hybridisation step to optimise the detection of *cerberus* transcripts. Dorsal is to the right.

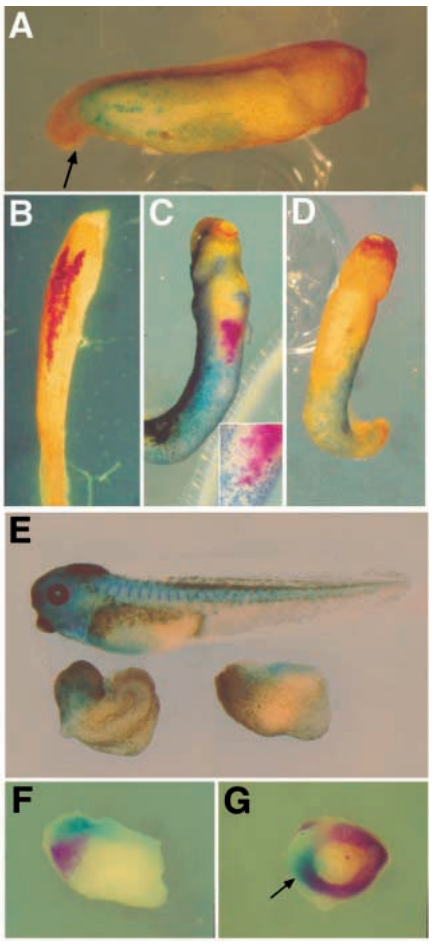

Fig. 4. Mix.1 represses terminal mesodermal differentiation. (A) Lateral view of a stage 32 embryo that had been injected at the 4-cell stage in the ventral marginal zone with 200 pg of *Mix.1* mRNA. The arrow indicates posterior defect. (B-D) Ventral view of whole-mount in situ hybridisation analysis of the expression of the blood marker α-T4 globin in stage 33 control embryos (B), or in embryos injected ventrally at the 4-cell stage with 50 pg (C) or 200 pg (D) of *Mix.1* mRNA per blastomere. Note that in C, cells expressing globin do not express the lineage tracer NLS-β gal (inset). (E) Embryos injected at the 4-cell stage in the dorsal marginal zone with mRNA coding for nuclear β-galactosidase (NLS-β-gal) alone (top embryo) or with 100 pg of *Mix.1* mRNA (bottom embryos). The position of injected cells is indicated by the position of the X-gal-stained nuclei. (F,G) Whole-mount in situ hybridisation with a α-T4 globin probe on stage 33 embryos injected dorsally at the 4-cell stage with mRNA for *Mix.1* (200 pg/blastomere, (F) or *Bmp4* (400 pg, G). In G, the arrow points to the overlap between the lineage tracer and the expanded expression domain of α-T4 globin.

of both dorsal blastomeres at the 4-cell stage severely disrupted the embryonic body plan (Fig. 4E; Table 1). The blastopore of embryos that had been injected with 100 pg of *Mix.1* mRNA did not close, leading to the phenotypes shown in Fig. 4E. A majority of embryos had reduced muscle and notochord structures to which *Mix.1*-expressing cells did not contribute (Fig. 4E, bottom left and data not shown). In a minority of injected embryos (Fig. 4E, bottom right embryo and Table 1), no axial structures formed and muscle and notochord were absent (not shown). Increasing the amount of injected *Mix.1* mRNA led to an increase in the proportion of embryos showing this extreme phenotype (Table 1). Mead et al. (1996) obtained similar results which they attributed to the ventralisation of dorsal mesoderm by *Mix.1*. However, in embryos injected dorsally with *Mix.1* mRNA, the domain of expression of the ventral mesodermal marker α–T4 globin was not expanded to include the cells that received *Mix.1* mRNA (Fig. 4F). In contrast, ventralisation of embryos by dorsal overexpression of *Bmp4* led to an increase in the size of the domain of expression of α-T4 globin, which included cells that had received *Bmp4* mRNA (Fig. 4G).

We conclude that overexpression of *Mix.1* in the marginal zone causes the repression of both dorsal and ventral mesoderm differentiation. The progressive exclusion of *Mix.1* transcripts from the expression domain of *Xbra* is therefore required for mesoderm to form in the marginal zone.

Table 1. Effect of the expression of *Mix.1* and *Mix.2* in the dorsal marginal zone

Gene overexpressed	Amount of mRNA injected per dorsal blastomere	*n*	Normal embryos (%)	Reduced axial structures (%)	Axis-less embryos (%)
mix.1	100 pg	75	24	68	8
	200 pg	50	4	52	44
	500 pg	21	9.5	28.5	62
mix.2	500 pg	5	0	0	100

Embryos were scored for axial deficiencies at the talbud stage. Reduction of the axial structures was always accompanied by a failure of blastopore closure.

Mix.1 acts as a transcriptional activator to suppress mesoderm formation

Mix.1 contains a C-terminal acidic domain, suggesting that this protein may act as a transcriptional activator (Rosa, 1989; Mead et al., 1996). To test this hypothesis, we constructed a mutant protein, VP16Mix.1, in which the homeodomain (HD) and a few flanking amino acids of Mix.1 were fused to the strong activator domain of Herpes virus VP16 (Fig. 5A). This protein was expected to behave as a transcriptional activator recognising the same target genes as *Mix.1*. A potential problem with this approach is that HDs have overlapping DNA-binding specificities and may not be sufficient to determine the specificity of target recognition by homeodomain proteins (Biggin and McGinnis, 1997). To address this issue, we compared the effects of overexpressing VP16Mix.1 and VP16Sia, a second fusion protein in which the VP16 activator domain was fused to the paired-like homeodomain of Siamois. In spite of the high degree of relatedness between the two HDs (65% of conserved aminoacids, including Q50), overexpression of VP16Mix.1 and VP16Sia led to very different phenotypes. While ventral expression of VP16Sia induced a secondary axis, thus behaving as wild-type Siamois (Fig. 5C, and Kessler, 1997), ventral expression of VP16Mix.1, like Mix.1, had little effect on axial patterning (Fig. 5B). Conversely, dorsal overexpression of VP16mix gave a phenotype similar to Mix.1 (Fig. 5D) and the cells expressing VP16Mix.1 did not contribute to the reduced axial structures (not shown). Furthermore, like *Mix.1*, overexpression of *VP16Mix.1* led to the downregulation of *Xbra* and *chordin* (Fig. 5E-H).

These results suggest that Mix.1 behaves as a transcriptional activator and that its homeodomain and a few flanking amino acids are sufficient for the specific recognition of downstream targets.

Effect of ectopic *Mix.1* expression on *endodermin* and *cerberus*

While ectopic expression of *Mix.1* in the marginal zone negatively influences mesoderm differentiation, the vegetal expression of this gene suggested that it plays a role in endoderm differentiation. To test this hypothesis, we studied in mid-gastrulae the effect of *Mix.1* overexpression on *cerberus* (*cer*), expressed in the anterior mesendoderm (Bouwmeester et al., 1996), and endodermin (*edd*), a marker of endoderm and dorsal mesoderm (Sasai et al., 1996).

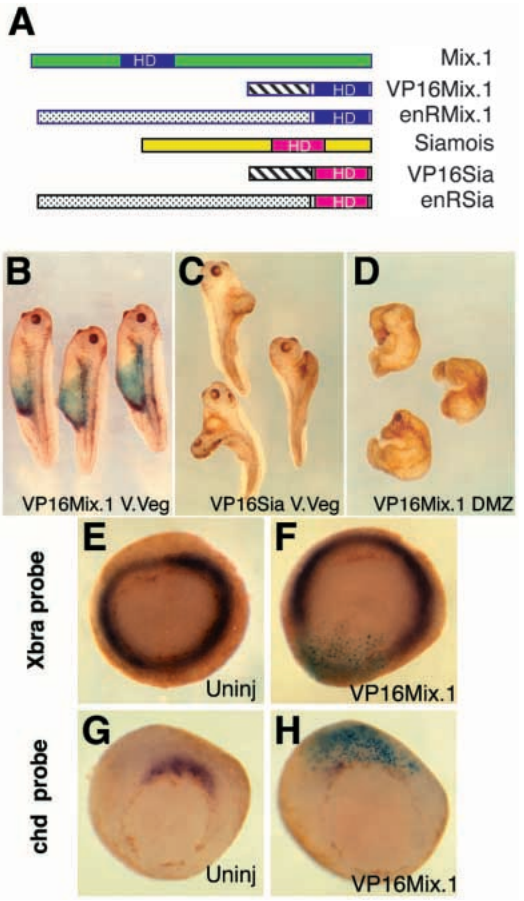

Fig. 5. Mix.1 acts as a transcriptional activator. (A) Structure of the fusion proteins used in Figs 6-8. Blue, Mix.1 homeodomain (HD, aminoacids 96-155); magenta, Siamois HD, aminoacids 142-201); stippled box, activation domain of VP16 (aminoacids 413-490); dotted box, repressor domain of *Drosophila* Engrailed (aminoacids 1-298). The sequences of Mix.1 and Siamois located outside the homeodomain are shown in green and yellow respectively. (B-D) Effect at the tadpole stage of the injection of *VP16Mix.1* (100 pg) and *VP16Sia* (10 pg) mRNA in the dorsal marginal zone (DMZ, D) or in the ventral vegetal region (V. Veg, B,C). Ventral vegetal injection of 100 pg of *VP16Sia* mRNA also induced complete secondary axes (not shown). (E-H) Vegetal views of mid-gastrula embryos injected with *VP16Mix.1* (100 pg) and *NLSβ-gal*. mRNAs and subjected to whole-mount in situ hybridisation with *Xbra* or *chd* probes.

Consistent with the coexpression of these two genes in the anterior endomesoderm (AEM) during normal embryogenesis (Fig. 1), *cerberus* expression was not suppressed by dorsal-vegetal overexpression of *Mix.1* (Fig. 3H,I). Interestingly, overexpression of *Mix.1* was not sufficient to cause ectopic *cer* expression (Figs 3I and 7J).

In contrast, ectopic *edd* expression was detected in the majority of marginal-zone cells that had received *Mix.1* mRNA (Fig. 3J,K). Although *edd* is expressed at the mid-gastrula stage in dorsal mesoderm as well as in endoderm, the mesoderm-suppressing activity of *Mix.1* suggests that, in our assay, ectopic expression of *edd* reflects ectopic endoderm formation.

To test this idea further, we analysed the effect of ectopic animal expression of *Mix.1* on late endodermal markers.

Control and *Mix.1*-expressing animal cap explants were cultured until stage 30 and assayed for the expression of *edd*, which at this stage is a pan-endodermal marker (Sasai et al., 1996). A weak but reproducible activation of *edd* in *Mix.1*-expressing caps was detected by RT-PCR (Fig. 6A) and corresponded to the presence of small foci of cells strongly expressing *edd* (Fig. 6C). However, activation of *edd* was not paralleled by the activation of the posterior endodermal marker *IFABP* (Shi and Hayes, 1994) or the more anterior endodermal marker *Xlhbox8* (pancreas, Wright et al., 1994) (Fig. 6A). Likewise, *Cerberus* was not activated in *Mix.1*-injected caps (Fig. 6B). Like *Mix.1*, *VP16 Mix.1* could induce *edd* but not the other endodermal markers (not shown).

We conclude that, although *Mix.1* ectopically activates *edd* in the marginal zone and the ectoderm, this activation is weak in animal caps, suggesting that expression of *Mix.1* alone is not sufficient to account for the formation of embryonic endoderm. We therefore looked for cofactors that could cooperate with *Mix.1* to promote endoderm formation.

Mix.1 synergises with *Siamois* to convert ectoderm into dorsoanterior endoderm

Siamois codes for a paired-like homeobox protein that can form heterodimers with Mix.1 (Mead et al., 1996) and is coexpressed with *Mix.1* in the dorsal vegetal cells of early gastrulae (Lemaire et al., 1995). To test if the two proteins could cooperate in endoderm formation, animal cap explants expressing *Mix.1*, *Siamois* or both were cultured to the tailbud stages 30-33 and assayed for the expression of markers for endoderm and mesoderm.

Activation of *muscle actin* or *globin* was never detected in injected caps, while a very low level of the notochord marker *collagen II* could be detected in some experiments (Fig. 6A). This expression of *collagen II* probably does not reflect notochord formation as histological analysis of the injected caps did not reveal the presence of vacuolated notochord cells (Fig. 6C). Ectopic ectodermal expression of *Mix.1*, *Siamois* or both thus does not lead to mesoderm formation.

While *Siamois* did not activate *edd* expression in animal caps, coexpression of *Mix.1* and *Siamois* led to a strong *edd* activation in this tissue (Fig. 6A). *Edd*-positive cells formed one or two compact domains containing most of the progeny of the injected cells and segregating from the uninjected ectoderm (Fig. 6C). Activation of *IFABP*, a marker of posterior endoderm also required the presence of both transcripts, and was very weak. In contrast, the dorsal endodermal marker *XlHbox8* was strongly activated by the coexpression of *Sia* and *Mix.1* but not by either gene alone (Fig. 6A). Finally, while neither *Siamois* nor *Mix.1* alone were able to activate *cerberus* in ectoderm, coinjection of both mRNAs led to a clear, though weak, activation of this gene (Fig. 6B).

Siamois has previously been shown to repress Bmp signalling (Carnac et al., 1996). To test whether *Mix.1* could cooperate with Bmp4 antagonists to induce endoderm, animal cap explants expressing *noggin*, *Mix.1* or both were analysed for endodermal differentiation at the tailbud stage. In contrast to a previous report (Sasai et al., 1996), caps injected with *noggin* mRNA alone (12.5-50 pg) formed well-differentiated cement glands but failed to express elevated levels of *edd* (Fig. 6A and not shown). Similar results were obtained following injection of 200-1000 pg of *chordin* mRNA (not shown). In addition to its inability to induce *edd* on its own, *noggin* failed to strongly cooperate with *Mix.1* to activate *edd*, *IFABP* and *Xlhbox8* (Fig. 6A). Hence, cooperation of *Siamois* and *Mix.1* in endoderm formation cannot be accounted for by the repression of the Bmp4 pathway by *Siamois*.

We conclude that *Mix.1* and *Siamois* cooperate to induce dorsoanterior endoderm, a finding consistent with the coexpression of these genes in dorsal vegetal cells. The weak

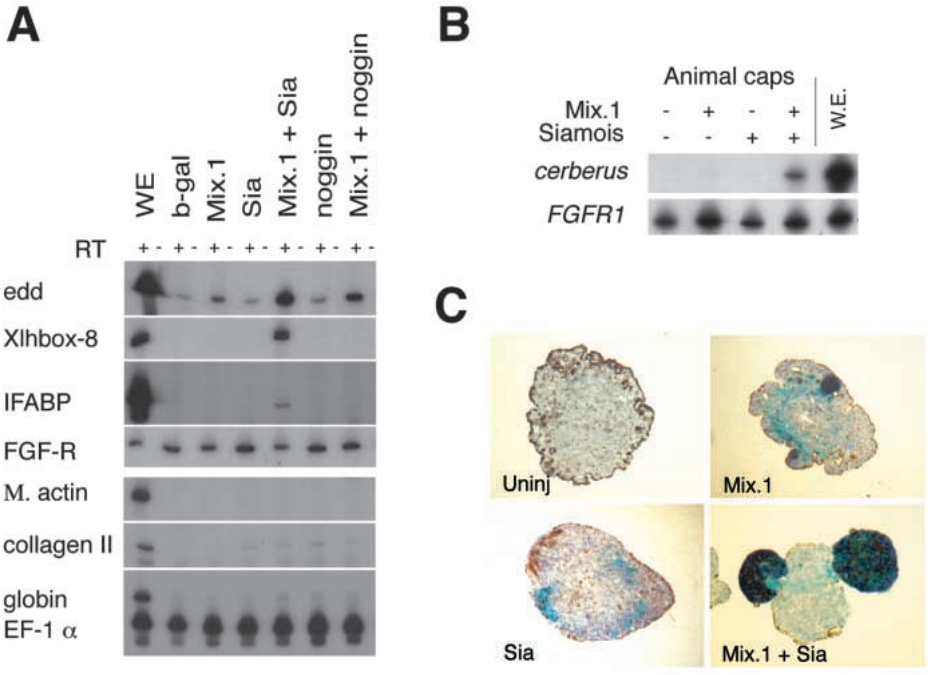

Fig. 6. Mix.1 and Siamois synergise to induce endoderm differentiation in ectoderm. (A) RT-PCR analysis of the expression of *endodermin* (*edd*, 25 cycles), *XlhBox8* (25 cycles) and *IFABP* (29 cycles), and of the mesodermal markers muscle actin (M. actin, 21 cycles), α-T4 globin (21 cycles) and Collagen II (21 cycles). The ubiquitously expressed *FGF-R* (25 cycles) and EF1-α (21 cycles) transcripts are used as loading controls. Analysis performed at the tailbud stage on uninjected whole embryos (WE) or on animal cap explants cut at stage 9 from embryos injected animally with the indicated mRNAs (*Mix.1*, 100 pg; *noggin*, 12.5 pg; *Sia*, 20 pg). (B) RT-PCR analysis at stage 11 of *cerberus* (*cer*, 25 cycles) expression in control whole embryos (WE) or in animal cap explants from embryos injected animally with mRNAs for *Mix.1* (100 pg) and/or *Siamois* (50 pg). *FGFR 1* (25 cycles) was used as a loading control. (C) In situ hybridisation with an *edd* probe on sections from stage 30 animal caps injected with mRNA for NLS-β-gal alone (top left), or with *Mix.1* (250 pg, top right), *Siamois* (50 pg, bottom left) or *Mix.1* (250 pg) and *Siamois* (50 pg) (bottom right).

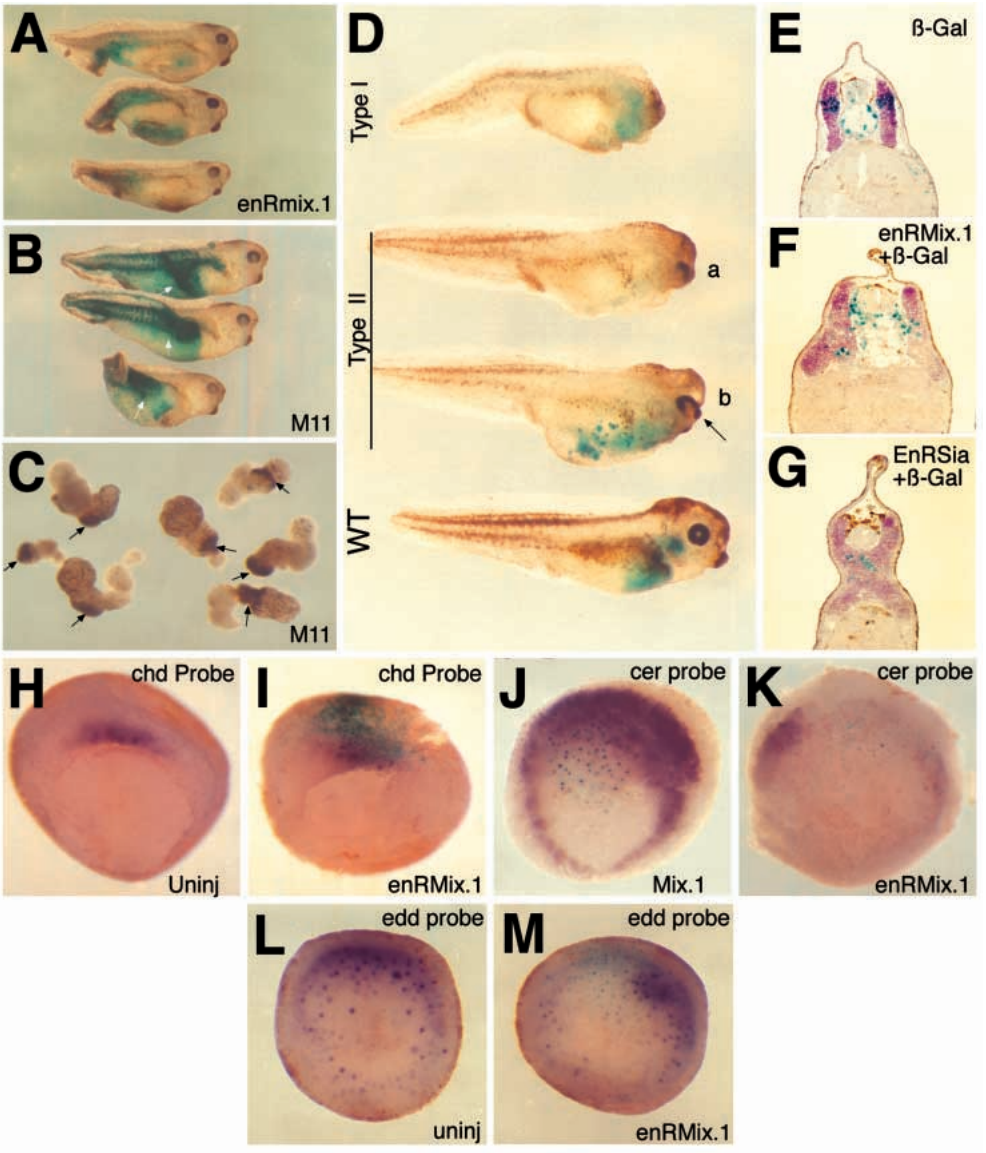

Fig. 7. Mix.1 activity is required for anterior head development. (A,B) Effect at stage 37-38 of the injection of NLS-β-gal mRNA with 250 pg of *enRMix.1* or *M11* mRNAs. The progeny of the injected cells is marked by nuclear X-gal staining. In B, white arrows point to secondary axes. (C) Stage 35 animal caps injected at the 2-cell stage with 500 pg of *M11* mRNA. Black arrows point to sticky cement glands. A similar induction of cement glands was obtained following injection of 250 pg of *M11* mRNA whereas uninjected control animal caps formed atypical epidermis (not shown). (D) Range of anterior truncations obtained following dorsal vegetal expression of enRMix.1. Type I embryos lack both eyes and cement gland; type II embryos are cyclopic without (a) or with (b, arrow) cement gland. (E-G) Transverse sections through the trunk of advanced tailbud embryos injected at the 4-cell stage in the vegetal part of both dorsal blastomeres with mRNA for NLS-β-gal (β-gal) alone (E) or with 250 pg of *enRMix.1* mRNA (F) or 100 pg of *enRSia* mRNA (G). The expansion of axial mesoderm seen in F was also obtained following injection of 25 or 100 pg of *enRMix.1* mRNA (not shown). (H-M) Whole-mount in situ hybridisation with *chd* (H,I), *cer* (J,K) or *edd* (L,M) probes on mid-gastrula embryos either uninjected (H) or injected with *enRMix.1* (100 pg, I,K,L) or *Mix.1* (50 pg, J) mRNAs. In J and K-M, the embryos were bissected along the equator prior to hybridisation to help with the detection of the *cer* and *edd* signals.

induction of *cerberus*, however, suggests that additional factors are involved in the formation of the anterior-most endoderm.

A Mix.1-like activity is required for anterior endoderm formation

Mead et al. (1996) have shown that overexpression of a mutant form of Mix.1, M11, which inhibits *Mix.1* function, antagonises the Bmp4 pathway, suggesting that *Mix.1* acts downstream of Bmp4. As our data on the overexpression of *Mix.1* did not support this view, we reanalysed the effect of M11 overexpression. Consistent with the idea that M11 blocks the Bmp4 pathway, we find that ventral overexpression of M11 leads to the formation of incomplete secondary axes (Fig. 7B). Inhibition of Bmp4 signalling in animal caps leads to the formation of prominent cement glands (Hemmati-Brivanlou and Melton, 1997). Likewise, overexpression of M11 leads to the same phenotype (Fig. 7C). However, as *Mix.1* is not expressed in animal cells (Rosa, 1989; and Fig. 1B), this effect cannot be due to the inhibition of *Mix.1* activity. Thus, while M11 may repress of Bmp4 signalling, this is unlikely to result from the specific inhibition of *Mix.1* activity.

As Mix.1 acts as a transcriptional activator to suppress mesoderm formation and induce *edd* in animal caps, we constructed a second mutant protein (enRMix.1) in which the homeodomain of Mix.1 and a few flanking amino acids were fused to the repressor domain of *Drosophila* Engrailed (Fig. 5A). By analogy with work done on the transcriptional activators *Siamois* and *Xbra* (Conlon et al., 1996; Fan and Sokol, 1997), enRMix.1 should antagonise *Mix.1* activity by repressing its target genes.

In contrast to M11, ventral overexpression of *enRMix.1* mRNA perturbed posterior axial development but did not lead to the induction of secondary axes (Fig. 7A). A more striking axial phenotype was obtained following dorsal-vegetal expression of enRMix.1. Head structures were severely reduced, the majority of injected embryos displaying a headless (type I) or cyclopic (type II) phenotype (Fig. 7D; Table 2). Dorsal-vegetal overexpression of *enRSia* can also lead to a loss of anterior head structures, which is accompanied

Table 2. Effect of dorsal-vegetal injection of *enRMix.1* mRNA

RNA injected			Headless	Cyclops	WT	Gastrulation defects
enRmix.1	mix.1	*n*	(%)	(%)	(%)	(%)
100 pg	-	77	31	52*	13	2.5
100 pg	50 pg	30	13	49	36	-
100 pg	100 pg	65	14	26†	54	1.5

Embryos were scored for axial deficiencies at stage 37-38. Two classes of cyclopic embryos could be distinguished on the basis of the presence or absence of a cement gland (CG).
*A majority of cyclops had no CG.
†A majority of cyclops had a CG.

by a loss of the notochord (Fig. 7G; Fan and Sokol, 1997; Darras et al., 1997; Kessler, 1997). In contrast, embryos injected with *enRMix.1* mRNA showed an enlarged set of axial structures (notochord and somites) (Fig. 7E,F), indicating that enRmix and enRSia interfere with the function of different subclasses of paired-like homeodomain proteins.

A further indication of the specificity of enRMix.1 was provided by the ability of Mix.1 to rescue enRMix.1-affected embryos. Coinjection of equimolar amounts of *Mix.1* and *enRMix.1* mRNA resulted in normal anterior head development in a majority of injected embryos (Table 2). These findings suggest that enRMix.1 specifically interferes with *Mix.1* or closely related genes.

Head structures are thought to be induced by signals coming from prechordal mesoderm or anterior endoderm. To characterise further the origin of the anterior truncations observed following dorsal injection of *enRMix.1* mRNA, we analysed the expression of *edd*, as well as that of *cerberus* and *chordin*, which mark anterior endomesoderm and dorsal mesoderm, respectively. Dorsal vegetal injection of *enRMix.1* mRNA led to a repression of *edd* and *cerberus*, while dorsal marginal injection of *enRMix.1* mRNA had no effect on *chordin* expression (Fig. 7H-M), suggesting that enRMix.1 affects the formation of anterior endoderm, but not that of dorsal mesoderm. The inability of enRMix.1 to repress axial mesoderm formation was also demonstrated by the presence of injected cells in the enlarged notochord and somites of embryos overexpressing enRMix.1 (Fig. 7F).

The differentiation of more posterior endoderm was also affected by the injection of *enRMix.1* mRNA. Tadpoles obtained from embryos injected with *enRMix.1* mRNA at their vegetal poles developed reduced gut structures (Fig. 8A-C). This correlated at the tailbud stage with a repression of *edd* in *enRMix.1*-expressing cells (Fig. 8D-F). This repression was not accompanied by the activation of muscle α-actin or α-T4 globin, suggesting that overexpression of enRMix.1 did not convert endoderm into mesoderm (Fig. 8G and not shown).

DISCUSSION

Mix.1 acts as a suppressor of mesoderm formation

Mead et al. (1996) recently proposed that *Mix.1* acts downstream of Bmp4 to ventralise *Xenopus* embryos. This conclusion relied on three arguments: (i) Mix.1 is activated in Bmp4-treated ectodermal explants, (ii) overexpression of Mix.1 represses dorsal mesodermal markers and induces ventral markers, and (iii) M11, a mutated form of Mix.1 with dominant negative effect, promotes dorsal development and rescues axial development in Bmp4-injected embryos. Our results suggest a different role for *Mix.1* in mesoderm patterning.

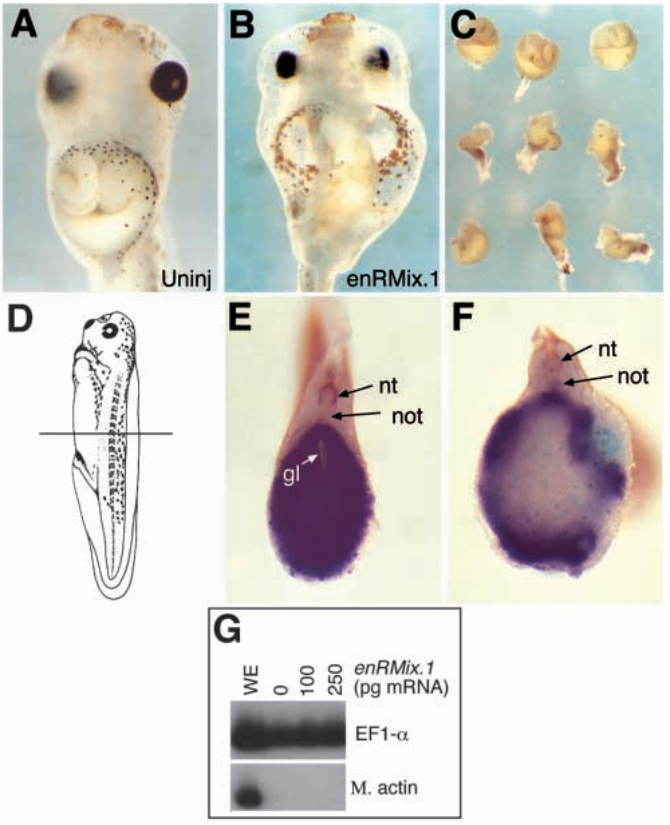

Fig. 8. *enRMix.1* suppresses posterior endoderm formation. (A-C) Embryos injected with 100 pg of *enRMix.1* mRNA at the vegetal pole of each blastomere at the 4-cell stage were left to develop until stage 44. (A,B) Ventral view of uninjected (A) or *enRMix.1*-injected (B) embryos showing a reduction of the gut size. (C) Dissected guts of uninjected (top row) or *enRMix.1*-injected embryos (two bottom rows). A marked reduction of the gut was observed in 9/13 injected embryos in the experiment shown. (D-F) In situ hybridisation with an *edd* probe on cross sections of stage 35 embryos injected with mRNA for the lineage tracer NLS-β-galactosidase with or without 100 pg of *enRMix.1* mRNA per vegetal blastomere at the 8-cell stage. The position along the A-P axis of the sections presented in E and F is shown in D. In embryos injected with β-gal mRNA alone, the nuclear X-gal staining is masked by the *edd* staining. The gut lumen (gl) is not present in *enRMix.1*-injected embryos. nt, neural tube; not, notochord. (G) RT-PCR analysis of muscle actin (M. actin) expression in stage 33 vegetal caps injected with the indicated amount of *enRMix.1* mRNA.

Firstly, there is no good correlation between the expression domains of *Mix.1* and the intensity of Bmp4 signalling in the embryo. *Mix.1* is expressed in the dorsoanterior mesendoderm, in which Bmp 4 signalling is not thought to be active (Harland and Gerhart, 1997). Conversely, *Bmp4,* and its targets *msx1*, *Xvent*-1 and *Xvent-2*, are expressed in the ventral part of the animal cap (Fainsod et al., 1994; Gawantka et al., 1995; Maeda et al., 1997; Suzuki et al., 1997), while *Mix.1* expression is not detected in this tissue. Activation of *Mix.1* in animal caps treated with high concentrations of Bmp4 may therefore not reflect the endogenous regulation of this gene.

Secondly, we find that *Mix.1* is able to repress both dorsal (*chordin*) and ventral (*Xpo*, *Xvent-1*) early mesodermal markers, which later leads to the absence of dorsal (muscle, notochord) and ventral (blood) differentiated mesoderm. Consistent with the idea that *Mix.1* acts as a general suppressor of mesoderm, overexpression of this gene blocks both dorsal and ventral expression of *Xbra* (Fig. 2 and Latinkic et al., 1997). In contrast, overexpression of Bmp4 or its target gene *msx1* does affect *Xbra* expression at the early gastrula stage. Interestingly, our finding that Mix.1 behaves as a transcriptional activator suggests that the repression of mesodermal markers by this gene is indirect.

Thirdly, we found that M11, like Bmp4 antagonists, induces cement glands in naive ectoderm. However, this tissue does not express *Mix.1*, indicating that at least some of the effects of M11 are independent of *Mix.1*. Consistent with our proposition that *Mix.1* does not act downstream of Bmp4, ventral overexpression of *enRMix.1* does not induce secondary axes, while dorsal overexpression of this molecule leads to anterior truncations that are not observed by inhibiting Bmp4 signalling but can be rescued by overexpressing Mix.1.

Taken together, we would like to propose that *Mix.1* acts as an indirect suppressor of mesoderm formation rather than as a ventralising molecule. The sustained expression of *Mix.1* in vegetal cells suggests that this gene may be important for the restriction of mesodermal differentiation to the marginal zone. Yet, interference with *Mix.1* function by overexpressing *enRMix.1* does not lead to the activation of late mesodermal markers in vegetal explants or to an extension of the domain of expression of *Xbra* (not shown). As several other transcription factors with paired-like homeodomains have been shown to repress *Xbra* (Artinger et al., 1997; Latinkic et al., 1997), it may be necessary to interfere with the function of several members of this family to derepress *Xbra* in the vegetal cells.

Mix.1 and endoderm formation

To analyse an involvement of the transcriptional activator Mix.1 in endoderm formation, we have tested the effect of the vegetal overexpression of the antimorphic mutant form *enRmix.1*. Vegetal expression of *enRMix.1* leads to a severe reduction in gut structures. This reduction is paralleled by a decreased expression of *edd* in the progeny of the injected cells. Therefore, the function of *Mix.1* or closely related genes is required for endoderm differentiation.

Conversely, overexpression of *Mix.1* alone is sufficient to drive a strong expression of *edd* in a minority of ectodermal cells but fails to activate IFABP or Xlhbox8. This suggests that, during normal development, the formation of endoderm results from a collaboration between *Mix.1* and other factors.

Siamois is coexpressed with *Mix.1* in the dorsovegetal cells (Lemaire et al., 1995). Our results indicate that *Siamois* and *Mix.1* strongly synergise to induce dorsoanterior endoderm marked by the pancreas marker *XlHbox8* and the anterior endomesodermal marker *cerberus* (Figs 6, 7). Conversely, overexpression of enRMix.1 or enRSia leads to the loss of *cerberus* expression (Fig. 7K and Darras et al., 1997). The presence of *Mix.1* and *Siamois* transcripts therefore appear to be necessary and sufficient for anterior endoderm development. In a previous study, we showed that ectopic *Siamois* expression activated *cerberus* in vegetal cells but not in animal cells, while *chordin* was activated in animal cells but not in vegetal cells (Darras et al., 1997). This differential competence to respond to Siamois may, at least in part, be due to the presence of Mix.1 in vegetal but not in animal cells. As Mix.1 and Siamois have been shown to form heterodimers in vitro (Mead et al., 1996), it is tempting to suggest that such heterodimers may have a different specificity of DNA recognition from Mix.1 or Siamois monomers or homodimers.

Finally, a large number of endoderm cells are derived from ventral vegetal cells that do not express *Siamois*. Expression of *edd* in these cells also requires a *Mix.1*-like activity (Fig. 8D-F) and it will be interesting to look for factors that cooperate with *Mix.1* to activate posterior endodermal markers such as IFABP.

Regionalisation of the organiser: a role for the anterior endoderm in anterior head formation

Several arguments have recently led to the suggestion that, in the mouse, the rostral head structures are induced by a signal emitted by the anterior visceral endoderm rather than the head mesoderm as previously thought (reviewed in Bouwmeester and Leyns, 1997).

Overexpression of enRMix.1 leads to a repression of the anterior endodermal marker *cerberus* and of *edd*, without affecting the expresssion of *chordin*, a marker of head and trunk axial mesoderm. Hence, embryos expressing enRMix.1 in their dorsal-vegetal cells specifically lack anterior endoderm. This allowed us to analyse the consequences on head patterning of the specific ablation of this tissue. The severe reduction in the rostral head territories that we observed suggests that in amphibia, as in amniotes, anterior head structures are induced by the anterior endoderm.

We thank R. Carballada, M-A O'Reilly, and H. Yasuo for helpful suggestions, and A. Ribas and G. Tétart for keeping our frog colony. We gratefully acknowledge the gift of reagents by J. Brockes, A. Hemmati-Brivanlou, M.-L. King, M. Maeno, F. Rosa, Y. Sasai, J. Smith, F. Stennard, T. Sykes, P. Vize, F. Watt and L. Zon. We thank H. Woodland for sharing unpublished results. This work was supported by the CNRS and the French Ministry of Research (ACC 4).

REFERENCES

Artinger, M., Blitz, I., Inoue, K., Tran, U. and Cho, K. W. (1997). Interaction of goosecoid and brachyury in Xenopus mesoderm patterning. *Mech. Dev.* **65**, 187-196

Biggin, M. D. and McGinnis, W. (1997). Regulation of segmentation and segmental identity by Drosophila homeoproteins: the role of DNA binding in functional activity and specificity. *Development* **124**, 4425-4433

Bouwmeester, T., Kim, S., Sasai, Y., Lu, B., and De Robertis, E. M., (1996).

Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. *Nature* **382**, 595-601

Bouwmeester, T. and Leyns, L. (1997). Vertebrate head induction by anterior primitive endoderm. *BioEssays* **19**, 855-863

Carnac, G., Kodjabachian, L., Gurdon, J. B. and Lemaire, P. (1996). The homeobox gene Siamois is a target of the Wnt dorsalisation pathway and triggers organiser activity in the absence of mesoderm. *Development* **122**, 3055-3065

Conlon, F. L., Sedgwick, S. G., Weston, K. M. and Smith, J. C. (1996). Inhibition of Xbra transcription activation causes defects in mesodermal patterning and reveals autoregulation of Xbra in dorsal mesoderm. *Development* **122**, 2427-2435

Darras, S., Marikawa, Y., Elinson, R. P. and Lemaire, P. (1997). Animal and vegetal pole cells of early *Xenopus* embryos respond differently to maternal dorsal determinants: implications for the patterning of the organiser. *Development*, In press

Fainsod, A., Steinbeisser, H. and De Robertis, E. M. (1994). On the function of BMP-4 in patterning the marginal zone of the Xenopus embryo. *EMBO J* **13**, 5015-5025

Fan, M. and Sokol, S. Y. (1997). A role for Siamois in Spemann organizer formation *Development* **124**, 2581-2589

Gawantka, V., Delius, H., Hirschfeld, K., Blumenstock, C. and Niehrs, C. (1995). Antagonizing the Spemann organizer: role of the homeobox gene Xvent-1. *EMBO J* **14**, 6268-6279

Harland, R. M. and Gerhart, J. (1997). Formation and function of Spemann's organizer. *Ann. Rev. Cell Dev. Biol.* **13**, 611-667

Hemmati-Brivanlou, A. and Melton, D. (1997). Vertebrate neural induction. *Annu. Rev. Neurosci.* **20**, 43-60

Henry, G. L., Brivanlou, I. H., Kessler, D. S., Hemmati-Brivanlou, A. and Melton, D. A. (1996). TGF-beta signals and a pattern in Xenopus laevis endodermal development. *Development* **122**, 1007-1015

Hudson, C., Clements, D., Friday, R. V., Stott, D. and Woodland, H. R. (1997). Xsox17alpha and -beta mediate endoderm formation in Xenopus. *Cell* **91**, 397-405

Kessler, D. S. (1997). Siamois is required for formation of Spemann's organizer. *Proc. Natl. Acad. Sci.USA* **94**, 13017-13022

LaBonne, C. and Whitman, M. (1997). Localization of MAP kinase activity in early Xenopus embryos: implications for endogenous FGF signaling. *Dev. Biol.* **183**, 9-20

Latinkic, B. V., Umbhauer, M., Neal, K. A., Lerchner, W., Smith, J. C. and Cunliffe, V. (1997). 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. *Genes Dev.* **11**, 3265-3276

Lemaire, P., Garrett, N. and Gurdon, J. B. (1995). Expression cloning of Siamois, a Xenopus homeobox gene expressed in dorsal-vegetal cells of blastulae and able to induce a complete secondary axis. *Cell* **81**, 85-94

Maeda, R., Kobayashi, A., Sekine, R., Lin, J.-J., Kung, H.-F. and Maeno, M. (1997). Xmsx-1 modifies mesodermal tissue pattern along dorsoventral axis in Xenopus laevis embryo. *Development* **124**, 2553-2560

Mead, P. E., Brivanlou, I. H., Kelley, C. M. and Zon, L. I. (1996). BMP-4-responsive regulation of dorsal-ventral patterning by the homeobox protein Mix.1. *Nature* **382**, 357-360

Papaioannou, V. E. and Silver, L. M. (1998). The T-box gene family. *BioEssays* 20, 9-19

Rao, Y (1994). Conversion of a mesodermalizing molecule, the Xenopus Brachyury gene, into a neuralizing factor. *Genes Dev.* **8**, 939-947

Rosa, F. M. (1989). Mix.1, a homeobox mRNA inducible by mesoderm inducers, is expressed mostly in the presumptive endodermal cells of Xenopus embryos. *Cell* **57**, 965-974

Sasai, Y., Lu, B., Piccolo, S. and De Robertis, E. M. (1996). Endoderm induction by the organizer-secreted factors chordin and noggin in Xenopus animal caps. *EMBO J.* **15**, 4547-4555.

Shi, Y. B. and Hayes, W. P. (1994). Thyroid hormone-dependent regulation of the intestinal fatty acid-binding protein gene during amphibian metamorphosis. *Dev. Biol.* **161**, 48-58

Slack, J. M. (1994). Inducing factors in Xenopus early embryos. *Curr. Biol.* **4**, 116-126

Stennard, F., Ryan, K. and Gurdon, J. B. (1997). Markers of vertebrate mesoderm induction. *Curr. Opin. Genet. Dev.* **7**, 620-627

Suzuki, A., Ueno, N. and Hemmati-Brivanlou, A. (1997). Xenopus msx1 mediates epidermal induction and neural inhibition by BMP4. *Development* **124**, 3037-3044

Vize, P. D. (1996). DNA sequences mediating the transcriptional response of the Mix.2 homeobox gene to mesoderm induction. *Dev. Biol.* **177**, 226-31

Walmsley, M. E., Guille, M. J., Bertwistle, D., Smith, J. C., Pizzey, J. A. and Patient, R. K. (1994). Negative control of Xenopus GATA-2 by activin and noggin with eventual expression in precursors of the ventral blood islands. *Development* **120**, 2519-2529

Watabe, T., Kim, S., Candia, A., Rothbacher, U., Hashimoto, C., Inoue, K. and Cho, K. W. (1995). Molecular mechanisms of Spemann's organizer formation: conserved growth factor synergy between Xenopus and mouse. *Genes Dev.* **9**, 3038-3050

Wright, C. V., Schnegelsberg, P. and De Robertis, E. M. (1994). XlHbox 8: a novel Xenopus homeo protein restricted to a narrow band of endoderm. *Development* **105**, 787-794