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Cloning of Mix-related homeodomain proteins using fast retrieval of gel shift activities, (FROGS), a technique for the isolation of DNA-binding proteins

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ABSTRACT **We have developed a technique, fast retrieval of gel shift activities (FROGS), that allows for the rapid isolation of proteins that interact with DNA. Using this technique, we have isolated two proteins that are structurally similar to Mix.1, a PAX class homeodomain protein with ventralizing activity in *Xenopus*. The Mix family of proteins are expressed during late blastula and gastrula stages of *Xenopus* development. During gastrulation, these genes are expressed at high levels in distinct, yet overlapping regions in mesoderm and endoderm. The members of the Mix family heterodimerize with each other and overexpression of each results in severe axial abnormalities. Mix.3 and Mix.4 can directly induce primitive ectoderm to become endoderm whereas Mix.1 cannot. Injection of Mix.3 or Mix.4 RNA in the whole embryo results in extensive ectopic endodermin mRNA expression. The expression of the Mix family homeoproteins is differentially regulated by activin, Vg1, BMP-4, and fibroblast growth factor, supporting a model in which the Mix homeoproteins are downstream effectors of growth factor signaling during endoderm and ventral mesoderm formation.**

Key regulatory events during development are modulated by transcription factor complexes which bind *cis*-elements and direct the correct temporal and tissue-specific expression of target genes. During early vertebrate embryonic development, inductive events lead to the formation of three germ layers: ectoderm, mesoderm, and endoderm. Soluble factors, such as the fibroblast growth factor (FGF), Wnt, and transforming growth factor β (TGF-β) families and their antagonists, have been shown to play a key role in these events (refs. 1 and 2 and references therein). Defining the transcriptional targets of growth factor signaling will provide important clues to the molecular basis of pattern formation in early development.

Blood is derived from ventral mesoderm (reviewed in ref. 3). The TGF-β family member BMP-4 is a potent inducer of ventral mesoderm (reviewed in ref. 4) and is thus likely to participate in the induction of the hematopoietic program (3). Downstream targets of BMP signaling include the Smad proteins (5) and a variety of homeodomain-containing proteins including Mix.1 (6, 7), Xvent1 (8), Xvent2 (9), and Msx1 (10, 11). The hematopoietic transcription factors GATA-2 (12) and SCL (13, 14) are also up-regulated by BMP-4 expression.

We recently described a BMP-4-responsive cascade that is important for ventral mesoderm formation in *Xenopus* embryos (7). BMP-4 induces the expression of the PAX-like homeoprotein Mix.1, which leads to ventralization of the embryo. In support of this model, overexpression of a dominant negative Mix.1 mutant homeoprotein (M11) blocks BMP-4-induced ventralization of embryos. Mix.1 can heterodimerize with other members of the PAX family such as goosecoid (15) and siamois (16), and in so doing antagonizes the function of these dorsalizing factors (7). Thus, the heterodimerization of Mix.1 with other DNA-binding proteins may directly regulate fate decisions throughout the mesodermal layer of the developing embryo.

In an effort to clone heterodimeric partners of Mix.1 that may participate in ventral mesoderm formation, we have developed a technique called fast retrieval of gel shift activities (FROGS). FROGS uses coupled transcription-translation of small pools of cDNA clones to generate pools of proteins. DNA-binding activity in protein pools is assayed by gel mobility shift analysis. Proteins that interact with DNA as dimers can be isolated by supplementing the pool with heterodimeric partner proteins. We have used the FROGS assay to isolate Mix.3 and Mix.4, two novel members of the Mix family of PAX-like homeoproteins. These factors heterodimerize with Mix.1, pattern mesoderm to a ventral (hematopoietic) fate, and participate in the formation of endoderm.

MATERIALS AND METHODS

Construction of Library and Protein Pools. *Xenopus* embryos were UV-ventralized (DAI 0–4 (17)) and harvested at stage 11 (18). Size-selected (>1.5 kb), double-stranded cDNA was directionally cloned into pCDNA3 (Invitrogen). Plasmid from pools of 100 independent clones was prepared by Wizard Prep (Promega). Plasmid DNA (1 μg) from each pool was used to generate pools of protein (T7 TnT rabbit reticulocyte lysate transcription/translation kit, Promega). Gel mobility shift assays on 5 μl of each protein pool were performed as described (19). Briefly, a 20-μl gel shift reaction contains 10 mM Hepes (pH 7.8), 40 mM potassium glutamate, 5 mM magnesium chloride, 1 mM EDTA, 5% glycerol, 0.5 mM DTT, 50 μg/ml poly(dI-dC) (Sigma) and approximately 5×10^4 cpm ^{32}P-labeled double- stranded oligonucleotide probe. After a 20-min incubation on ice, gel mobility shifts were resolved on nondenaturing 5% (wt/vol) polyacrylamide gels in 0.5× TBE at 235 V for approximately 2 hr at 4°C.

Sib Selection of Clones. Single cDNA clones with specific gel shift activities were isolated from cDNA pools by sib selection. Positive pools were replated on nitrocellulose and transferred to 96-well plates. DNA was prepared from rows of wells and

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Abbreviations: FGF, fibroblast growth factor; TGF-β, transforming growth factor β; FROGS, fast retrieval of gel shift activities; RT, reverse transcription; HA, homeodomain alone; edd, endodermin.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AF081350 (Mix.3) and AF081351 (Mix.4)].

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the gel shift assay was repeated. Rows of clones containing the original gel shift activity were subjected to further sib selection.

Xenopus Embryo Manipulations. Individual cDNAs were characterized by sequence analysis, *in situ* hybridization (20), gel mobility supershift assays, and for biological activity by ectopic expression in developing *Xenopus* embryos. Injection of *Xenopus* embryos, explant assays, and reverse transcription (RT)-PCR analysis was as previously described (7, 21). Ornithine decarboxylase and EF-1α were used as controls for RNA recovery. RT-PCR primers unique to this study were as follows: Mix.3 (forward 5′-GGAGGCACCCAGGAGAAAG-T-3′ and reverse 5′-TAGCGTGAGGTTTAGAGATG-3′ amplify a 373-bp fragment), Mix.4 (forward 5′-CAATGCCTA-CTCTACACAGG-3′ and reverse 5′-GTGTCCAAGTGCCA-CAAGG-3′ amplify a 239-bp fragment), and GATA-5 (forward 5′-GCCAGACGAATACACCTACAG-3′ and reverse 5′-AGAAACCCAAGATACCACCAT-3′ amplify a 332-bp fragment).

RESULTS

FROGS, an Expression Cloning Strategy for Transcription Factors. To isolate transcription factors that interact with Mix.1, we have developed a technique called FROGS. Pools of 100 cDNA clones from a *Xenopus* gastrula library were assembled. DNA from each pool was used to generate pools of proteins using a coupled *in vitro* transcription and translation system (22–25). Seventy-five such pools of translated proteins were subjected to gel mobility shift assays in the presence or absence of the Mix.1 homeodomain peptide homeodomain alone (HA) (7) using a radiolabeled DNA-binding site [P3: an inverted ATTA repeat separated by three nucleotides (26)]. Since PAX family members exhibit cooperative binding on DNA, the presence of HA facilitates factors in the pools to bind to the P3 DNA target. Detection of an additional gel shift complex indicates that the factor in the pool can form a stable heterodimer with Mix.1 (Fig. 1*A*). Gel mobility shifts from primary pools were readily detectable, and the activity and relative ability of proteins to heterodimerize became more easily visualized after sib selection.

Using the FROGS assay, we have isolated four independent proteins that interact with the truncated Mix.1 homeodomain construct (HA) (Fig. 1*B*). Two were independent clones of Mix.2, which has previously been isolated and shares 95% amino acid identity with Mix.1 (27). Mix.2 has an identical pattern of expression and functions as Mix.1 (data not shown). In addition to Mix.2, two new members of this family of homeoproteins were isolated, Mix.3 and Mix.4 (Fig. 2). The Mix family of homeodomain proteins shares extensive sequence similarity (Fig. 2, dark shading) in the region of the DNA-binding domain (Fig. 2, bar) but diverges outside the homeodomain. Each of the Mix family proteins interacts with the Mix.1 homeodomain to give a gel mobility shift activity, representing the formation of a heterodimer complex on DNA (Fig. 3*A*, arrowheads). The Mix genes activate transcription in transient transfection assays in NIH 3T3 cells cotransfected with a growth hormone reporter construct with concatemerized P3 sites upstream of a minimal globin promoter (data not shown). Thus, using the FROGS assay we have identified new

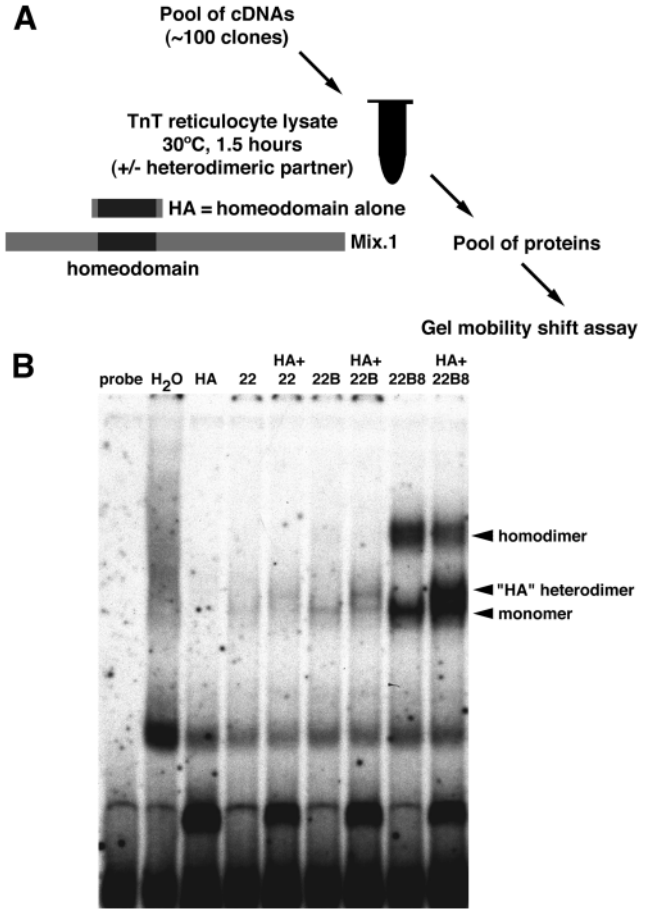

FIG. 1. (*A*) FROGS. Protein from pools of 100 cDNA plasmids was synthesized *in vitro* by coupled transcription/translation. A gel mobility shift assay on radiolabeled P3 site was performed in the presence or absence of HA, a truncated mutant of Mix.1 that encodes just the homeodomain. Pools with a specific gel mobility shift were sib-selected to isolate pure clones. (*B*) Sib selection of a sample pool. The gel mobility shift is detected in the primary pool (pool 22). The activity becomes more prominent with sib selection to pools of 12 cDNA clones (22B) and finally with the pure cDNA clone (22B8) when homodimers are evident. The HA heterodimer is marked. Note: the unprogrammed lysate (labeled H_2O) contains no added plasmid and characteristically gives high background on gel mobility shift analysis.

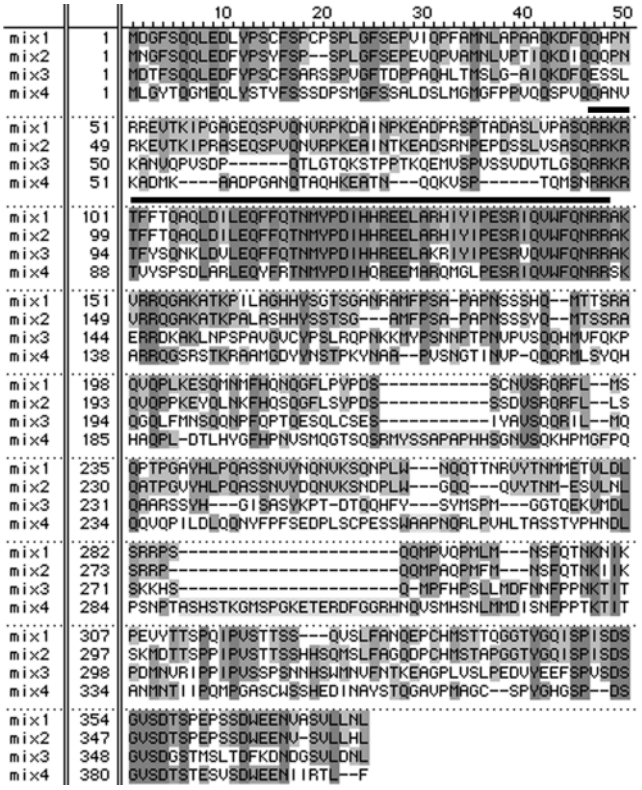

FIG. 2. Sequence analysis of the Mix family of homeoproteins. Conserved residues are shaded and the homeodomain is indicated by the solid bar.

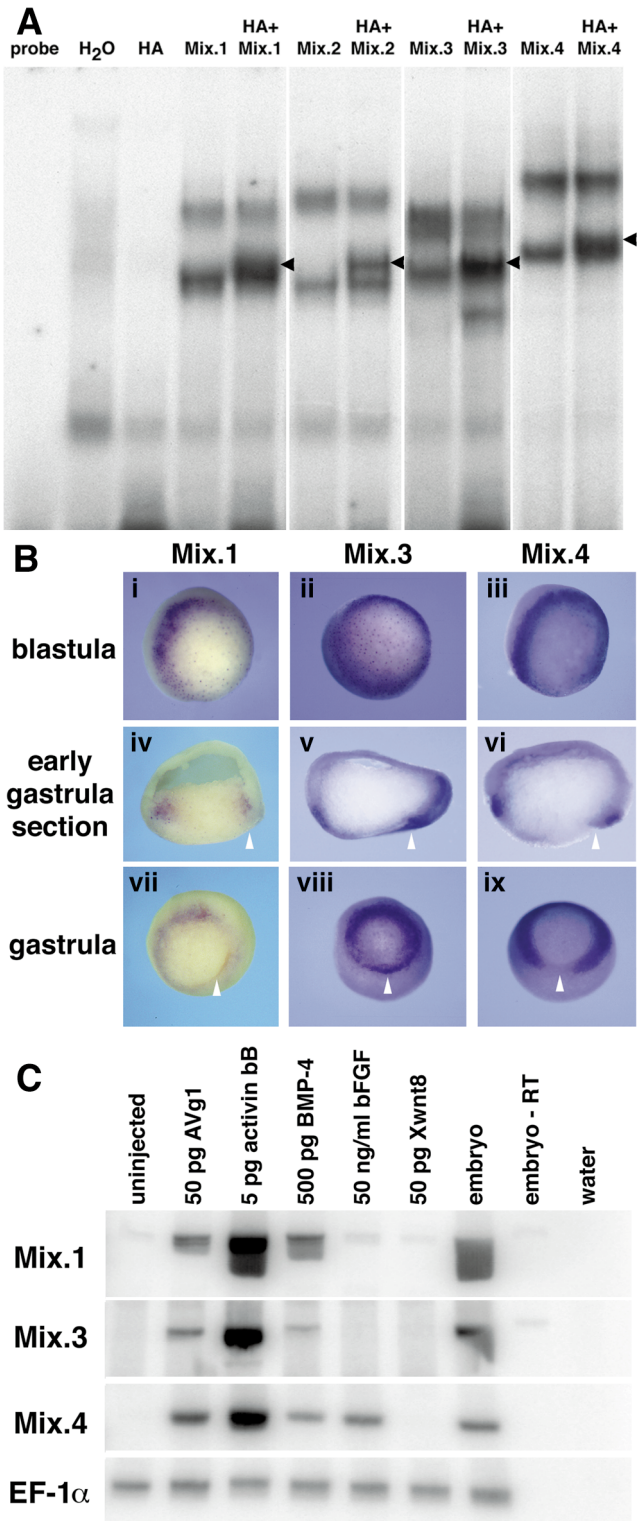

FIG. 3. (*A*) Mix family members can heterodimerize with each other. The full-length clones were tested for their ability to heterodimerize on a candidate P3 site with the truncated Mix.1 homeodomain construct HA. Each combination yields an extra band demonstrating dimerization (arrowheads). (*B*) Expression patterns of the Mix family of homeoproteins. (*Top*, i–iii) Vegetal view of blastula (stage 9). The embryos are slightly tilted to show a portion of the marginal zone. (*Middle*, iv–vi) Transverse section of early gastrula (stage 10^+). (*Bottom*, vii–ix) Vegetal view of mid-late gastrula (stage 10.5–11). White arrows indicate the dorsal axis. (*C*) Growth factor signaling upstream of the Mix homeoproteins. Embryos were injected with growth factor mRNA at the one-cell stage, animal pole explants were dissected at stage 8, and the expression of each Mix family member was analyzed at stage 11 by RT-PCR. The TGF-β family members activin, AVg1, and BMP-4 induce expression of each of the Mix family of homeoproteins. Basic FGF induced expression of Mix.4 alone. Expression of the Mix genes was not stimulated by Xwnt-8.

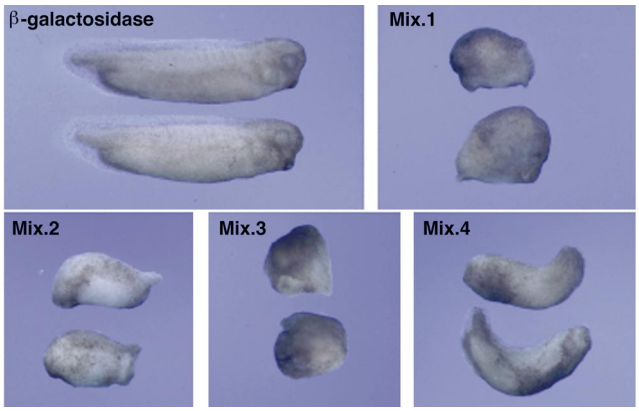

FIG. 4. Ectopic expression of Mix.1, Mix.2, Mix.3, and Mix.4 leads to axial abnormalities. Embryos were injected at the one-cell stage with 1 ng of each synthetic mRNA. β-galactosidase RNA was included as a negative control.

members of a family of PAX-like homeoproteins which includes Mix.3 and Mix.4.

The patterns of expression of Mix.1, 3 and 4 are distinct, suggesting different roles for these factors during early development (Fig. 3*B*). During blastula stages (Fig. 3*B*, i–iii), the Mix genes are expressed in both endoderm and mesoderm in a radial pattern. During gastrula stages, Mix.1 becomes predominantly localized on the ventral axis in both endoderm and mesoderm (Fig. 3*B*, iv and vii). Mix.3 is expressed in the leading edge mesoderm in a radial pattern (Fig. 3*B*, viii). By mid-gastrula stage, Mix.4 is expressed in a ring around the embryo but is excluded from the organizer region (Fig. 3*B*, ix) in a pattern reminiscent of that observed with Xwnt-8 (28, 29). The ring of Mix.4 expression extends more laterally than that of Mix.3 and does not include the leading edge (Fig. 3*B*, vi and ix). By late gastrula, the Mix family members are highly expressed in the ventral mesendoderm (Fig. 3*B*, vii–ix). These studies demonstrate that Mix.1, 3, and 4 have distinct, yet overlapping patterns of expression and suggest a potential role for Mix family members in the genesis of endoderm as well as mesoderm.

TGF-β Signaling Induces Expression of the Mix Family. Mix.1 was originally identified as an immediate-early response gene to activin signaling (30). To identify signaling molecules that may regulate the expression of Mix factors, we examined levels of Mix transcripts in animal cap explants treated with growth factors (Fig. 3*C*). Expression of the Mix genes was not stimulated by Xwnt-8. All of the Mix family members were stimulated by the TGF-β family members activin, Vg1 and BMP-4, albeit at differing levels. In addition, Mix.4 expression was stimulated by basic FGF treatment. This suggests that distinct environments within the developing embryo can lead to the differential expression of the Mix homeoproteins. Recent studies have described FGF and activin/Vg1 signal cascades that regulate mesoderm induction and a BMP cascade that regulates ventral patterning. The SMAD proteins are downstream targets of TGF-β signaling (31); XMAD1 is activated upon BMP signaling (5) whereas XMAD2 is activated upon activin signaling (32). SMAD-2 binds a forkhead protein, FAST1, in the activin response element of the Mix.2 promoter (33). Thus, the Mix family members are likely downstream effectors of TGF-β-triggered signal transduction cascades which regulate the formation of mesoderm and endoderm.

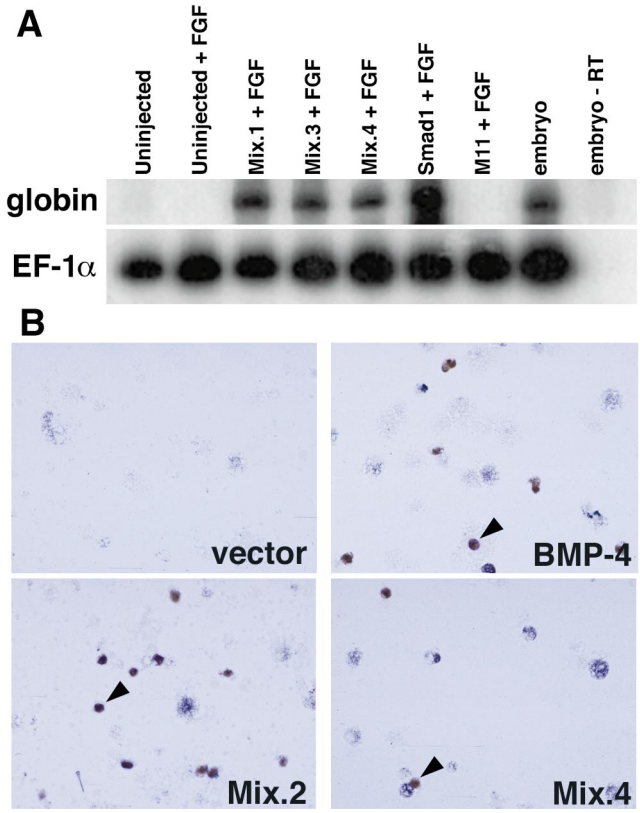

FIG. 5. Mix genes and hematopoiesis. (*A*) Mix genes pattern mesoderm to a ventral (hematopoietic) fate. Embryos were injected with mRNA at the one-cell stage in the animal pole (1 ng of each RNA), animal caps were explanted at stage 8 and cultured to sibling stage 36 in the presence of bFGF, and expression of globin RNA was examined by RT-PCR. M11 is a dominant negative mutant of Mix.1. (*B*) Mix genes induce erythroid cells in animal pole explants. Embryos were injected with plasmid DNA (300 pg, pcDNA3.0) at the one-cell stage in the animal pole and animal caps were explanted at stage 8 and cultured in the presence of basic FGF (20 ng/ml) to sibling stage 36. Disaggregated animal caps were stained with *O*-dianisidine (T.L.H., Y.Z., P.E.M., and L.I.Z., manuscript submitted), cytocentrifuged onto glass slides, and photographed at ×200.

Overexpression of Each Mix Gene Ventralizes Whole Embryos and Induces Blood. To address the function of these homeoproteins, we injected synthetic mRNA into embryos at the one-cell stage and examined the whole embryo phenotype after 2 days of development (Fig. 4). Ectopic expression of each Mix protein led to the ventralization of the embryo, including excessive blood formation (ref. 7 and data not shown). Injection of mRNA or an expression plasmid (pcDNA3.0, Invitrogen) encoding Mix family members in animal caps stimulated with basic FGF led to the production of globin mRNA (Fig. 5*A*) and red blood cells by *O*-dianisidine staining (Fig. 5*B*). Based on the overlapping expression domains of the Mix genes, their ability to heterodimerize, and the known role of Mix.1 in ventral mesoderm formation, these data suggest that Mix.3 and Mix.4 may cooperate with Mix.1 to regulate ventral patterning and the early stages of hematopoiesis in the vertebrate embryo.

Role of the Mix Genes in Endoderm. Intrigued by the high level of expression of the Mix genes in endoderm, we next examined the ability of Mix family members to induce endoderm in animal pole ectoderm (Fig. 6*A*). Mix.1 expression alone resulted in very little or no endodermal marker expression. Ectopic expression of Mix.3 or Mix.4 induced high levels of endodermal markers including endodermin [edd; a pan-endodermal marker (34)], GATA-5 [a primitive embryonic gut and heart marker (35, 36, 37)], IFABP [intestinal fatty acid-binding protein, a mid-gut marker (38)], XSox17α [a presumptive endoderm marker shown to mediate endoderm formation in *Xenopus* (39)], and XlHbox8 [a pancreatic marker (40)]. In contrast, expression of the Mix genes alone, without added growth factors, was not sufficient to induce mesodermal markers, including Xbra (41) (at stage 12.5), cardiac actin, Xnot [a notochord marker (42)], and α-globin (at stage 36, Fig. 6*A* and data not shown). The BMP-signaling molecule Smad1 (5) and the dominant negative Mix.1 construct M11 (7) were included as negative controls and did not lead to expression of either endoderm or mesoderm markers in the animal pole explants.

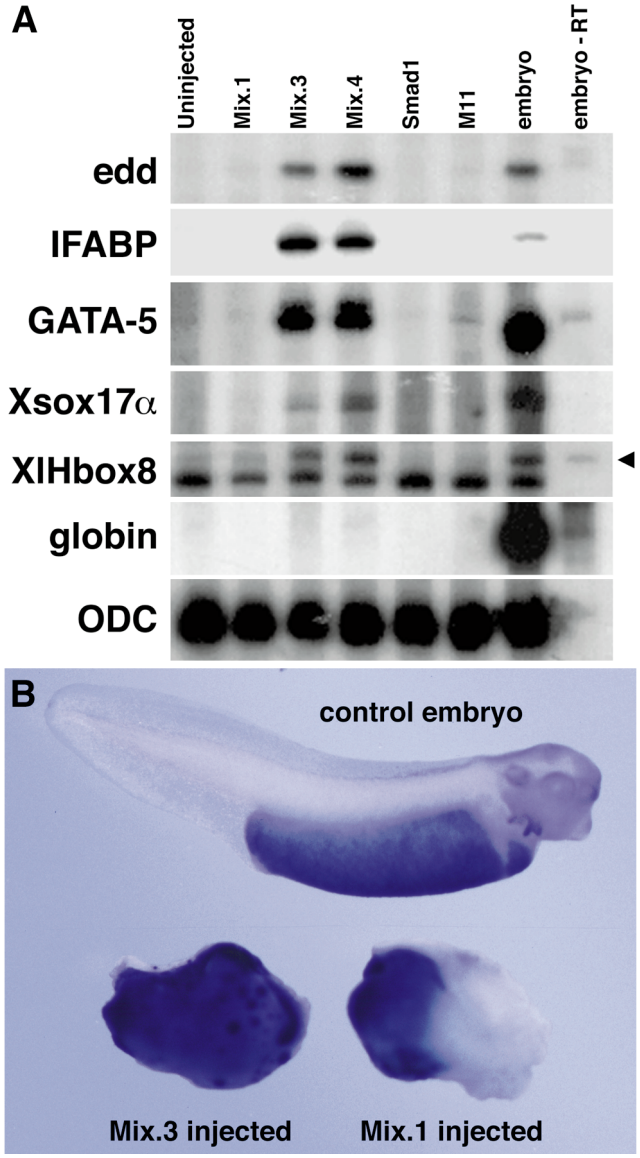

FIG. 6. Expression of Mix family proteins and markers of endoderm differentiation in animal pole explants and whole embryos. (*A*) Expression of Mix family members leads to the induction of endoderm. Embryos were injected at the one-cell stage with 1 ng of each RNA. Smad1 and M11 are included as negative controls. Animal pole explants were dissected at stage 8 and cultured to sibling stage 36. RT-PCR analysis demonstrated that Mix.3 and Mix.4 induce endodermal markers edd, IFABP, GATA-5, XSox17α, and XlHbox8. Overexpression of Mix family members, without added growth factors, failed to induce mesoderm markers such as globin. (*B*) Whole embryo *in situ* hybridization for edd. Embryos were injected with Mix RNA (500 pg/blastomere) at the two-cell stage, cultured to sibling control stage 38, and then fixed and stained for edd expression by *in situ* hybridization. Mix.3 led to ectopic edd expression (54/55 injected embryos) whereas Mix.1 did not (0/37 injected embryos).

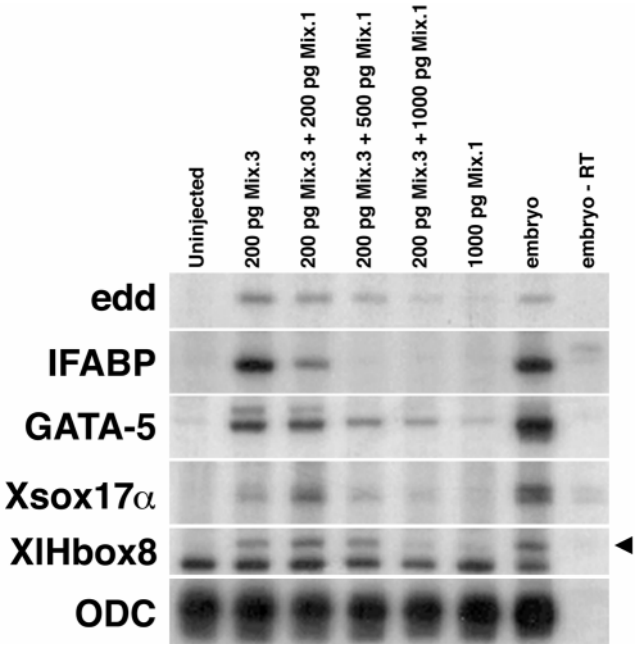

FIG. 7. Mix.1 antagonizes Mix.3 induction of endoderm markers. Embryos were injected at the one-cell stage with a constant dose of Mix.3 RNA (200 pg) and an increasing amount of Mix.1 RNA (200–1000 pg). Animal poles were explanted at stage 8 and cultured for 2 days (stage 36). Endoderm marker expression was determined by RT-PCR analysis.

To examine the effects of overexpressing Mix.3 (or Mix.4) on endoderm formation in the whole embryo, we injected Mix.3 RNA at the two-cell stage and performed *in situ* hybridization for endodermin at stage 38. Ectopic expression of Mix.3 resulted in characteristic axial abnormalities (see Fig. 4), with extensive endodermin staining, often in a patchy distribution. In contrast, ectopic expression of Mix.1 did not lead to extensive ectopic endodermin expression (Fig. 6*B*). Thus, Mix.3 and Mix.4 are potent and direct inducers of endoderm markers.

Heterodimerization of Mix Proteins and Embryonic Patterning. PAX class homeodomains bind DNA cooperatively (26, 43, 44) and our data suggest that Mix.3 or Mix.4 homodimers can regulate gene expression in endoderm. The expression cloning assay (FROGS) used to isolate the Mix genes indicates that these genes can form heterodimers with the Mix.1 homeodomain on DNA (Figs. 1*B* and 3*A*). As Mix.1 expression alone did not induce expression of endodermal markers, we tested whether coexpression of Mix.1 could affect the ability of Mix.3 to induce endoderm in animal pole explants. Coinjection of increasing doses of Mix.1 RNA with a constant level of Mix.3 RNA led to a decrease in expression of endoderm markers edd, IFABP, GATA-5, XSox17α, and XlHbox8 (Fig. 7). High levels of Mix.1 are likely to favor the formation of Mix.1 homodimers on target sites. Mix.1 on its own is insufficient to induce endoderm and may block Mix.3 or Mix.4 interaction with a target site. IFABP expression is blocked at a lower dose of Mix.1 than the other endodermal markers examined. As IFABP is a specific marker of mid-gut, this may indicate a role for the Mix.1/Mix.3 heterodimer in patterning endoderm. Thus, analogous to the regulation of dorsal-ventral mesoderm patterning by the interaction of Mix.1 with siamois (7), Mix.1 may regulate the formation of endoderm by interacting with Mix.3 (or Mix.4).

DISCUSSION

FROGS Assay. We have developed an expression cloning technique called FROGS to isolate transcription factors and their heterodimeric partners. The technique makes use of small pools of protein as "bait" for a gel mobility shift analysis. PAX-like homeoproteins bind to and bend DNA, which allows cooperative interaction of heterodimeric partners (44). Using the FROGS assay, we isolated two new partners for Mix.1. The FROGS assay can also be used to isolate transcription factors without the need for a heterodimeric partner. For example, using the activin-responsive element [goosecoid distal element (45)] as a probe for the FROGS technique, we cloned the transcription factors Xotx2 (46) and Xdll2 (47) (data not shown). Targeted disruption of the mouse Otx2 gene abolishes goosecoid expression in the developing embryo (48). Xdll2, a frog homolog of *Drosophila distalless*, is down-regulated in ectoderm by activin (47, 49). These data underscore the FROGS assay as an excellent tool for identifying transcription factors that are biologically relevant.

Growth Factor Signaling and Mix Proteins. Each TGF-β family member studied induces the expression of the Mix factors, albeit at different levels. FGF does not induce Mix.1, 2, or 3 expression; however, it does induce Mix.4 expression. Given the overlapping pattern of Mix gene expression in the embryo and the overlapping induction by growth factors, it remains to be defined which signaling cascades in the embryos lead to the induction of Mix.1. Activin stimulates the induction of Mix.2 by activating cytosolic Smad2 which then interacts with Smad4, translocates to the nucleus, and binds to the activin response element with a forkhead protein called FAST1 (33). Activin and Vg1 are potent inducers of all of the Mix genes and, yet, each of the Mix genes leads to ventralization upon overexpression. This suggests that activin stimulation not only leads to dorsal mesoderm formation but also to a spectra of ventralizing activities. The ventralizing effects of Mix expression may be overcome by the prominent dorsalization effects of activin.

Role of the Mix Family in Ventral Mesoderm Formation and Hematopoiesis. In concert with mesoderm induction by either FGF or activin, each Mix protein is able to induce erythroid differentiation in animal pole explants. We recently showed that Mix.1 induces the expression of SCL (14), a basic helix–loop–helix transcription factor thought to be a master regulator of vertebrate hematopoiesis (50). Dorsal marginal zones injected with Mix.1 typically lose dorsal structures, whereas dorsal marginal zones injected with SCL develop blood cells, but maintain dorsal structures. Thus, a cascade of events from Mix family members to SCL is likely to regulate early embryonic hematopoiesis.

Heterodimerization and PAX Activity. Our previous studies on the Mix.1 and siamois interaction demonstrated how heterodimerization of this PAX family can have profound consequences on embryonic patterning. Mix.1 increases cell adhesion and polarization in blastocoel roof cells and interaction of other PAX family members (such as goosecoid and siamois) can modulate these activities (51). Here, we demonstrate the functional consequence of Mix family member dimerization on blood and endoderm formation *in vivo*. Similar to Mix.1, overexpression of Mix.3 and 4 ventralizes embryos and induces hematopoiesis in FGF-treated animal caps. As each of these factors is expressed in ventral mesoderm, the formation of homo- and heterodimers is likely to regulate the initiation of the blood program. We have also demonstrated that Mix.1 can heterodimerize with Mix.3 (and Mix.4) and alter Mix.3-directed endoderm marker expression. Thus, TGF-β signaling may stimulate cooperative and antagonistic effects of homo- and heterodimerization of Mix family members, regulating the production of blood and endoderm.

It will be of interest to identify the transcriptional targets of the Mix homeoproteins, particularly with regard to endoderm formation. GATA-1 and GATA-2, genes required for normal hematopoiesis (52, 53), are potential targets of Mix.1. GATA-4, -5, and -6, which are expressed in primitive gut, are

potential targets of Mix.3 and Mix.4. We show that both Mix.3 and Mix.4 induce GATA-5 expression in animal pole ectoderm (Fig. 6*A*), and preliminary evidence suggests that expression of GATA-binding proteins leads to the induction of endoderm markers in animal pole explants (Y. J. Lee and R. Shivdasani, personal communication). Evans and colleagues (54) demonstrate that GATA-4, -5, and -6 directly stimulate expression of IFABP. Furthermore, mouse embryonic stem cells null for the GATA-4 gene have a block in visceral endoderm formation *in vitro* (55), while a recent study in *Caenorhabditis elegans* has shown a major role for the GATA-binding proteins in the genesis of gut (56). Thus, transcriptional targets of Mix proteins may include the GATA-binding proteins, suggesting a model of similar molecular pathways (TGF-β signaling $\rightarrow$ Mix expression $\rightarrow$ GATA-binding protein expression) for the development of both ventral mesoderm and endoderm.

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