The role of *Mixer* in patterning the early *Xenopus* embryo

Matt Kofron, Chris Wylie and Janet Heasman*

Division of Developmental Biology, Cincinnati Children's Hospital Research Foundation, 3333 Burnet Avenue, Cincinnati, OH 45229-3039, USA

*Author for correspondence (e-mail: heabq9@chmcc.org)

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Summary

The transcription factor VegT, is required in early *Xenopus* embryos for the formation of both the mesoderm and endoderm germ layers. Inherited as a maternal mRNA localized only in vegetal cells, VegT activates the transcription of a large number of transcription factors, as well as signaling ligands that induce cells in the vegetal mass to form endoderm, and the marginal zone to form mesoderm. It is important now to understand the extent to which transcription factors downstream of VegT play individual, or overlapping, roles in the specification and patterning of the endoderm and mesoderm. In addition, it is important to understand the mechanism that specifies the boundary between endoderm and mesoderm. One of the downstream targets of VegT, the homeodomain protein Mixer, is expressed at high levels at the mesoderm/endoderm boundary at the late blastula stage. We therefore examined its functions by blocking its translation using morpholino oligos. In Mixer-depleted embryos, the expression of many signaling ligands and transcription factors was affected. In particular, we found that the expression of several genes, including several normally expressed in mesoderm, was upregulated. Functional assays of Mixer-depleted vegetal cells showed that they have increased mesoderm-inducing activity. This demonstrates that Mixer plays an essential role in controlling the amount of mesoderm induction by the vegetal cells.

Key words: Mixer, VegT, Endoderm, Mesoderm, Antisense oligo, Morpholino

Introduction

In vertebrate embryos, the spatially restricted expression of specific zygotic genes presages the movements of gastrulation and the establishment of the three germ layers. In *Xenopus*, the first step in this process occurs at the mid-blastula transition (MBT), when the vegetally localized T-box transcription factor VegT activates the expression of transcription factors and TGFβ signaling molecules in vegetal cells (Kofron et al., 1999; Xanthos et al., 2002; Xanthos et al., 2001; Zhang et al., 1996; Zhang et al., 1998). Within 2 hours of MBT, VegT activates members of the GATA, homeobox and HMG box families of transcription factors, as well as FGF and TGFβ class signaling molecules. As a result, the equatorial region of the late blastula stage embryo becomes specified toward mesodermal fates, while vegetal cells become specified for endodermal fates (Heasman et al., 1984; Nieuwkoop, 1969; Smith et al., 1985). One question that remains to be understood about this process is how the boundary between prospective mesoderm and endoderm is established and maintained.

The homeodomain transcription factor Mixer is a good candidate for a role in boundary formation in the *Xenopus* gastrula (Henry and Melton, 1998). Its expression is dependent on maternal VegT and nodal related proteins and is confined to an 8-hour period during gastrulation (Henry and Melton, 1998; Xanthos et al., 2002; Xanthos et al., 2001). *Mixer* mRNA is expressed in the prospective endoderm cells of the vegetal hemisphere in the early gastrula stage embryo, but is particularly concentrated at the boundary between the equatorial, prospective mesoderm and the vegetal, prospective endoderm regions (Engleka et al., 2001; Henry and Melton, 1998). Previous functional studies show that *Mixer* is required for endoderm development. Overexpression in animal caps leads to the ectopic induction of endodermal molecular markers including *endodermin*, *cerberus*, *DKK-1* and *Xsox17* and their expression is blocked by a dominant inhibitory form of *Mixer*, Mixer-ENR (Henry and Melton, 1998). *Mixer-ENR* mRNA-injected embryos develop with gastrulation defects, as well as gut and head abnormalities, and vegetal explants from such embryos lack the expression of late endodermal markers *IFABP* and *Xlhbox8* (Henry and Melton, 1998). However, the expression of *Mixer* mRNA in maternal VegT-depleted embryos rescues the expression of *Xsox17* to only a limited extent, suggesting that its inducing activity relies on other VegT-dependent co-factors (Xanthos et al., 2001). Mixer interacts with phospho-Smad2 through a Smad interaction motif and acts as a transducer of Xnr signals (Germain et al., 2000).

There are six other *Paired*-like homeobox family members expressed at the same time as *Mixer* in *Xenopus* gastrulae, including *Mix.1*, the founding member, together with *Mix.2*, *Bix1*/*Mix4*, *Bix2*, *Bix3*/*Milk* and *Bix4* (Casey et al., 1999; Ecochard et al., 1998; Henry and Melton, 1998; Latinkic and Smith, 1999; Mead et al., 1996; Rosa, 1989). All have roles in endoderm formation and gastrulation, and *Mix.1* can also repress the mesodermal marker *Xbra* (Latinkic and Smith, 1999). Two zebrafish *Mix.1*-related genes, *bon* and *mezzo* have been described (Kikuchi et al., 2000; Poulain and Lepage, 2002). Although neither are homologs of *Xenopus* Mixer, they have been shown to act redundantly with each other in the

formation of endoderm, as well as in prechordal plate mesoderm formation (Poulain and Lepage, 2002). Recently, Bon was also demonstrated to function in precursors of the axial mesoderm to regulate anterior neural patterning (Trinh et al., 2003). In mice and humans, only one family member, *Mixl1* has been identified (Pearce and Evans, 1999; Robb et al., 2000). Mouse *Mixl1*$^{-/-}$ mutants have defects in gastrulation and axial mesoderm patterning, including increased expression of the mesodermal gene *Brachyury*, and of the *nodal* signaling molecule (Hart et al., 2002). Relatively normal early expression of endodermal markers *Sox17* and *Cer1* occurs, but the embryos die at the early somite stage (8.5 dpc), without forming a heart or gut tube (Hart et al., 2002).

To establish the roles of *Mixer* in endoderm and mesoderm specification in *Xenopus*, we used antisense morpholino oligos (MixerMO) to block the translation of Mixer protein throughout the entire period of *Mixer* expression during gastrulation. We find that Mixer-depleted embryos develop with severe abnormalities of the head and gut, which are partially rescued by the expression of a non-complementary *Mixer* mRNA. The pattern of early zygotic gene expression is reproducibly altered at the gastrula stage, and the effects of Mixer-depletion are gene specific rather than germ layer specific. We show that *Mixer* loss-of-function results in overexpression of the mesodermal markers *eomesodermin, Bix3* and *Fgf8* in their usual equatorial location, as well as the spread of their expression into deeper endodermal territory. We also show that Mixer acts cell autonomously, and represses some genes (*Bix3*, *Xnr5*, *Xnr1* and *Fgf8*) while activating others (*cerberus*, *Xsox17*). In functional assays of Mixer-depleted vegetal cells, we show that a major biological role of Mixer is to control the degree to which cells induce the formation of mesoderm.

Materials and methods

Oocytes and embryos

Eggs were obtained by injecting *Xenopus laevis* with 1000 U of chorionic gonadotropin (Sigma Aldrich, Milwaukee, WI), and fertilized using a sperm suspension in 1×MMR. Embryos were maintained in 0.1×Marc's Modified Ringer's Solution (MMR), and dejellied using 2% cysteine (Sigma Aldrich, Milwaukee, WI) at pH 7.8. Staging was according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). For injections of morpholino oligos or mRNA, embryos were transferred to 2% Ficoll (Amersham Bioscience, Uppsala, Sweden) in 0.5×MMR, and then maintained in 0.1×MMR after the blastula stage. The sites of MO and mRNA injection are described in the text.

Explant culture

Mid-blastula (stage 8) wild-type and Mixer MO-injected embryos were devitellined and dissected with tungsten needles on agar coated dishes in 1×MMR. After washing away dead cells, vegetal and animal pieces were placed alone (Fig. 4A) or together in the combinations described in Fig. 6 and cultured on agar in OCM for 2 hours. The recombinants were separated using tungsten needles and stray vegetal cells were identified by their larger size and whitish, opaque color and removed from the explants. Animal caps were then cultured in fresh OCM on agar until sibling uninjected embryos were stage 11 and frozen in batches of 10 caps for analysis

Cell transplantation, sectioning and immunohistochemistry of embryos

Vegetal cells were transplanted to host blastulae as described previously (Heasman, 1984). Briefly, vegetal masses were dissected from late-blastula stage embryos, dissociated in 67 mM phosphate buffer, washed once with 1×Ca/Mg-free MMR, transferred to Ca/Mg-free 1×MMR with 10 μg/ml TRITC (Sigma T-3163) and labeled for 5 minutes. Cells were washed twice in 1×Ca/Mg-free MMR and transplanted to recipient blastulae. Embryos were cultured to the tailbud stage and were fixed in 2% TCA for 2 hours at room temperature. Embryos were embedded in wax, mounted, and cut into 20 μm sections and mounted on positively charged slides. Slides were dewaxed and stained with 12/101 antibody at 1:500 dilution in PBS/Tween 0.1% for 3 hours at room temperature. Slides were washed three times for 20 minutes at room temperature. Secondary antibody was 1 hour 1:250 GAM Cy5 conjugate (Jackson Immuno). Slides were washed, mounted in 90% glycerol and imaged on LSM 510 confocal microscope (Zeiss).

Oligos and mRNAs

Three overlapping morpholino oligos were designed complementary to the junction between the *Mixer* 5′UTR and the open reading frame. Fig. 1A shows the reverse complement of each oligo sequence aligned with *Mixer*, a *Mixer* pseudo-allele from the EST sequencing project, the related genes *Mix.1* and *Mix.2*, and a morpholino-resistant mRNA (*Mixer* MO-R) designed for the rescue experiments. Mixer MO-1 was most efficient in blocking the in vitro translation of *Mixer* mRNA, but did not block translation of *Mix.1* mRNA (Fig. 1B and data not shown). However, it also caused cell disaggregation and apoptosis at the late gastrula stage in doses above 15 ng. To determine whether this was a specific effect, the oligo was injected into the four animal blastomeres of eight-cell stage embryos to target cells that do not express *Mixer* mRNA. MO-1 caused disaggregation and apoptosis of epidermal cells at the neurula stage, as evidenced by TUNEL staining, indicating that disaggregation was not due to blocking *Mixer* translation (data not shown). MO-2 was shifted eight nucleotides 5′ of the region targeted by *Mixer* MO-1 and caused more severe apoptosis beginning at gastrula stage. *Mixer* MO-3 targeted a region four nucleotides 3′ of *Mixer* MO-1. MO-3 caused no disaggregation effects (data not shown) and blocked translation of *Mixer* mRNA, although with less efficiency than MO-1 (Fig. 1B). We confirmed by in vitro translation that neither oligo blocked the translation of the most closely related family member *Mix.1* (data not shown). The experiments described here were performed using both MO-1 and MO-3 oligos.

A morpholino resistant mRNA (*Mixer MO-R* mRNA) was synthesized by PCR amplification of the *Mixer*-coding region using a 5′ primer designed to introduce conserved substitutions into this sequence. The PCR amplification was performed using the proofreading Advantage-2 HF polymerase (Clontech). The PCR amplified product was subcloned into pCS2+ and was sequenced to ensure no PCR induced mutations were introduced. These base changes introduce 10 mismatches for *Mixer* MO-1, 18 mismatches for *Mixer* MO-2, and six mismatches for *Mixer* MO-3.

In vitro translation

Wild-type or *Mixer MO-R* mRNA was used in a Biotin in vitro Translation kit (Roche). Reactions were assembled on ice and then incubated at 30°C for 1 hour. Aliquots of the reactions were separated in a 10% Tris-HCl electrophoresis gel and blotted to PVDF. The membranes were blocked then incubated with streptavidin-POD and developed with Chemiluminescence substrate (Roche). Membranes were then exposed to X-ray film.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed on pigmented or albino embryos as described (Harland, 1991) using BM Purple as substrate (Roche). After satisfactory color development, embryos were fixed in MEMFA for 1 hour at room temperature, washed and stored in 100% methanol. Embryos for half-mount in situ hybridization were prepared by fixing whole gastrulae for 1 hour in MEMFA, bisecting the embryos along the dorsoventral axis with a scalpel blade, fixing 1 additional hour in MEMFA, washing and storing in 100% ethanol. The in situ hybridization were performed

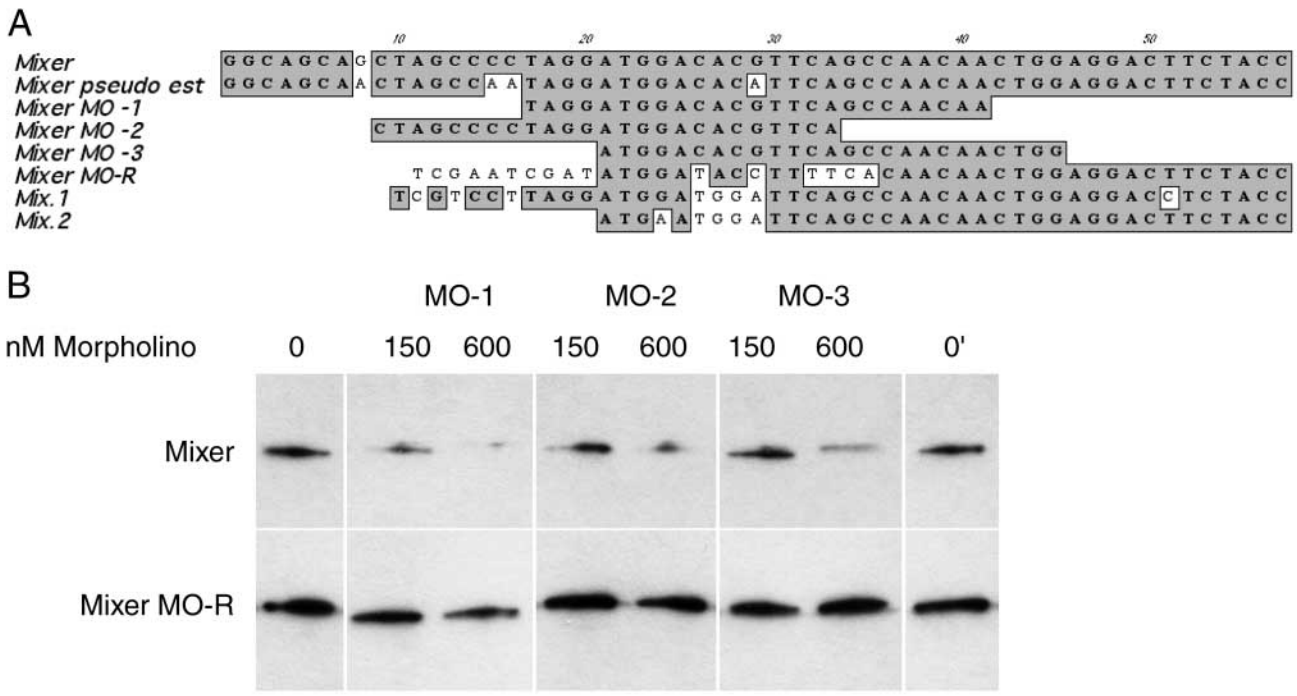


Fig. 1. Alignment of *Mixer* morpholinos against *Mixer* and related *Xenopus* sequences (A) and morpholino inhibition of in vitro translation (B) of wild-type and MO-R *Mixer* mRNA. (A) *Xenopus Mixer* aligned against the reverse complement of the three morpholinos used in this study using Clustal alignment algorithem in Macvector. These sequences were also compared to a potential pseudo-allele identified by the Sanger EST sequencing project and the most closely related homeodomain proteins *Mix.1* and *Mix.2*. (B) Mixer MO-1, MO-2 and MO-3 block translation of wild type but not MO-R *Mixer* mRNA in an in vitro translation assay as described in the Materials and methods.

exactly as above with the exception of reducing the proteinase K treatment to 5 μg/ml for 10 minutes to reduce damage to embryos.

Analysis of gene expression using real-time RT-PCR

Total RNA was prepared from oocytes, embryos and explants using proteinase K and then treated with RNase-free DNase as described (Zhang et al., 1998). Approximately 0.5 μg RNA was used for cDNA synthesis with oligo (dT) primers followed by real-time RT-PCR and quantitation using the 'LightCycler' System (Roche) as described previously (Kofron et al., 1999). The primers and cycling conditions used are listed in Table 1. Relative expression values were calculated by comparison to a standard curve generated by serial dilution of uninjected control cDNA. Samples were normalized to levels of ornithine decarboxylase (ODC), which was used as a loading control. Samples of water alone or controls lacking reverse transcriptase in the cDNA synthesis reaction failed to give specific products in all cases.

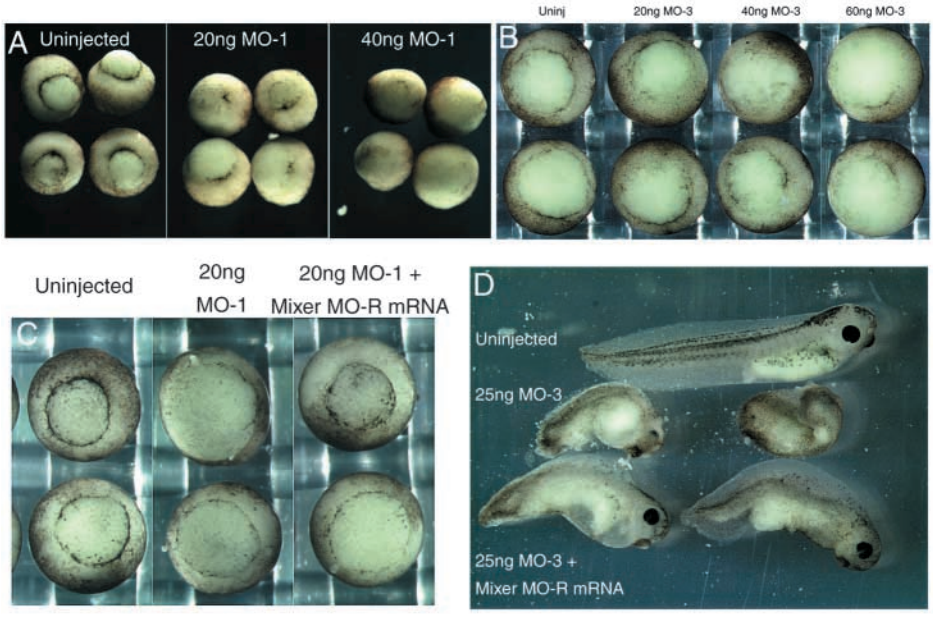
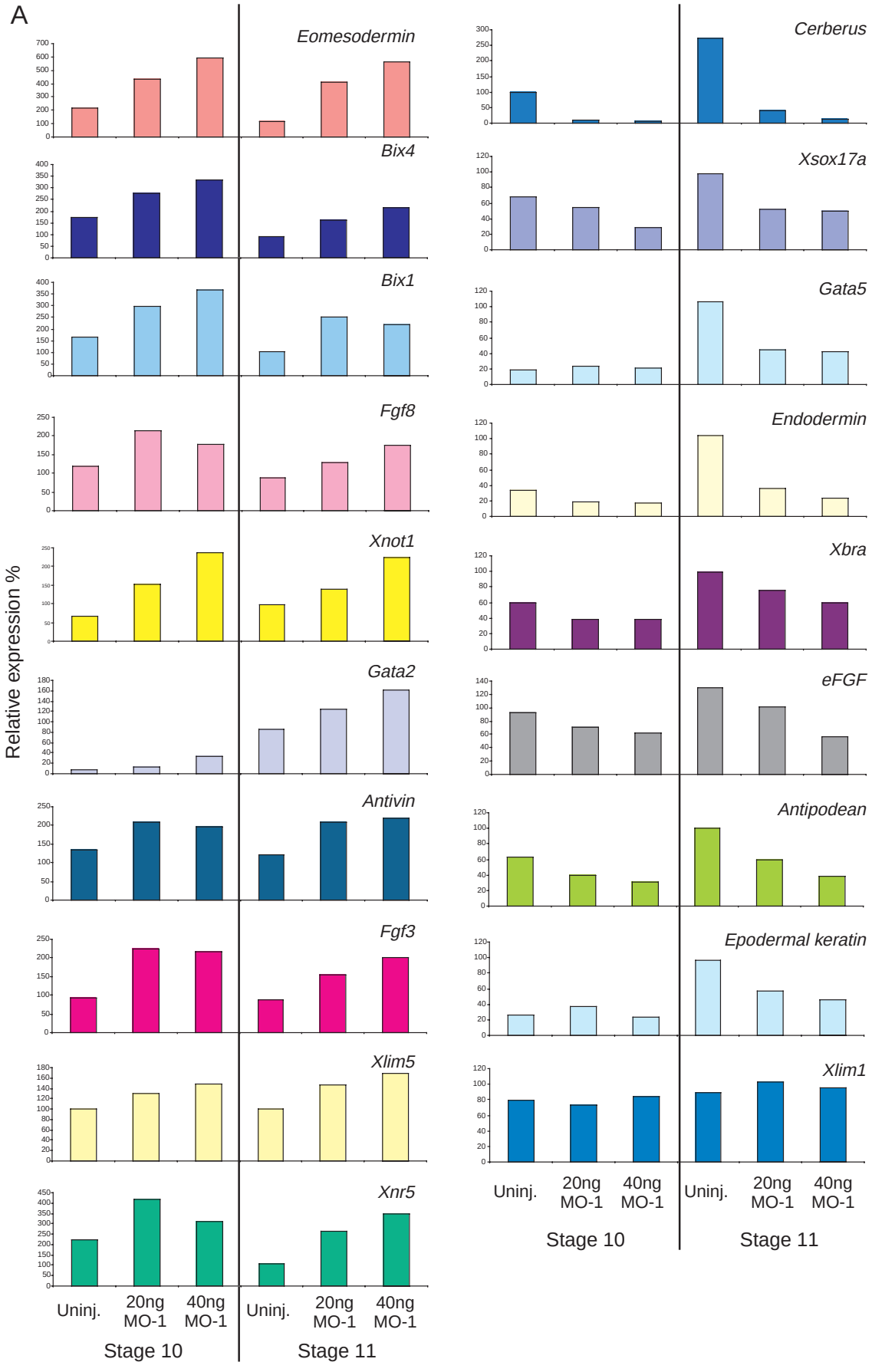


Fig. 2. Non-complementary *Mixer* mRNA rescues the effects of *Mixer* depletion. Gastrulation is delayed in embryos injected with MO-1 (A) and MO-3 (B) as marked by formation of the dorsal blastopore lip. The gastrulation delay is partially rescued by the injection of Mixer MO-R mRNA into the four vegetal cells of eight-cell MO-1-injected embryos (C). Anterior and gut abnormalities in *Mixer*-depleted embryos is partially rescued by injection of *Mixer* MO-R mRNA into the four vegetal cells of the eight-cell embryo (D).


A
Relative expression %
Eomesodermin
Bix4
Bix1
Fgf8
Xnot1
Gata2
Antivin
Fgf3
Xlim5
Xnr5
Cerberus
Xsox17a
Gata5
Endodermin
Xbra
eFGF
Antipodean
Epodermal keratin
Xlim1
Uninj.
20ng MO-1
40ng MO-1
Stage 10
Stage 11

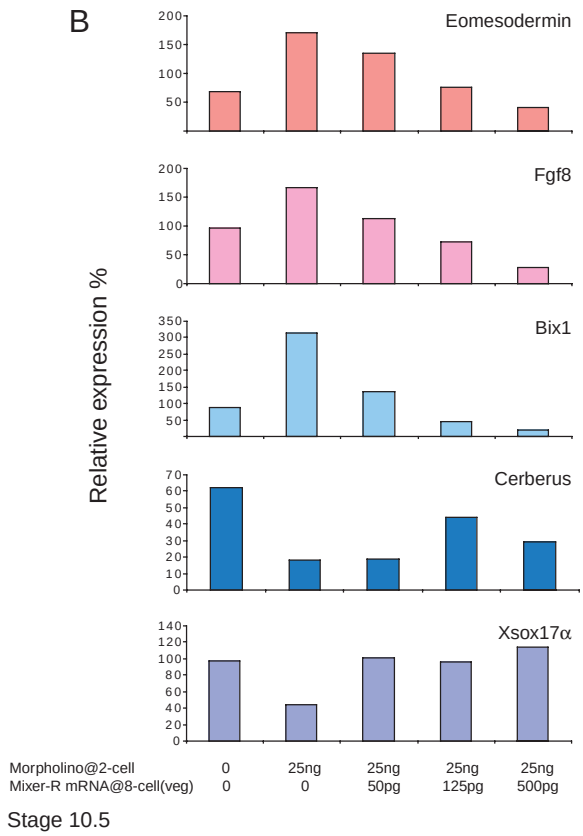


Fig. 3. Analysis of gene expression in Mixer-depleted gastrulae (A) and in mRNA rescued gastrulae (B). (A) Embryos were injected at the two-cell stage with 20 ng or 40 ng of MO-1 and cultured until the early or mid-gastrula stage. The resulting Mixer-depleted embryos were collected at indicated stages and analyzed by real time RT-PCR for the expression of various mesodermal markers *Eomesodermin*, *Fgf8*, *Xnot1*, *Gata2*, *Fgf3*, *eFGF*, *antipodean*, mesendodermal markers *Xnr1*, *Xnr5*, *Antivin*, *Bix1*, *Bix4*, endodermal markers *Xsox17α*, *Gata5*, *Endodermin*, *Cerberus*, *Xlim-1* and Ectodermal markers *Xlim-5* and *epidermal keratin.* Expression levels are normalized to ODC. (B) The rescue experiment was carried out by injecting MO-1 into the vegetal region of both cells of the two-cell embryos. Embryos were cultured until the eight-cell stage and injected into the four vegetal blastomeres with either 50, 125 or 500 pg of *Mixer* MO-R mRNA. Embryos were cultured until the mid-gastrula stage (stage 10.5), frozen and assayed by real-time RT-PCR. Expression levels were normalized to ODC.

Results

Mixer-depleted embryos have gastrulation and tailbud abnormalities

To block the translation of endogenous *Mixer* mRNA, which is expressed in the vegetal hemisphere of embryos at the gastrula stage, *Mixer* MO-1 or MO-3 was injected into the vegetal region of two-cell stage embryos. Embryos injected with *Mixer* MO-1 or MO-3 developed normally to the gastrula stage. There was a dose-dependent delay in the appearance of the blastopore and gastrulation was slower than in controls, although neural folds eventually formed normally (Fig. 2A,B). At the tailbud stage, embryos failed to elongate, had curved body axes and reduced head size compared with uninjected controls (Fig. 2D; Table 2). These effects were due specifically to the depletion of *Mixer* mRNA because they were significantly rescued by the introduction of *Mixer* MO-R mRNA at the eight-cell stage (Fig. 2C,D; Table 2). These results show that Mixer has a non-redundant function in early development in *Xenopus*.

Mixer regulates the expression of mesodermal and endodermal zygotic genes in a gene-specific manner

As *Mixer* mRNA is particularly concentrated at the boundary between mesoderm and endoderm at the gastrula stage (Henry and Melton, 1998), we asked whether its depletion affected the expression of early zygotic genes expressed in this region. Uninjected control and Mixer-depleted embryos were frozen at the early and mid-gastrula stages and the mRNA expression levels of early zygotic genes were compared using real-time RT-PCR (Fig. 3A; data not shown).

Zygotic gene expression was altered in a highly reproducible fashion in these embryos (7/7 experiments). Molecular markers could be placed into three categories according to whether they were downregulated, upregulated or not altered by Mixer depletion. The downregulated category included the general endodermal markers *Xsox 17*, *Gata5*, *endodermin* (Hudson et al., 1997; Sasai et al., 1994; Weber et al., 2000), the anterior endodermal marker *cerberus* (Bouwmeester et al., 1996), and the mesodermal markers, *Xbra*, *antipodean*, *eFGF* (Slack, 1994; Smith et al., 1991; Stennard et al., 1999). Surprisingly, several mesodermal markers that are normally expressed in the same domain as *Xbra*, including *eomesodermin*, *Fgf3*, *Fgf8*, *Xnot* and *Gata2* (Ryan et al., 1996; Christen and Slack, 1997; Lombardo et al., 1998; Yasuo and Lemaire, 2001; Zon et al., 1991) were upregulated by Mixer depletion. Similarly, the homeobox genes *Bix1* and *Bix4* (Casey et al., 1999; Tada et al., 1998), which are normally expressed in both prospective mesoderm and endoderm, were overexpressed in Mixer-depleted embryos, as were the nodal related genes *Xnr1* and *Xnr5* (Jones et al., 1995; Takahashi et al., 2000). By comparison, the expression of the anterior endodermal gene *Xlim1* was not affected by Mixer depletion (Fig. 3A). This indicates that *Mixer* regulates the level of expression of many zygotic genes in a complex fashion, and is not simply an endodermal determinant.

To confirm that these changes in gene expression were specific, we analyzed the effects of the introduction of the non-complementary *Mixer* mRNA (*Mixer MO-R* mRNA) into Mixer-depleted embryos. Fig. 3B shows that both upregulation and downregulation of target genes caused by the Mixer depletion were rescued by subsequent *Mixer MO-R* mRNA injection into the four vegetal blastomeres at the eight-cell stage.

As several mesodermal genes were upregulated in Mixer-depleted embryos, this suggested that a normal function of Mixer may be to keep these genes off in the developing endoderm. To test this, control and Mixer-depleted embryos were dissected at the mid-blastula stage into animal, equatorial and vegetal parts, which were cultured separately until the mid-gastrula stage and analyzed for the expression of *eomesodermin*, *Fgf8*, *Bix1*, *Bix4* and *Xsox17*. Fig. 4A shows that Mixer depletion caused an increase in the expression of the mesodermal markers in vegetal explants, indicating that *Mixer* normally inhibits the ectopic expression of these

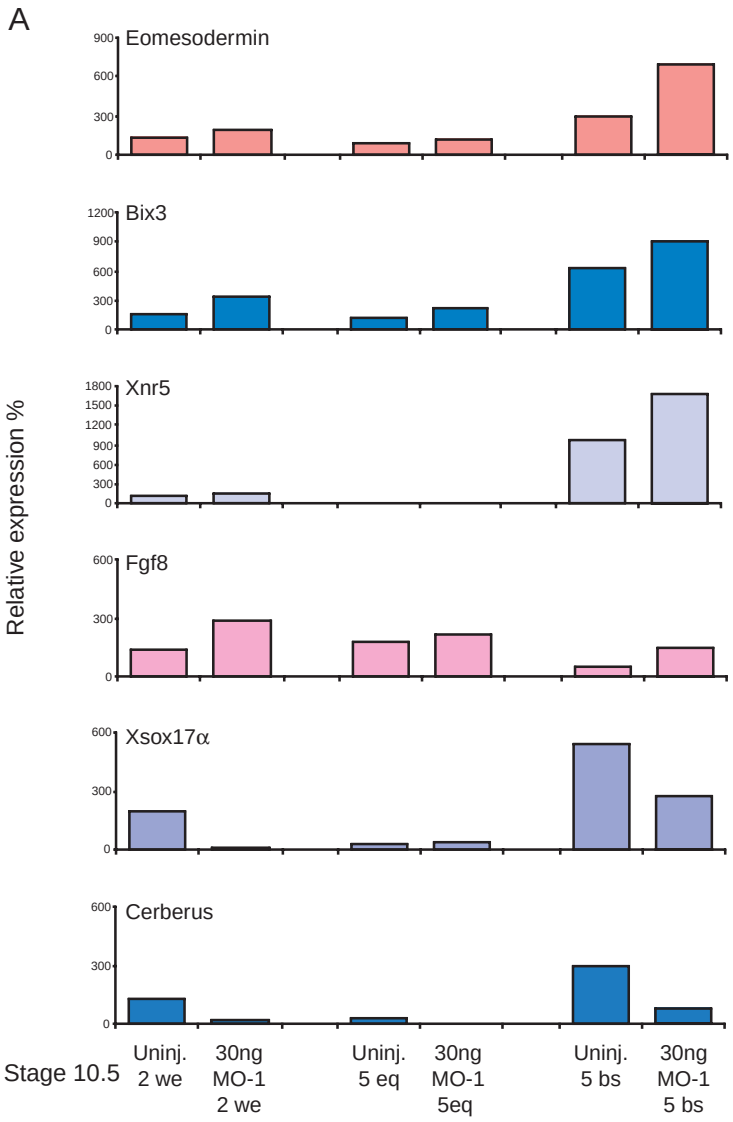
Table 1. Primer pairs and cycling conditions for Lightcycler real-time PCR

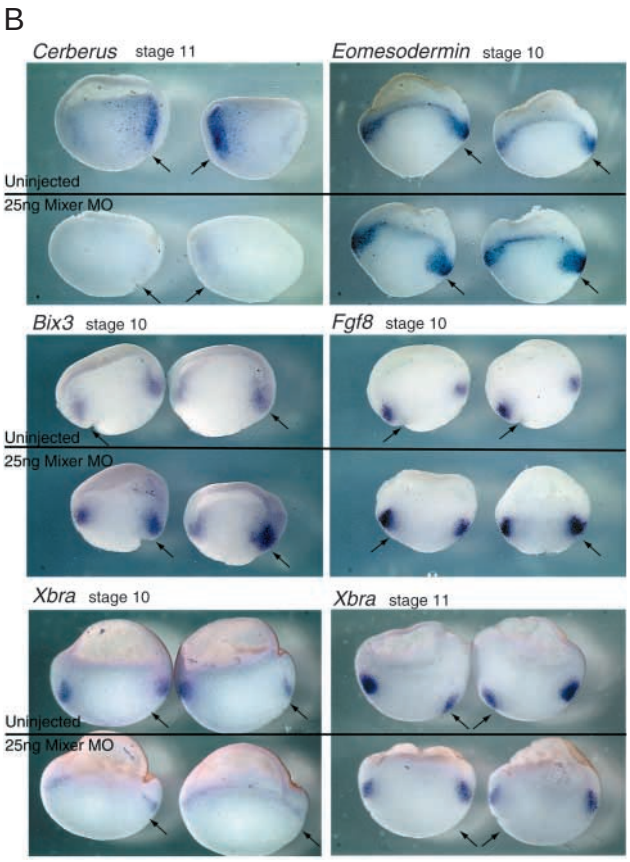
PCR primer pair	Origin	Sequence	Denaturing temperature	Annealing temperature (°C)/time	Extension temperature (°C)/time	Acquisition temperature (°C)/time
Antipodean	Stennard et al., 1999	U: 5′-TGG ATT AGT TTA GGA ACA -3′ D: 5′-CGG ATC TTA CAC TGA GGA -3′	95	55/5	72/16	83/3
Antivin	New	U: 5′-GCC TCA AAG GGA TGT GGA G-3′ D: 5′-TGT GGC TGA GTC AAC GAG AG-3′	95	55/5	72/11	81/3
Bix1	Xanthos et. al., 2001	U: 5′-AGA GAC TCC CAG TTC ATC TGA-3′ D: 5′-GGT AGG TGG GAA GTT GCT AAT-3′	95	60/5	72/8	83/3
Bix3	Xanthos et. al., 2001	U: 5′-TCT CGC ATT CAG GTT TGG TTC C-3′ D: 5′-ATC TCC TTG TTA GGG ATC ATA C-3′	95	60/5	72/11	83/3
Bix4	Xanthos et. al., 2001	U: 5′-AGA TGC TAC AGG CTG GAG CAA-3′ D: 5′-GTG TGT AAG GGG TGA GTC ATA-3′	95	60/5	72/11	84/3
Cerberus	Heasman et al., 2000	U: 5-GCT TGC AAA ACC TTG CCC TT-3′ D: 5-CTG ATG GAA CAG AGA TCT TG-3	95	60/5	72/20	81/3
Eomesodermin	New	U: 5′-TGG TCC TCA AGG TCA AGT CC-3′ D: 5′-GGG GAG TTT TCA TTG CTT GA-3′	95	56/6	72/10	83/3
Endodermin	Sasai et al., 1996	U: 5′-TAT TCT GAC TCC TGA AGG TG-3′ D: 5′-GAG AAC TGC CCA TGT GCC TCC-3′	95	55/5	72/8	81/3
Epidermal keratin	XMMR*	U: 5′- CAC CAG AAC ACA GAG TAC -3′ D: 5′- CAA CCT TCC CAT CAA CCA -3′	95	55/5	72/9	81/3
eFGF	New	U: 5′-TAT GAA TGC AAA GGG GAA GC-3′ D: 5′-ATA TCC GTG GCA AGA AAT GG-3′	95	55/5	72/10	83/3
Fgf3	Kofron et al., 1999	U: 5′-GTC ATT TGT TTC CAG ACT TC-3′ D: 5′-TAT CTG TAG GTG GTA CTT AG-3′	95	55/5	72/12	85/3
Fgf8	Kofron et al., 1999	U: 5′-CTG GTG ACC GAC CAA CTA AG-3′ D: 5′-ACC AGC CTT CGT ACT TGA CA-3′	95	55/5	72/14	86/3
Gata2	New	U: 5′-CTA AAC AGA GGA GCA AGA GC-3′ D: 5′-CCT AAG TTC CTC AAA AC-3′	95	55/5	72/15	86/3
Gata5	Xanthos et. al., 2001	U: 5′-ACC TTC AGA GCT GCG ACA CT-3′ D: 5′-CCA ACC GGG AGC CCC GAT A-3′	95	60/5	72/20	86/3
ODC	Heasman et al., 2000	U: 5′-GCC ATT GTG AAG ACT CTC TCC ATT C-3′ D: 5′-TTC GGG TGA TTC CTT GCC AC-3′	95	55/5	72/12	83/3
Sprouty2	New	U: 5′-TGC TCA GCC CAG GAA GTA GT-3′ D: 5′-ACC ACA AAC AGG GCA AGA AC-3′	95	55/5	72/10	83/3
Xbra	Sun et al., 1999	U: 5′-TTC TGA AGG TGA GCA TGT CG-3′ D: 5′-GTT TGA CTT TGC TAA AAG AGA CAG G-3′	95	55/5	72/8	75/3
Xlim1	New	U: 5′-CCCTGGCAGCAACTATGACT-3′ D: 5′-GGTTGCCATAACCTCCATTG-3′	95	55/5	72/11	85/3
Xlim5	Houston et. al., 2003	U: 5′ - CCA ACA GAC AGG CCC AAC- 3′ D: 5′- GTG GCT CCG GTG CTA CAG - 3′	95	60/5	72/8	84/3
Xnr1	Kofron et al., 1999	U: 5′-TGG CCA GAT AGA GTA GAG-3′ D: 5′-TCC AAC GGT TCT CAC TTT-3′	95	55/5	72/12	81/3
Xnr5	New	U: 5′-ATG AGG CCT CTG TCA ATG CT-3′ D: 5′-GCC CTG AAT GTC TTG CAT CT-3′	95	55/5	72/10	80/3
Xnot1	New	U: 5′-CTGCATTTGGCCACCACCTGGC-3′ D: 5′-GATGAGCCACACGGGTGGGTA-3′	95	55/5	72/15	86/3
Xsox17	Xanthos et al., 2001	U: 5′-GCA AGA TGC TTG GCA AGT CG-3′ D: 5′-GCT GAA GTT CTC TAG ACA CA-3′	95	58/5	72/8	85/3

**Xenopus* Molecular Marker Resource (http://www.xenbase.org/xmmr/Marker_pages/primers.html).

mesodermal genes. By contrast, the amount of expression of *Xsox17* is reduced in vegetal explants depleted of *Mixer* compared to control expression levels. This suggests that a normal role of Mixer is to repress mesodermal genes and activate endodermal genes in vegetal cells.

In order to study the effect of depleting Mixer on the regional expression of mesodermal and endodermal genes, wild-type and Mixer-depleted embryos were fixed and bisected at the early and mid-gastrula stages and the location of *eomesodermin*, *Bix3*, *Fgf8*, *Xbra* and *cerberus* mRNA was examined by in situ hybridization (Fig. 4B and data not shown). Although *cerberus* and *Xbra* expression was clearly reduced in Mixer-depleted embryos at both stages, the expression of *eomesodermin*, *Bix 3* and *Fgf8* was increased in equatorial cells and expanded into deeper cells. To examine the expansion of the domain of *eomesodermin* expression more closely, Mixer MO-1 was injected into single vegetal cells of eight-cell stage embryos, to block *Mixer* translation over only one quarter of the vegetal mass at the gastrula stage. In comparison with the control side, where *eomesodermin* mRNA is expressed in equatorial cells only, Fig. 4C shows an expansion of *eomesodermin* expression into the vegetal





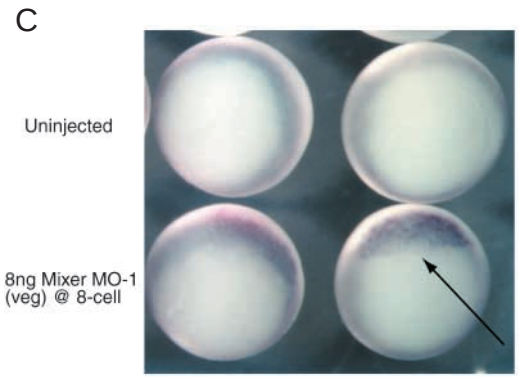


Fig. 4. *Mixer* depletion causes ectopic expression of VegT target genes. (A) Embryos injected with *Mixer* MO-1 were cultured until the blastula stage, and dissected into vegetal and equatorial explants. These explants were cultured until the mid-gastrula stage, frozen and analyzed for expression of mesodermal and endodermal markers by real-time RTPCR. Expression levels were normalized to ODC. we, wild-type embryo; eq, equatorial explant; bs, vegetal explant. Expression levels were normalized to ODC. (B) Half-mount in situ for *Cerberus*, *eomesodermin*, *Bix3*, *Fgf8* and *Xbra* in Mixer MO-1-injected embryos. Arrows indicate dorsal. Embryos were injected with 25 ng of Mixer MO-1 into both vegetal cells of a two-cell embryo. These embryos were cultured until stage 10 and 11, fixed in MEMFA, bisected, re-fixed in MEMFA and assayed for gene expression by whole-mount in situ hybridization for the indicated gene. (C) Whole-mount in situ for *eomesodermin* in Mixer MO-1-injected embryos. Embryos were injected with 6 ng of Mixer MO-1 into one vegetal cell of an eight-cell embryo. These embryos were cultured until stage 10.5, fixed in MEMFA and analyzed for *eomesodermin* expression by whole-mount in situ hybridization. The arrow indicates the expanded region of *eomesodermin* mRNA. The pinkish color is due to β-gal staining from co-injection of β-galactosidase mRNA as a lineage tracer.

territory in the Mixer-depleted area (arrow). These data show clearly that a normal role of Mixer is to repress the expression of mesodermal genes in the endoderm.

Mixer regulates the capacity of the vegetal mass of the late blastula-stage embryo to induce mesoderm

Both gain- and loss-of-function experiments show that Mixer is required for the increased expression of some genes and the decreased expression of others, both in the mesoderm and endoderm. What is the biological role of Mixer? To test this, we used recombination experiments (Nieuwkoop, 1969) in which wild-type animal caps from mid-blastula stage embryos were placed in contact with vegetal explants from uninjected control or Mixer-depleted late blastula stage embryos. After a

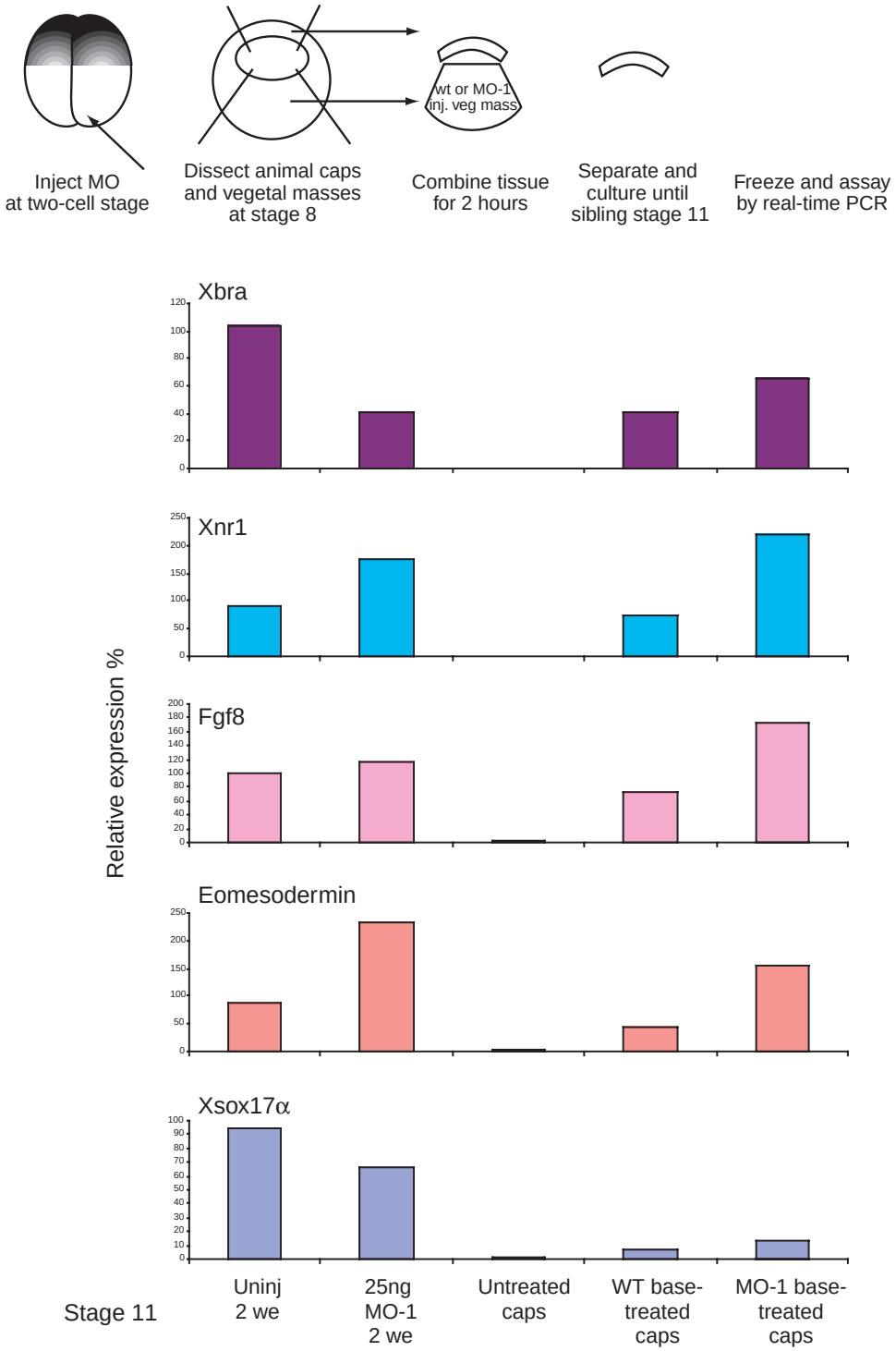


Fig. 5. Mixer-depleted vegetal masses have increased mesoderm inducing activity in Nieuwkoop recombinants. (Top) Schematic of real-time RT-PCR of Nieuwkoop recombinant experiment in which the capacity of wild-type and *Mixer*-depleted vegetal masses to induce mesoderm in animal caps is compared. *eomesodermin*, *Fgf8*, *Xbra* and *Xnr1* are induced more efficiently in animal caps by Mixer-depleted vegetal masses than control vegetal masses. we, wild-type embryos; WT base-treated caps, 10 animal caps co-cultured with wild-type vegetal masses; MO-1 base-treated caps, 10 animal caps co-cultured with *Mixer*-depleted vegetal masses. Expression levels were normalized to ODC.

2 hour co-culture period, animal caps were peeled off the vegetal masses, stripped of any adherent vegetal cells, cultured until sibling embryos had reached the mid-gastrula stage and analyzed for mesodermal markers, including *Xbra*, *Xnr1*, *Fgf8*, *eomesodermin* and the endodermal marker *Xsox 17*. Fig. 5 indicates that Mixer-depleted vegetal masses induced higher levels of expression of all the mesodermal markers in animal caps than did control vegetal masses. By comparison, the endodermal marker *Xsox 17* was not significantly induced in caps by either control or Mixer-depleted vegetal masses. The experiment was repeated with a similar result (data not shown). This suggests that Mixer normally functions to control the amount of mesoderm-inducing activity in the vegetal mass.

We next tested the effect of Mixer-depletion on the ability of vegetal cells to induce mesoderm in vivo. To do this, we disaggregated vegetal masses from control and Mixer-depleted late blastulae and transplanted small groups (12-15) of cells to the blastocoel cavities of untreated host embryos. Previous studies have shown that transplanted vegetal cells from late blastulae give rise to descendants mostly in endoderm and occasionally in the lateral plate mesoderm (Wylie et al., 1987). We found that descendants of the Mixer-depleted groups were found only in the endoderm (11/16 cases), or in both lateral plate mesoderm and endoderm (5/16 cases) (Fig. 6B). In three out of 16 cases, an ectopic somite was induced near to the descendant cells (Fig. 6D,E) and identified using the somite-specific 12.101 antibody. Induction of an ectopic somite was not seen when control vegetal cells were transplanted (Fig. 6C, 0/13 cases). These data, together with the Nieuwkoop recombinant experiments show that Mixer-depleted vegetal cells have an increased capacity to induce mesoderm.

Discussion

Mixer, unlike the other six paired homeodomain proteins of the Mix/Bix family expressed during gastrulation in *Xenopus*, is most strongly expressed at the boundary between prospective

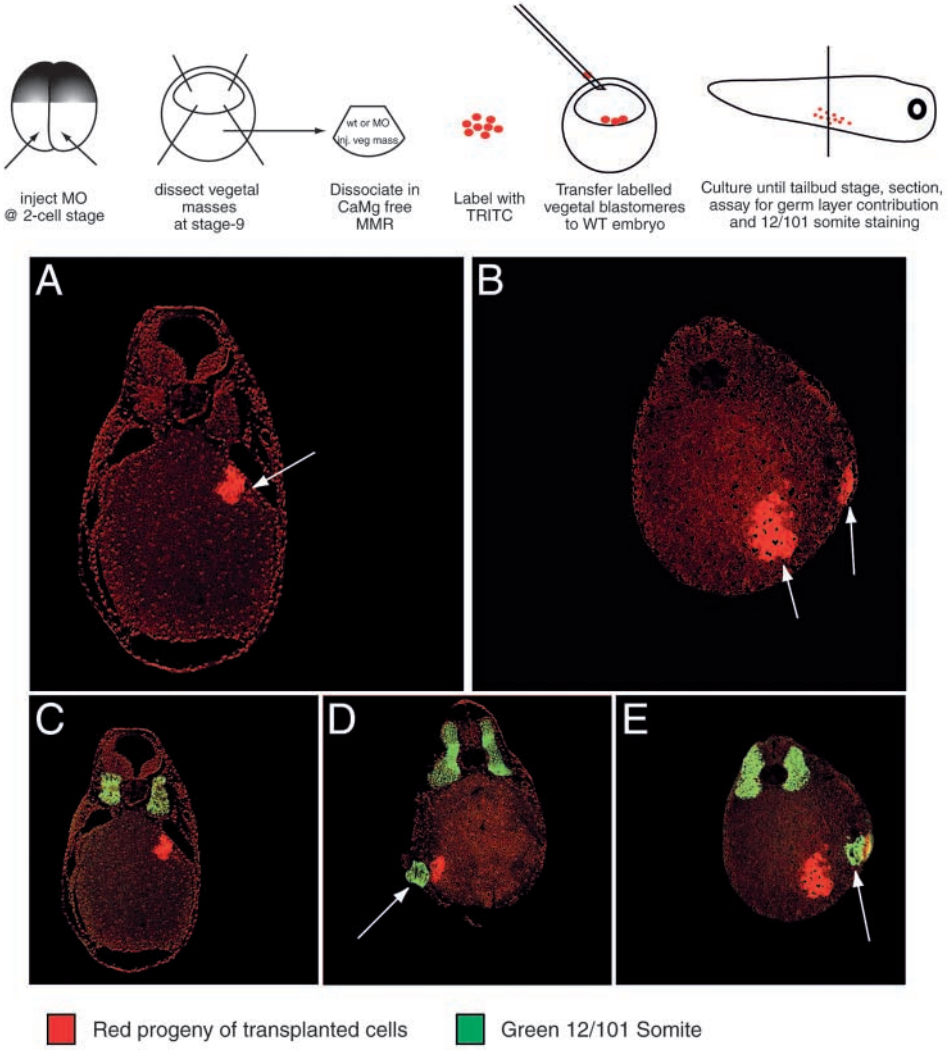


Fig. 6. Vegetal cells from Mixer-depleted embryos can induce ectopic somite tissue. Schematic of vegetal-cell transplantation experiment shown at top of figure. (A) Progeny from transplanted vegetal cells from control late blastula embryos become endoderm in tailbud stage host embryos (TRITC labeled cells in red, arrows). (B) The progeny of transplanted vegetal cells from Mixer-depleted embryos are found in endoderm and lateral plate mesoderm (arrows). (C) Control host embryo with progeny of wild-type vegetal cells (red) and somite staining (green, 12/101 MAB). (D,E) Ectopic patches of 12/101 positive cells are found near to the progeny of transplanted Mixer-depleted vegetal cells (arrows).

mesoderm and endoderm (Henry and Melton, 1998). In order to test the essential roles for Mixer in normal development, we have depleted it and assayed the effects first on gene expression and second on biological function of the Mixer-expressing cells. The morpholino antisense oligos chosen for these studies, are complementary to all isoforms of *Mixer* mRNA but have at least four mismatches with other family members, and do not block translation of Mix.1. We confirm that the loss of function is Mixer-specific by rescuing both phenotypically and in terms of molecular markers with non-complementary *Mixer* mRNA.

These studies show that the roles of Mixer are more complex than was suggested previously. We find that Mixer negatively regulates several early zygotic genes both in the mesoderm and endoderm, while positively regulating others. Further experiments are required to determine whether these effects of Mixer are direct or indirect. One possible scenario is that Mixer activates genes such as *cerberus* directly and these then block Xnr5 signaling activity, and thus the Xn5 autoregulatory loop reducing Xnr5 mRNA expression. This seems unlikely because of the timing of expression of these two genes. *Xnr5* mRNA is already overexpressed in Mixer-depleted embryos at the early gastrula stage when *cerberus* mRNA expression is only just beginning [Fig. 3A; see Xanthos et al. (Xanthos et al., 2002) for a more extensive temporal series of *cerberus* mRNA expression]. Another possibility is that Mixer activates some genes and repress others directly. As one dose of oligo upregulates some target genes, and downregulates others in the same area, it seems unlikely that the type of activity is concentration dependent. More likely, the behavior of Mixer is context dependent and gene specific, its combinatorial activity with other transcription factors, as well as with co-repressors and co-activators, determining the final outcome of Mixer binding. Detailed analysis of the regulatory regions of these target genes, and the identification of Mixer-associated co-repressors and co-activators is necessary to determine if Mixer acts as a transcriptional activator and repressor.

Table 2. mRNA rescue of head and body axis defects in Mixer-depleted embryos

	Normal	Curved body axis	Curved body axis/ reduced head
Control	50/50		
Mixer MO injected	1/50	10/50	39/50
Totals from three experiments using MO-3			
Experiment 1			
Control	15/15		
Mixer MO injected		0/10	10/10
Rescue	7/15	3/15	5/15
Experiment 2			
Control	25/25		
Mixer MO injected		5/22	17/22
Rescue	10/19	5/19	4/19

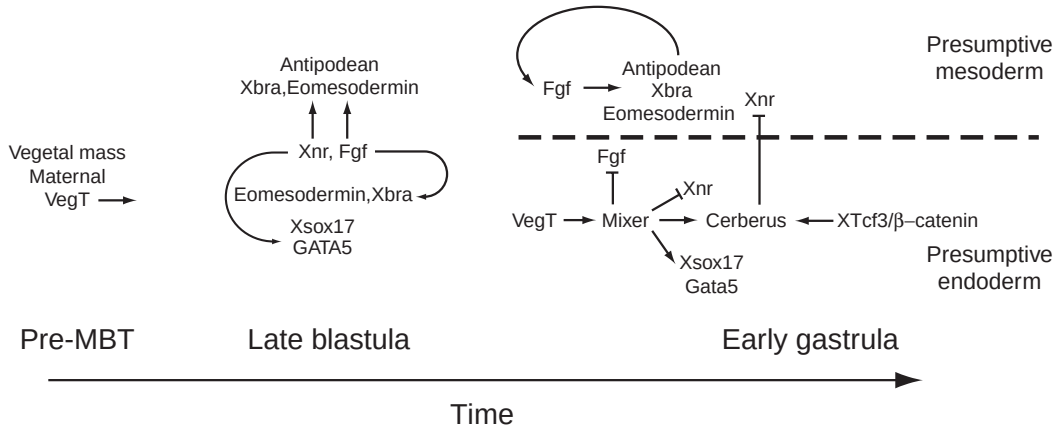


Fig. 7. Model for *Mixer* function in the early *Xenopus* embryo. This and previous work suggest the following model for formation of boundaries of expression of several VegT target genes. After MBT, VegT activates the expression of Xnr genes, *Xsox17*, *Gata4*, *Gata5* and *Gata6*, and Fgf genes in vegetal cells. The secreted mesoderm inducing molecules induce adjacent marginal cells, which do not express VegT, to express more of themselves as well as the presumptive mesodermal genes including *eomesodermin* and *Xbra*. At the early gastrula stage, *Mixer* is cell autonomously induced by VegT-expressing cells. It reduces, directly or indirectly, the expression of *Xnr1* and *Xnr5*, *Fgf3* and *Fgf8* and *eomesodermin* by vegetal cells and therefore prevents further mesoderm inducing signals being released by the vegetal mass. Mixer also activates a second round of *Xsox17* and *Gata5* expression, consolidating endodermal fates for vegetal cells. Together with maternal XTcf3/β-catenin, Mixer initiates *cerberus* expression and cerberus protein blocks further Xnr, Wnt and BMP signaling in the anterior endoderm.

It is interesting that, although the T-box transcription factors eomesodermin, Xbra and Antipodean are all recognized as pan-mesodermal markers and have very similar expression patterns, they are not regulated in the same way by Mixer, as *eomesodermin* expression increases, while the other genes are downregulated after Mixer depletion. Thus, similarity of expression patterns is not necessarily indicative of similar mechanisms of gene regulation, and Mixer cannot be viewed as a simple mesodermal repressor.

Xnr1, *Xnr*5, *Fgf3* and *Fgf8*, which are repressed by Mixer, encode secreted proteins that act as mesoderm inducing signals in *Xenopus* embryos. Xnr1 and Xnr5 have been shown to rescue mesoderm and axis formation in VegT-depleted embryos (Kofron et al., 1999; Takahashi et al., 2000), while FGF proteins are implicated in muscle cell precursor formation (Standley et al., 2001). We show in Nieuwkoop assays and by carrying out cell transplantation experiments, that that the loss of Mixer activity results in excessive production of mesoderm inducing signals. This suggests that one important role for Mixer is in limiting the signaling that specifies mesodermal fates, and thus in dictating the size of the mesodermal territory. One interesting aspect of this possible role for Mixer is the timing of its expression. The temporal expression of *Mixer* mRNA lags considerably behind that of Xnr and Fgf genes. We and others have shown that *Xnr1*, *Xnr2*, *Xnr4*, *Xnr5* and *Xnr6* are strongly expressed downstream of VegT at the late blastula stage (Takahashi et al., 2000; Xanthos et al., 2002), while *Mixer* expression begins at this stage but peaks 4 hours later at the gastrula stage (Xanthos et al., 2002). The onset of *Mixer* activity thus corresponds to the time of 'loss of competence' of the vegetal mass, as defined by its ability to induce mesoderm in animal caps (Jones and Woodland, 1987), and is preceded by a 3-4 hour time window when mesoderm induction is uninhibited. It will be important to understand what, besides VegT, controls the temporal pattern of expression of Mixer.

Interestingly, transplantation experiments suggest that Mixer expression is not essential for endodermal fate specification. All individually transplanted and most small groups of Mixer-depleted vegetal cells transplanted onto the blastocoel floor of host embryos continued to develop in the endoderm germ layer and did not redistribute to mesoderm, suggesting that Mixer-depletion is insufficient to cause their relocation to mesoderm. Previous studies have shown that other transcription factors including GATA5 and other Mix family members have important roles and may act in parallel with Mixer in determining endodermal fate (Casey et al., 1999; Weber et al., 2000). It is also possible that the surrounding wild-type vegetal cells release inductive signals that maintain the endodermal fate of the Mixer-depleted cells.

A model for the role of Mixer in mesendoderm patterning

This and previous work suggest the following model for formation of boundaries of expression of several VegT target genes (Fig. 7). After MBT, VegT activates the expression of Xnr genes, *Xsox17*, *Gata4*, *Gata5* and *Gata6*, and Fgf genes in vegetal cells. The secreted mesoderm-inducing molecules induce adjacent marginal cells, which do not express VegT, to express more of themselves as well as the presumptive mesodermal genes including *eomesodermin* and *Xbra*. At the early gastrula stage, *Mixer* is cell autonomously induced by VegT-expressing cells. It represses further expression of *Xnr1*, *Xnr5*, *Fgf3* and *Fgf8*, and therefore prevents further mesoderm inducing signals being released. Mixer also activates *Xsox17* and *cerberus* expression, consolidating endodermal fates for vegetal cells and further limiting the range of signaling molecule activity by the antagonistic activity of cerberus.

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