

Xenopus nodal-related signaling is essential for mesendodermal patterning during early embryogenesis

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SUMMARY

Previously, we showed that *Xenopus nodal*-related factors (Xnrs) can act as mesoderm inducers, and that activin induces *Xnr* transcription, suggesting that Xnrs relay or maintain induction processes initiated by activin-like molecules. We used a dominant negative cleavage mutant Xnr2 (cmXnr2) to carry out loss-of-function experiments to explore the requirement for Xnr signaling in early amphibian embryogenesis, and the relationship between activin and Xnrs. cmXnr2 blocked mesoderm induction caused by *Xnr*, but not *activin*, RNA. In contrast, cmXnr2 did suppress mesoderm and endoderm induction by activin protein, while *Xnr* transcript induction was unaffected by cmXnr2, consistent with an interference with the function of Xnr peptides that were induced by activin protein treatment. The severe hyperdorsalization and gastrulation defects caused by Xnr2 in whole embryos were rescued by cmXnr2, establishing a specific antagonistic relationship between the normal and cleavage mutant proteins.

Expression of cmXnr2 resulted in delayed dorsal lip formation and a range of anterior truncations that were associated with delayed and suppressed expression of markers for dorsoanterior endoderm, in which the recently recognized head organizer activity resides. Reciprocally, Xnr2 induced dorsoanterior endodermal markers, such as *cerberus*, *Xhex-1* and *Frzb*, in animal cap ectoderm. The migratory behavior of head mesendoderm explanted from cmXnr2 RNA-injected embryos was drastically reduced. These results indicate that Xnrs play crucial roles in initiating gastrulation, probably by acting downstream of an activin-like signaling pathway that leads to dorsal mesendodermal specification, including setting up the head organizer.

Key words: Xnr, Nodal, Anterior endoderm, Activin, Organizer, Head induction, *Xenopus laevis*, Mesoderm induction, Gastrulation, Cell migration

INTRODUCTION

The TGF β -related *nodal*-related genes have been implicated in mesoderm formation based on analysis of the original mouse *nodal* null mutant and overexpression experiments in *Xenopus*. In homozygous *nodal* mutant mouse embryos, little or no mesodermal tissue is detected, a recognizable primitive streak is not formed, and embryonic development arrests at early gastrulation (Conlon et al., 1994). In *Xenopus*, four Xnrs (*Xnr1-4*) have been isolated (Jones et al., 1995; Joseph and Melton, 1997; Smith et al., 1995). All are expressed within the Spemann organizer region and can dorsalize ventral mesoderm. The four Xnrs have different functions in terms of mesoderm induction, neural induction and left-right axis determination. Xnr1, Xnr2 and Xnr4, like mouse *nodal*, can induce mesoderm in animal caps (Jones et al., 1995; Joseph and Melton, 1997). In contrast, Xnr3, which has a structurally divergent C terminus, lacks mesoderm inducing activity but induces anterior neural tissue (Hansen et al., 1997). The unilateral expression of *Xnr1* in lateral plate mesoderm, and the results of ectopic expression experiments suggest a function similar to mouse and chick *nodal* in regulating asymmetric morphogenesis (Levin and Mercola, 1998).

Mouse *nodal* displays transient expression in the extraembryonic anterior visceral endoderm (Conlon et al., 1994; Varlet et al., 1997b). By studying chimeric mouse embryos, *nodal* expression in this region was shown to be critical for anterior central nervous system development (Varlet et al., 1997b). *Xnr1/Xnr2* expression is also first observed in the endodermal region, before becoming localized to a subregion of the Spemann organizer (Jones et al., 1995). The significance of the initial endodermal expression remains an open question. Recently, data have been accumulating supporting the idea that the amphibian embryo has separate head and trunk organizers (Spemann, 1931). Noggin, chordin, and follistatin seem to be involved in trunk organizer activity by antagonizing BMP signals (Bouwmeester and Leyns, 1997): ventral misexpression in early *Xenopus* embryos induces secondary axes lacking the head. These genes are expressed in the classical Spemann organizer region, the dorsal blastopore lip (Harland and Gerhart, 1997). In contrast, *cerberus* and *dickkopf-1* (*dkk-1*) have head organizer activity (Bouwmeester et al., 1996; Glinka et al., 1998) and are expressed in the deep dorsoanterior endoderm, a possible functional equivalent of the mouse anterior visceral endoderm (Beddington and Robertson, 1998; Bouwmeester and Leyns, 1997). Little is known about

the mechanism controlling the formation of head organizer endoderm.

We have investigated the involvement of Xnrs in early amphibian embryogenesis. In *Xenopus*, although genetic tools for reducing or inactivating zygotic gene function are not readily available, dominant-negative approaches have allowed loss-of-function experiments (Lagna and Hemmati-Brivanlou, 1998). For TGF β -1, activin, and several BMPs, alteration of a proteolytic cleavage site dividing the pro- and mature ligand domains of the precursor peptide produces dominant-negative reagents that can heterodimerize with their normal counterparts in co-producing cells, thereby preventing the release of active dimers (Hawley et al., 1995; Lopez et al., 1992; Nishimatsu and Thomsen, 1998; Wittbrodt and Rosa, 1994). We generated a similar cleavage site-mutated Xnr2 mutant (cmXnr2) that fulfills several criteria expected of a specific inhibitor of Xnr signaling. Introduction of cmXnr2 RNA into early *Xenopus* embryos delayed dorsal lip formation and caused anterior truncations, accompanied by the delayed and suppressed induction of mesodermal markers, in particular those for head organizer endoderm. Reciprocally, these head organizer markers were induced by nodal and Xnrs in animal caps. Moreover, cmXnr2 blocked the induction of mesoderm and endoderm by activin protein in ectodermal explants. Considering that activin induces *Xnr* transcription (Jones et al., 1995), and that dominant-negative forms of the activin type IIB receptor and its intracellular mediator Smad2 also induce anterior defects similar to those seen in cmXnr2-injected embryos (Dyson and Gurdon, 1997; Hoodless et al., 1999), our results suggest that nodal-related signaling plays an essential role in early amphibian patterning, perhaps by mediating the signaling of an earlier expressed activin-like molecule.

MATERIALS AND METHODS

cmXnr2 construction

Clal and *XhoI* sites placed at the 5' and 3' ends of the Xnr2 protein-coding region by PCR allowed insertion into pCS2+. The putative Xnr2 cleavage site, RRPRR (AGAAGACCCCGGCCG), was altered to GVDGG (GGAGTCGACGGGGG) by overlapping PCR, similar to a previous strategy (Hawley et al., 1995): the 5' portion was amplified from pCS2+Xnr2 plasmid using SP6 primer and the downstream primer 5'-GACGGGTCGACTCTGGTATCTGTACCTGACTG-3' (*SalI* site underlined), and the 3'-portion was made using the upstream primer 5'-GACGGGTCGACGGGGCAACCAAAAACGAAAAT (*SalI* site underlined) and T7 primer. Gel-purified fragments were *SalI*-digested, ligated, and reamplified with SP6/T7 primers. The final gel-purified product was reinserted into pCS2+ using *Clal/XhoI*. High fidelity PCR used *Pfu* polymerase (Stratagene); sequencing detected no additional alterations.

Embryo manipulation

In vitro fertilization and culture of embryonic tissues were performed as reported by Kay and Peng (1991). Embryos were staged according to Nieuwkoop and Faber (1967). Blastula animal cap explants were dissected in 1 \times regular Barth's solution (RBS), and transferred to 0.75 \times normal amphibian medium (NAM) for development until later stages. For activin protein and retinoic acid (RA) treatment, purified human activin A (gift from Genentech), or all *trans*-RA (Sigma) dissolved in DMSO (0.1 M), was added to the culture medium to a final concentration of 1-3 ng/ml or 2 μ M, respectively. Dorsal and

ventral marginal zones (DMZs and VMZs) were dissected at stage 10.25-10.5 with the indentation of the blastopore lip marking the dorsal side.

Microinjection of synthetic mRNAs

Xnr1, *Xnr2*, *Xnr4* and cmXnr2 constructs in pCS2+ were linearized with *Asp718*, and capped RNA made using the mMessage mMachine Kit (Ambion). *Xnr3*, Cm-activin, BVg1, *noggin*, *chordin* and Xnr2^{C333S} RNAs were synthesized as described by the supplier. For animal cap assays, 10 nl (doses given elsewhere) were injected at the 1- to 2-cell stage. For marginal zone assays, 5 nl/blastomere were injected into two dorsal or ventral blastomeres at the 4-cell stage. For vegetal explant assays, 5 nl of RNA or DNA were injected vegetally into all blastomeres at the 4-cell stage.

Whole-mount in situ hybridization

In situ hybridization with digoxigenin-labeled *XOtx2*, *XEn2*, *Krox20* and *HoxB9* probes was performed according to Harland (1991) with the BM purple alkaline phosphatase substrate (Boehringer Mannheim).

Cell migration assay

Bovine plasma fibronectin (FN; Sigma) was suspended in 1 \times RBS (100 μ g/ml). 12-well plastic dishes (Falcon) were covered with 500 μ l/well of the solution (room temperature, two hours), then replaced with 1 \times RBS plus 10 mg/ml bovine serum albumin for at least 1 hour to block nonspecific cell attachment. Involved prospective head mesoderm was dissected at stage 10.25 as described by Keller (1991); Winklbauer (1990), allowed to heal (15 minutes), transferred onto an FN-coated well, and cultured in 1 \times RBS. Explant morphology was photomicroscopically recorded 1 and 3 hours after explantation.

RT-PCR

Total RNA was isolated from embryonic tissues at stages indicated, and RT-PCR carried out as described by Wilson and Melton (1994). Primers for *FGFR*, *Xbra*, *NCAM*, *XOtx2*, *HoxB9*, muscle *actin*, α *T1-globin*, *chordin*, *gooseoid*, *cerberus*, *Frzb*, *dkk-1*, *Xsox17 β* , *Mixer*, *Xnr1* and *endodermin* were synthesized according to previous reports (details available upon request). Other primers used here were: *XIHbox8* (F: 5'-GGCAGTCCTCCAGACATCTC-3'; R: 5'-AGCTCTACTCTCCTTGGTCT-3'), *Siamois* (F: 5'-CAACAGTCTGCAGTCAACAGG-3'; R: 5'-TGCTCTTGGCAGATGTCTGG-3'), and *Xlim-1* (F: 5'-ATGGTTCAGTGTGCTGGATGC-3'; R: 5'-TCGCCCAA-GGTTTTGGTTCG-3'). Conditions under which each primer pair provided amplification in the linear range were empirically determined. All RT-PCR data presented here are representative of at least 3 independent experiments.

RESULTS

cmXnr2 antagonizes Xnr signaling

To generate a putative dominant negative reagent suitable for exploring in vivo roles of Xnrs, we mutated the presumed Xnr2 cleavage site from RRPRR to GVDGG (cmXnr2; Fig. 1A), and tested its activity by animal cap assay (Fig. 1B). As previously reported (Jones et al., 1995), Xnr2 overexpression induced elongation and differentiation of dorsal mesoderm (e.g. notochord and muscle). In contrast, cmXnr2 did not induce cap elongation, but blocked elongation induced by Xnr2 or Xnr4. Significantly, even 200-fold excesses of RNA encoding an activin cleavage mutant (Cm-activin), which effectively prevents cleavage of co-expressed activin, and blocks animal cap elongation induced by *activin* RNA (Hawley et al., 1995), did not block Xnr2-induced cap extension. This result indicates

Table 1. cmXnr2 rescues normal development in Xnr2-injected embryos

RNA injected (pg)	<i>n</i>	Survived	Completely rescued	Partially rescued	Gastrulation defects	Others
Xnr2 (20) + β -globin (50)	44	42	0%	2%	94%	4%
+ cmXnr2 (20)	47	41	73%	19%	8%	0%
+ cmXnr2 (50)	45	40	52%	37%	3%	8%

Indicated amounts of RNA were injected into two dorsal blastomeres at the 4-cell stage, and the embryos were analyzed for rescue of the A-P axis at stage 35. Completely rescued axes have a cement gland and eyes, which were reduced or missing in partially rescued embryos.

that the pre-pro forms of Xnr and activin do not physically interact.

These findings were confirmed molecularly (Fig. 1C). Wild-type *Xnr1*, *Xnr2* and *Xnr4* RNAs all induced general (*Xbra*) and dorsal (muscle *actin*) mesodermal markers. cmXnr2 RNA induced neither marker, but blocked their induction by Xnr1, Xnr2 and Xnr4. As expected from its inability to block Xnr2-induced animal cap extension, Cm-activin did not block mesodermal marker induction by Xnr2 (not shown). We also tested another mutant, *Xnr2*^{C333S}, in which the second conserved cysteine of the mature region was altered to serine (Hansen et al., 1997). By analogy with activin (Mason, 1994), this residue is critical for the 'cysteine knot' structure of TGF β -related ligands. As previously shown (Hansen et al., 1997), *Xnr2*^{C333S} did not induce mesodermal markers in animal caps (Fig. 1C). Unlike cmXnr2, however, *Xnr2*^{C333S} did not block cap elongation (Fig. 1B) or marker induction (Fig. 1C) by Xnr2; hence, *Xnr2*^{C333S} is not a dominant-negative reagent. Another Xnr2 variant, *Xnr2*^{C368S}, was made with an altered fourth cysteine of the ligand domain. In activin, the equivalent residue is essential for ligand dimerization and biological activity (Mason, 1994). Surprisingly, Xnr2 and *Xnr2*^{C368S} have similar mesoderm-inducing activities (Fig. 1C), suggesting that disulfide dimerization through this residue is dispensable for Xnr2 activity. cmXnr2 was also tested for specificity against two other TGF β -related ligands implicated in early developmental processes: Vg1 (as the active chimera, BVg1) and activin. Marker induction by BVg1 or *activin* RNA was not blocked by cmXnr2 (Fig. 1C), even at large excess (500-

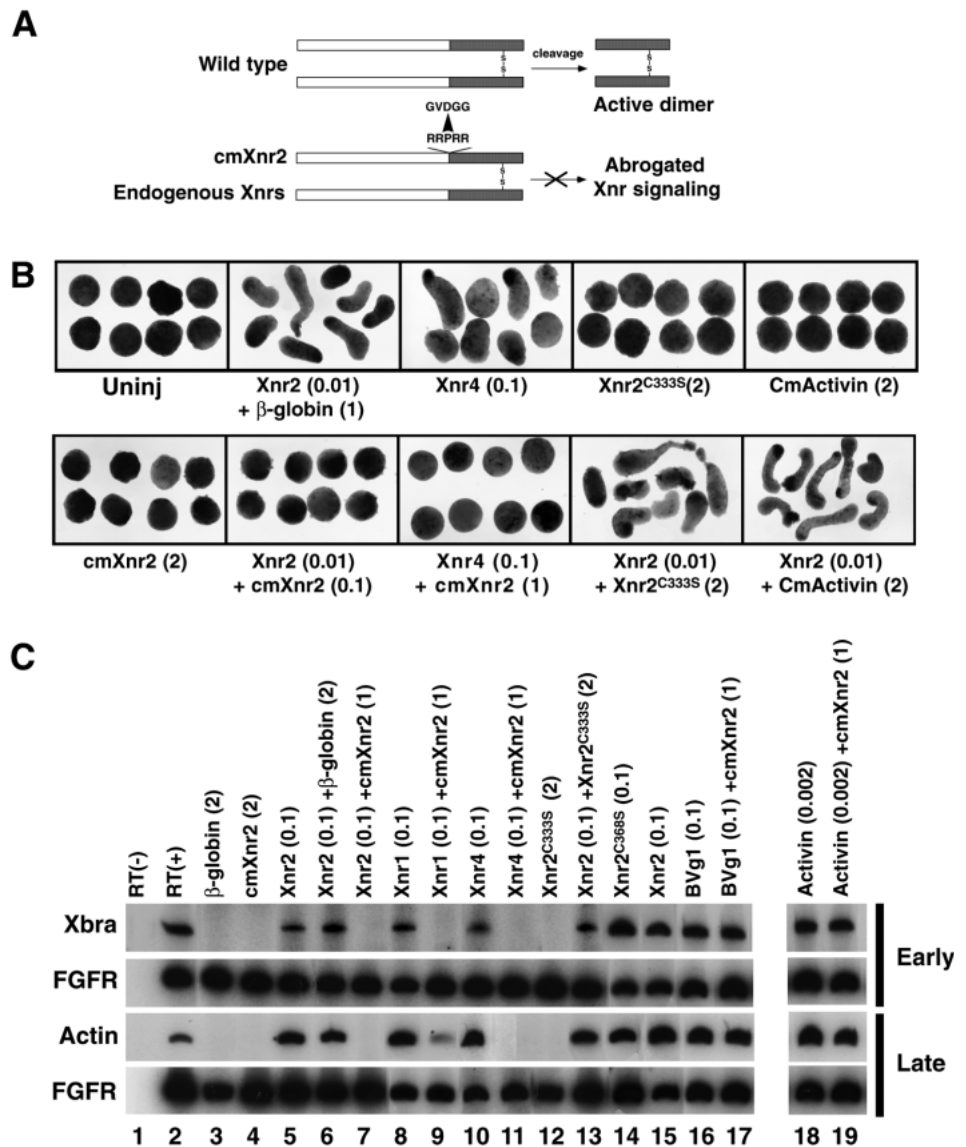


Fig. 1. cmXnr2 specifically blocks mesoderm induction by Xnrs. (A) Schematic representation of putative dominant negative block of Xnr function by cmXnr2 (open box, pro region; shaded box, mature ligand region). The cleavage site alteration is indicated. TGF β -type molecules form dimers (disulfide bond indicated). Protease cleavage releases active dimeric ligand; dimerization of cmXnr2 with Xnrs abrogates this process. (B) cmXnr2 blocks elongation of animal caps induced by Xnr2 and Xnr4 (stage 25). Parentheses: RNA dose (ng). β -globin was a control RNA. (C) cmXnr2 blocks mesoderm induction by *Xnr1*, *Xnr2* and *Xnr4*, but not *activin* or BVg1 RNA. RT-PCR analysis of animal cap RNA for the pan-mesodermal marker, *Xbra* (stage 10.5), and dorsal mesodermal marker, muscle *actin* (stage 25). Parentheses: RNA dose (ng). *Fibroblast growth factor receptor* (*FGFR*) RNA was assessed as a loading control. RT(+) and RT(-) indicate whole embryo RNA transcribed with or without reverse transcriptase (RT).

fold in the case of activin). These results agree with the inability of Cm-activin to block Xnr2-mediated induction, and provide evidence for cmXnr2 specificity.

We next tested whether cmXnr2 and Xnr2 are specific antagonists *in vivo*. As reported previously, dorsal injection of Xnr2 RNA into embryos severely disrupted morphogenesis (Fig. 2A). The resulting embryos showed no recognizable anteroposterior (A-P) or dorsoventral (D-V) axis, and often developed protrusions typical of hyperdorsalization. When cmXnr2 RNA was co-injected with Xnr2 RNA, A-P and D-V patterning were restored to various extents (Fig. 2B; Table 1). The dose range of cmXnr2 RNA (1-5 fold excess over Xnr2) rescuing Xnr2 RNA-injected embryos was similar to that which blocks Xnr2-mediated mesoderm induction in animal caps (Fig. 1B,C). As described later, cmXnr2 itself also causes gastrulation defects. Coupled with the potential for unequal partitioning or translation of coinjected RNAs throughout the embryo, and because these are secreted factors, it is unlikely that Xnr2/cmXnr2 would in every case precisely negate each other's effects. Nevertheless, our finding that the majority were completely rescued supports the view that cmXnr2 can block Xnr signals in a dominant negative manner.

cmXnr2 blocks mesendoderm induction by exogenous activin protein

Activin, Vg1 and Xnrs have been implicated in mesendoderm induction in *Xenopus* (Harland and Gerhart, 1997; Jones et al., 1995; Joseph and Melton, 1997, 1998; Thomsen and Melton, 1993), although it remains unclear whether these factors act in parallel or sequentially. The finding that activin protein induces Xnr1 and Xnr2 expression in animal caps (Jones et al., 1995) suggests that Xnrs relay activin-like induction *in vivo*. We therefore tested if cmXnr2 affected mesendodermal induction by activin protein, reasoning that interference would indicate that activin-initiated Xnr induction is a significant factor contributing to the cell fate changes in activin-treated caps. To make a fair comparison with the *activin* RNA injections described above, *activin* RNA (2 pg) and activin protein (1-3 ng/ml) doses were used that induced similar *Xbra* and *actin* expression levels.

cmXnr2 RNA dose-dependently suppressed the effects of activin protein on animal caps: both elongation (Fig. 3A), and mesodermal (*Xbra*, *chordin*, *Xlim-1*, *actin*) or endodermal (*cerberus*, *Mixer*, *endodermin*, *XIHbox8*) marker expression (Fig. 3B). In contrast, the level of Xnr1 RNA induced by activin was unaffected even by large amounts of cmXnr2 RNA. Since Xnr1 and Xnr2 induce all of these markers in caps (see below), we conclude that the suppression results from titration of Xnrs by cmXnr2. Although the sensitivity of animal caps to activin protein and cmXnr2 differed depending on embryo batch, substantial suppression of mesendodermal markers by cmXnr2 was observed under similar conditions in three independent experiments.

This effect clearly differs from the immunity of *activin* RNA-mediated mesendoderm induction to cmXnr2 (Fig. 1C, lanes 18, 19).

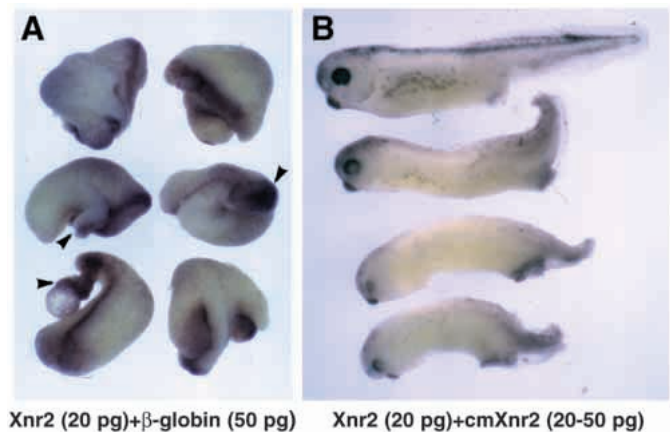


Fig. 2. Rescue of Xnr2-induced gastrulation defects by co-injected cmXnr2 RNA. (A) Embryos injected into two dorsal blastomeres with Xnr2 and control β -globin RNAs develop gross gastrulation defects, absence of recognizable axes and protrusions (arrowheads). (B) Examples of axial rescue by co-injected cmXnr2 RNA.

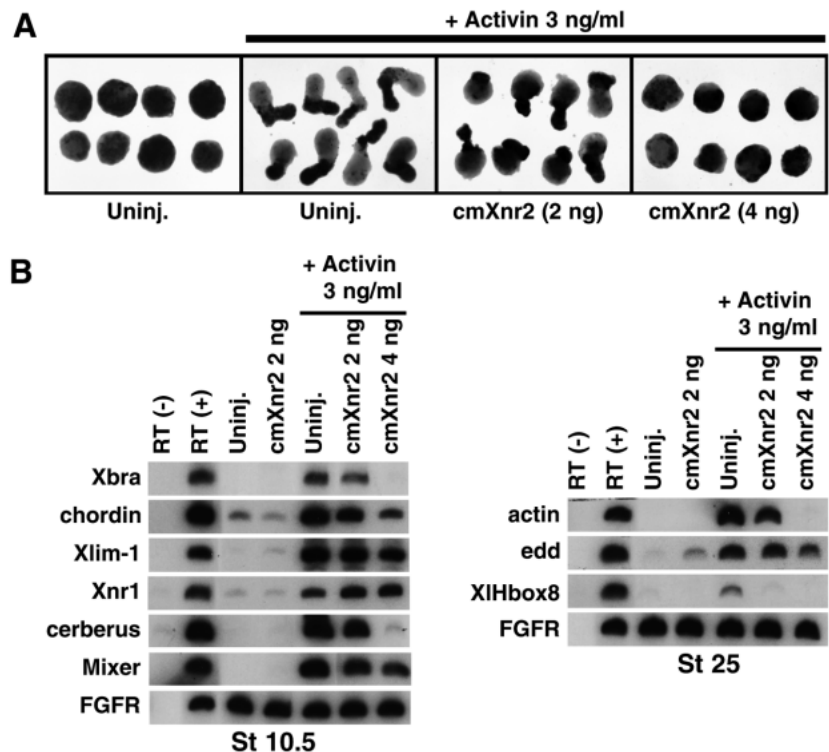


Fig. 3. cmXnr2 blocks mesendoderm induction by activin protein. (A) cmXnr2 blocks animal cap elongation induced by activin protein. Animal caps explanted from embryos injected with or without cmXnr2 RNA were dissected and treated with 3 ng/ml of human activin A protein, and analyzed at stage 22. (B) cmXnr2 blocks mesendoderm induction by activin protein. RT-PCR analysis of stage 10.5 and stage 25 animal cap RNA for mesodermal (*Xbra*, *chordin*, *Xlim-1*, *Xnr1* and muscle *actin*) and endodermal (*cerberus*, *Mixer*, *edd/endodermin*, and *XIHbox8*) markers. Note that induction of *Xnr1* by activin protein is not blocked by cmXnr2.

Fig. 4. cmXnr2 induces anterior truncations in *Xenopus* embryos. (A,B) cmXnr2 delays dorsal lip formation and (C) impairs neurulation. Control (left) or cmXnr2 (right) RNA was injected into two dorsal blastomeres at the 4-cell stage (2×1 ng). White arrowheads: dorsal lip. Asterisk: normal blastopore. Red arrowheads: elevating neural folds in control embryos. (D) Series of anterior truncations induced by cmXnr2 RNA (500 pg). I, normal uninjected embryo at stage 35; II-VI, embryos injected with cmXnr2 RNA into two dorsal blastomeres. The distribution of resultant embryos between the classes is indicated (%). Characteristic features and estimated anteroposterior deficiencies index (APDI) of the classes are: I, normal embryo (APDI 5); II, reduced forehead and slightly cyclopic (APDI 4); III, cyclopia and reduced cement gland (APDI 3); IV, no eye pigment or no cement gland (APDI 2); V, truncation of posterior and anterior axial structures (APDI 1), and VI, significant A-P axis shortening (APDI 0). (E) In situ hybridization of cmXnr2 RNA-injected embryos with region-specific neural markers (*XOtx2*, forebrain/midbrain; *XEn2*, midbrain/hindbrain boundary; *Krox20*, hindbrain rhombomeres 3 and 5). Yellow arrowheads indicate expression domains.

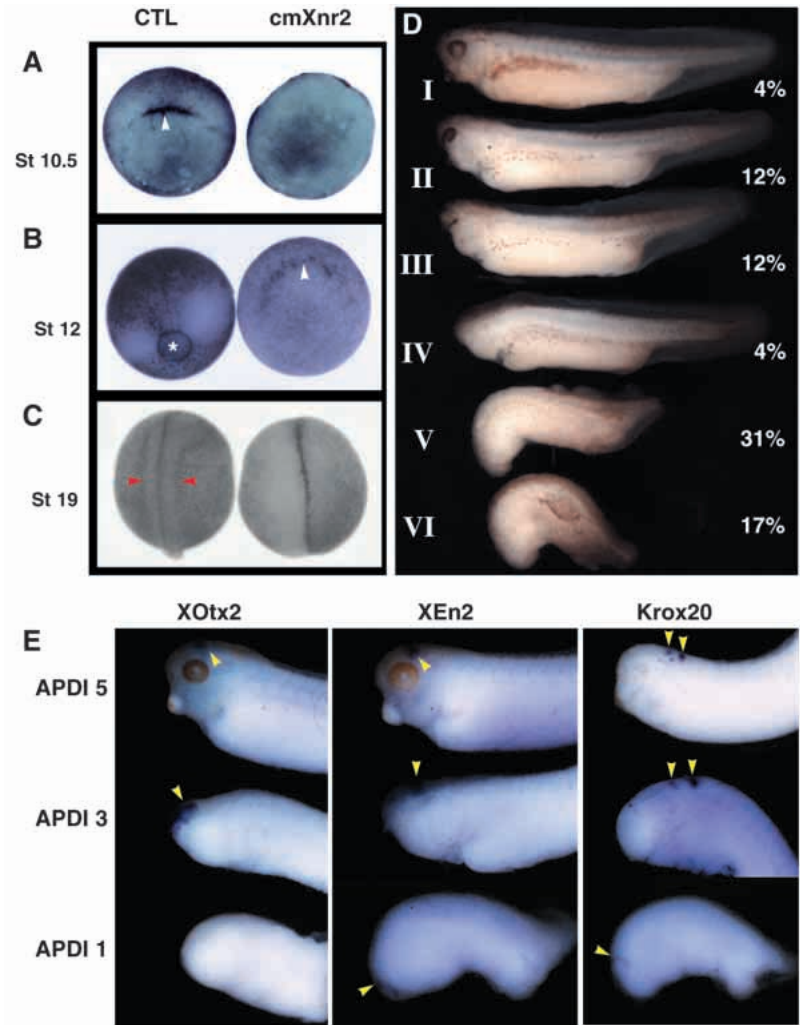
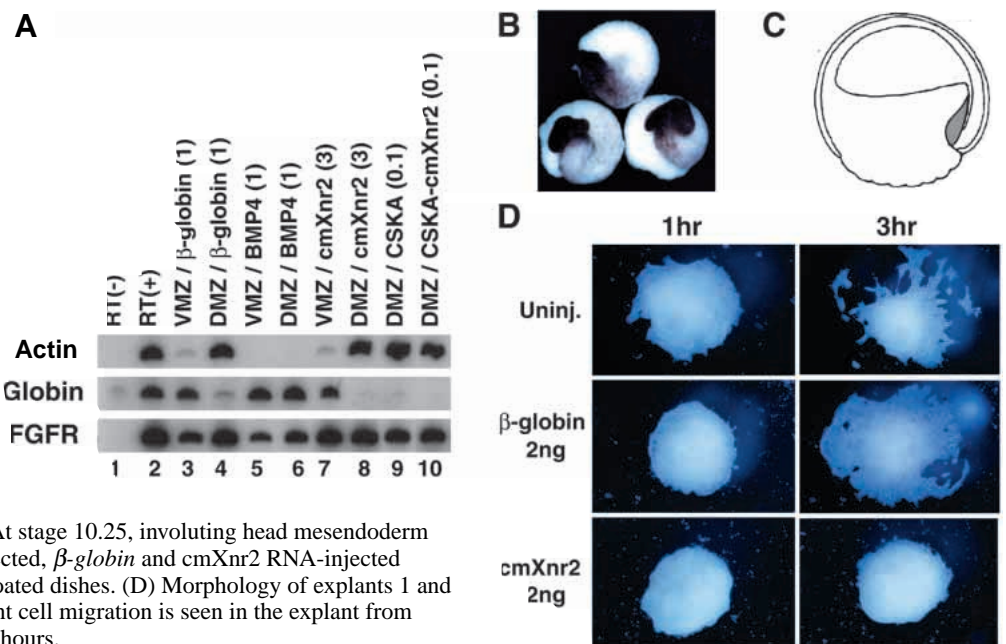


Fig. 5. cmXnr2 does not grossly ventralize mesoderm, but affects gastrulation movements. (A) Dorsal and ventral marginal zones (DMZ, VMZ) were analyzed at stage 25 by RT-PCR for expression of the dorsal mesodermal marker, muscle *actin*, and the ventral mesodermal marker, *globin*. Unlike *BMP4* RNA, neither cmXnr2 RNA nor pCSKA-cmXnr2 induced *globin* or reduced *actin* expression in DMZs. Parentheses: RNA or DNA dose (ng). *FGFR* was used as a loading control. (B) 'Exogastrulae' induced by cmXnr2 RNA injected into two dorsal blastomeres (500 pg/blastomere) at the 4-cell stage. (C) At stage 10.25, involuting head mesendoderm (gray region) was dissected from uninjected, β -globin and cmXnr2 RNA-injected embryos, and cultured on fibronectin-coated dishes. (D) Morphology of explants 1 and 3 hours after explantation. No significant cell migration is seen in the explant from cmXnr2 RNA-injected embryos after 3 hours.



Xnr interference causes anterior truncations

We next injected cmXnr2 RNA into two dorsal or ventral blastomeres at the 4-cell stage to examine the effects of interfering with Xnr function during *Xenopus* embryogenesis. The first visible effect was a delayed dorsal lip formation. When control-injected embryos had already reached late gastrula, a dorsal lip was first detected in cmXnr2 RNA-injected embryos (Fig. 4A,B); an approximately 2-hour delay. At higher cmXnr2 doses, the blastopore often did not close (not shown). When well-formed neural folds were observed in neurula stage controls, they were malformed in cmXnr2 RNA-injected embryos, indicating impaired neurulation, although a partial neural groove was discernible (Fig. 4C). At these stages, cmXnr2 RNA-injected embryos often displayed absence or severe reduction of the cement gland, a marker of very anterior induction (not shown).

At tadpole stages, cmXnr2 RNA-injected embryos were variously affected in anterior structures (Fig. 4D). In the absence of an extant scoring system for cmXnr2-specific defects, we categorized the injected embryos from type I to VI. This grouping is more similar to the anteroposterior deficiencies index (APDI) (Wallingford et al., 1997) than the more familiar dorsoanterior index (DAI) (Kao and Elinson, 1988). Since cmXnr2 did not grossly ventralize embryos (see below), the DAI scoring system, which includes reductions of dorsoventral and anteroposterior axes, seemed less appropriate. The severity of anterior truncations was dose-dependent (Table 1). At modest RNA doses (200–500 pg/embryo), a wide range of anterior truncations (APDI 4–0) was observed, while higher doses (1 ng per embryo or more) increased the incidence of microcephaly or anencephaly. At the extreme, anterior and posterior structures were both affected, shortening the body axis. Unlike ventralizing agents that cause anterior truncations only when misexpressed dorsally, ventral injection of cmXnr2 RNA caused gastrulation defects superficially similar to those caused by dorsal injections (Table 1). Neither Cm-activin nor Xnr2^{C333S} affected embryonic development (Table 1), suggesting that the anterior truncations arose via specific dominant-negative effects on Xnr signaling.

The anterior truncations were characterized by in situ hybridization for region-specific neural markers (Fig. 4E). *XOtx2* (a forebrain/midbrain marker) expression, was reduced in moderately affected (APDI 3), but absent from severely affected (APDI 1) embryos. *XEn-2* (a midbrain/hindbrain boundary marker) adopted a progressively more forward location in cmXnr2-injected embryos, and finally disappeared (not shown). In severely affected embryos, one or both bands of *Krox-20* expression (a hindbrain rhombomere 3 and 5 marker) were absent (not shown). The spinal cord marker *HoxB9*, however, was present in all embryos. These results show that anterior neural tissues extending back to the hindbrain failed to form in cmXnr2 RNA-injected embryos.

cmXnr2 does not ventralize mesoderm but affects gastrulation movements

Several possibilities can be considered to explain the anterior truncations induced by cmXnr2: (1) ventralization of dorsal mesoderm, (2) anti-neuralization, (3) posteriorization of neural tissues, (4) impairment of gastrulation movements, and (5) suppression of head organizer function. The following

experiments were carried out to discriminate among these possibilities.

Dorsal expression of ventralizing agents like BMP4 induces severe anterior truncations (Dale et al., 1992; Jones et al., 1992). In addition, dominant negative Vg1-specific reagents ventralize embryos (Joseph and Melton, 1998). The presence of notochord and muscle in cmXnr2 RNA-injected embryos (not shown) argues against such a global ventralization effect. In confirmation, dorsal marginal zone (DMZ) explants were not ventralized by cmXnr2 RNA or pCSKA-cmXnr2 (a plasmid in which the cytoskeletal *actin* promoter drives expression after early gastrula stage), unlike control injections of *BMP4* RNA (Fig. 5A).

We next addressed whether cmXnr2 affected either general neural induction or the A-P character of neural specification. The secreted factors noggin, chordin, follistatin and Xnr3 can neuralize animal caps by antagonizing BMPs (Harland and Gerhart, 1997); we tested whether cmXnr2 can block these effects. Coinjection of cmXnr2 RNA with *Xnr3*, *noggin*, *chordin*, or *follistatin* RNA did not attenuate general neural induction as tested by the pan-neural marker, *NCAM* (not shown). Anterior truncations are also induced by retinoic acid (RA) (Durstun et al., 1989; Sive et al., 1990), through the posteriorization of anterior neural tissue (Ruiz i Altaba and Jessell, 1991; Sive et al., 1990), and we tested whether cmXnr2 might work similarly. *Noggin* RNA induced anterior (*XOtx2*), but not posterior (*HoxB9*) neural markers in animal caps, as reported by Lamb et al. (1993). Addition of RA to noggin-treated explants induced *HoxB9* at the expense of *XOtx2*, reflecting efficient posteriorization. Coinjection of cmXnr2 RNA or pCSKA-cmXnr2 with *noggin* RNA, however, failed to induce *HoxB9* and did not affect *Xotx2* expression (not shown). We conclude that the cmXnr2-induced anterior truncations do not arise from gross ventralization of dorsal mesoderm, posteriorization of neural tissues, or anti-neuralization.

Animal cap extension induced by activin mimics gastrulation movements (Symes and Smith, 1987). The suppression of activin protein-induced animal cap extension by cmXnr2 (Fig. 3A), and the delayed blastopore closure caused by cmXnr2 (Fig. 4A) suggested that cmXnr2 affects gastrulation movements. We also noticed that the highest doses of cmXnr2 RNA induced what appeared similar to exogastrulation (Fig. 5B), an abnormal process in which mesoderm and endoderm fail to invaginate, leading to the formation of an epidermal ball connected to an elongated cord-like structure representing mesendodermal tissues. As shown in Table 2, the 'exogastrulation' phenotype incidence increased with cmXnr2 dose. Studies with agents like trypan blue, suramin, and sulfatase that disrupt cell-substratum interactions show that progressive reductions in the morphogenetic movements of gastrulation cause increasingly severe A-P patterning defects (Gerhart et al., 1989; Wallingford et al., 1997), suggesting that this mechanism could explain the truncations caused by cmXnr2.

We therefore compared the migratory activity of head mesendoderm explanted from control and cmXnr2 RNA-injected embryos (Fig. 5C,D). One hour after explantation, 75% of explants from uninjected ($n=24/31$) or β -globin RNA-injected embryos ($n=11/15$) showed an irregular margin reflecting outward cell migration, and many individual emigrating cells. At higher magnification, peripheral cells

Table 2. Summary of the phenotypes of cmXnr2-injected embryos

Injected RNA (pg)			n	Anteroposterior Deficiencies Index (%)						Exo (%)	Ave	Other (%)
				5	4	3	2	1	0			
Uninjected			50	100	0	0	0	0	0	0	5.0	0
β -globin	2000	DMZ	35	88	3	0	3	0	0	0	4.9	6
		VMZ	47	87	0	0	0	2	0	0	4.9	11
cmXnr2	50	DMZ	55	73	14	5	0	0	0	2	4.6	6
		VMZ	21	71	14	10	0	0	0	0	4.6	5
	100	DMZ	50	38	24	14	2	8	0	4	3.8	10
		VMZ	35	63	14	11	0	0	0	9	4.2	3
	200	DMZ	62	6	34	16	12	19	0	5	2.8	8
		VMZ	54	11	41	11	7	7	6	13	2.8	4
	500	DMZ	51	4	12	12	4	31	17	12	1.5	8
		VMZ	47	11	32	2	2	19	19	6	2.3	9
1000	DMZ	47	0	6	6	13	32	19	21	1.0	3	
	VMZ	56	4	14	9	14	30	23	6	1.6	0	
2000	DMZ	66	2	6	5	2	48	22	13	1.0	2	
	VMZ	48	6	6	6	10	10	31	29	1.0	2	
Xnr2 ^{C333S}	2000	DMZ	60	85	2	0	0	0	0	0	5.0	13
		VMZ	67	80	4	0	2	2	0	2	4.8	10
Cm-Activin	2000	DMZ	50	86	2	0	0	2	0	0	4.9	10
		VMZ	54	90	0	0	0	4	0	2	4.8	4

Indicated amounts of RNA were injected into two dorsal or ventral blastomeres at the 4-cell stage, and the phenotype scored at tadpole stages (stage 33-35) according to the anteroposterior deficiencies index (APDI; Wallingford et al., 1997). These data were pooled from several independent experiments. *n*, number of embryos tested; DMZ & VMZ, dorsal and ventral marginal zone, respectively; Exo, exogastrulated embryos. Other embryos had such severe defects as to prevent scoring by APDI. In calculating average APDI (Ave), exogastrulae were regarded as APDI 0, and non-scorable embryos were eliminated.

appeared well spread with numerous pseudopodia (not shown). In contrast, only 35% of explants from cmXnr2 RNA-injected embryos ($n=9/26$) showed a slightly irregular outline, and isolated migrating cells were seldom observed in any explant. Two hours later, the difference was more profound. Uninjected or control RNA-injected head mesendoderm showed many migratory cells (uninjected: 100%, $n=30/30$, 1 explant having become degraded in culture; β -globin-injected: 100%, $n=14/14$, 1 degraded). In contrast, 71% of the surviving explants ($n=17/24$; 2 became degraded) from cmXnr2 RNA-injected embryos showed almost no change in outline, and of the remainder (29%; $n=7/24$), the majority showed very little emigration activity.

cmXnr2 inhibits endoderm patterning

Several lines of evidence suggest that a 'head organizer' activity resides in the anterior deep endoderm in *Xenopus*, with the 'head-inducing factors' *cerberus* and *dickkopf-1* (*dkk-1*) representing functional components (Bouwmeester et al., 1996; Bouwmeester and Leyns, 1997; Glinka et al., 1998). We tested whether the cmXnr2-induced anterior truncations were correlated with suppressed expression of genes associated with Spemann organizer or head organizer activities. cmXnr2 RNA was injected equatorially into all cells of 4-cell stage embryos, and mesendodermal marker expression assessed in whole embryos (Fig. 6A). As shown in Fig. 6B, the alterations in expression profiles could be largely divided into two categories: (I), genes showing delayed expression, with suppression remaining throughout gastrulation or, (II), genes initially showing substantially delayed expression, but whose overall expression approached control levels during later gastrulation. The type I pattern was shown by *cerberus*, *Xhex-1* (a homeobox gene expressed in anterior endoderm; C. M. Jones, personal communication), *Frzb* (a secreted Wnt-antagonist expressed in anterior endoderm; Leyns et al., 1997), *dkk-1*, and *Mixer*, while the type II pattern was shown by *Xbra*,

Xlim-1, *chordin*, *XmyoD* and *Xwnt8*. Thus, cmXnr2 differentially affected the expression of genes in the organizer region, with prominent suppression of head organizer endoderm markers.

To investigate cmXnr2 effects on anterior endoderm/head organizer specification directly, we isolated vegetal explants (VEs) as depicted in Fig. 7A. To minimize any effect of general developmental delay, cmXnr2-injected VEs were explanted when undissected cmXnr2-injected embryos first displayed dorsal blastopore lips. cmXnr2 substantially suppressed early-expressed zygotic markers of general (*Mixer*, *Xsox17 β*) or anterior (*cerberus*, *Xhex-1*, *Frzb*) endoderm (Fig. 7B). In contrast, cmXnr2 did not affect expression of *Siamois*, a homeobox gene encoding a dorsal determinant expressed in dorsoanterior endoderm soon after zygotic transcription begins (Lemaire et al., 1995). At late stages, cmXnr2 also suppressed expression of *XIHbox8*, a posterior foregut marker expressed dorsoanteriorly in VEs (Gamer and Wright, 1995), but a general endodermal marker, *endodermin* (Sasai et al., 1996) was not affected. The suppression of both early and late markers was restored by *Xnr2* RNA coinjection.

In reciprocal experiments, *Xnr2* RNA dose-dependently induced anterior (*cerberus*, *Xhex1*, *Frzb*) and general (*Xsox17 β* ; Hudson et al., 1997) endodermal markers in animal caps (Fig. 8). These markers were also induced by mouse *nodal* and *Xnr1* RNAs. As for mesoderm induction, cmXnr2 RNA coinjection blocked this induction (not shown). Nodal-related signaling, however, was insufficient to induce *Siamois*. We conclude that cmXnr2-mediated interference with Xnr function in VEs directly affects endodermal patterning, including the induction of head organizer tissues.

DISCUSSION

While it is generally agreed that TGF β -related signaling

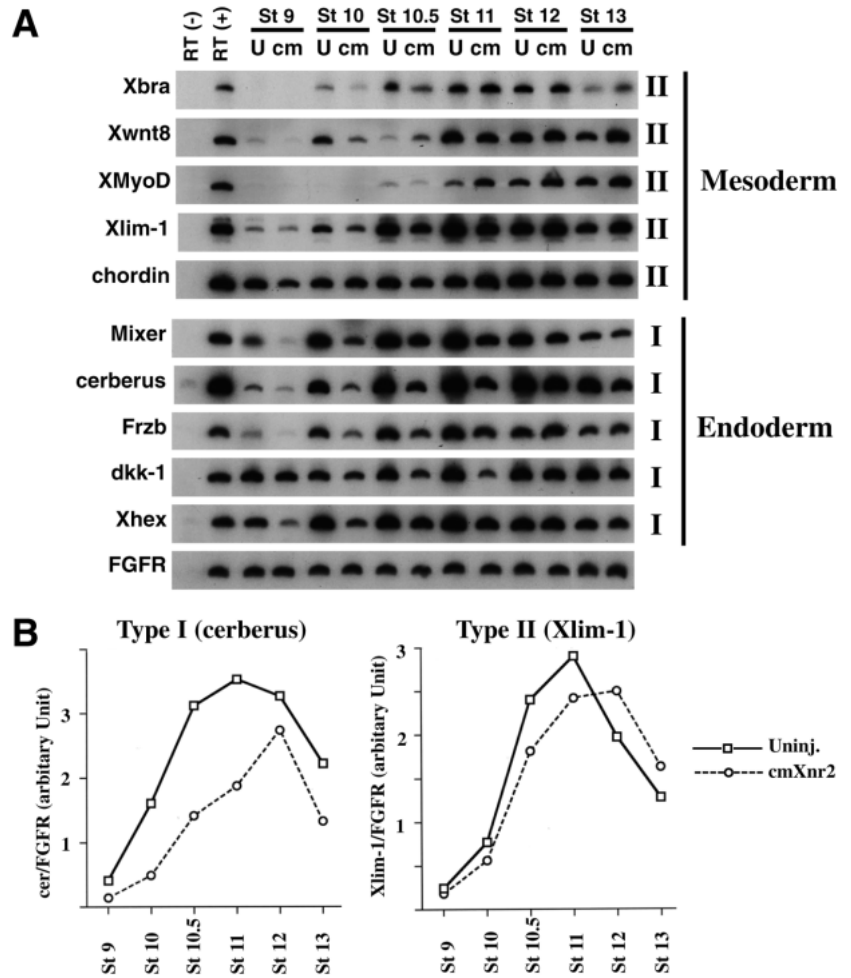


Fig. 6. cmXnr2 suppresses anterior mesendodermal specification. (A) Uninjected (U) and embryos injected equatorially at the 4-cell stage (4×1 ng) with cmXnr2 RNA (cm) were collected at the indicated stages, and analyzed by RT-PCR for *Xbra* (general mesoderm), *Xwnt8*, *XmyoD* (ventrolateral mesoderm), *Xlim-1*, *chordin* (dorsal mesoderm), *cerberus*, *Xhex*, *Frzb* and *dickkopf-1/dkk-1* (anterior mesendoderm). *FGFR*: loading control. (B) The signals obtained above were quantitated using 'NIH Image' software, and normalized to the intensity of *FGFR*. The normalized expression profiles of the markers fall into two categories, type I and II (represented by *cerberus* and *Xlim-1*, respectively), as indicated to the right of panel A. Note that the peak of expression in cmXnr2-injected embryos is delayed in both type I and II, but that overall induction (area under the line) is more suppressed in type I than type II.

pathways are involved in the induction of mesoderm and endoderm during vertebrate embryogenesis, the precise identity of the endogenous ligand(s) remains controversial (Harland and Gerhart, 1997). Here, we used an Xnr-specific inhibitory reagent, cmXnr2, to perform loss-of-function experiments addressing the requirement for nodal-like signaling in early amphibian embryogenesis. As well as using animal cap induction assays to show that cmXnr2 specifically abrogates Xnr signaling (Fig. 1B,C), we established an antagonistic relationship between cmXnr2 and Xnr2 both in whole embryos (Fig. 2) and vegetal explants (Fig. 7). Together, these results suggest that the effects of cmXnr2 in vivo result from selective interference with endogenous Xnr signaling.

Misexpression of cmXnr2 in whole embryos produced dose-dependent anterior truncations, and delay and/or suppression of the expression of many genes with functions associated with dorsoanterior mesendodermal specification. In contrast, expression of the dorsovegetally expressed zygotic homeobox gene *siamois*, which is a direct target for the maternally-initiated β -catenin D-V patterning pathway (Brannon et al., 1997; Heasman, 1997), was neither inhibited by cmXnr2 in VEs, nor induced by Xnr2 in animal caps. This implies that the effects of Xnrs on dorsoanterior mesendodermal patterning occur downstream of, or parallel to, *Siamois*.

Some zygotically expressed mesendodermal genes appear more sensitive to interference with Xnr signaling. Most

mesodermal markers had somewhat delayed expression, returning relatively rapidly to control levels (Type II; Fig. 6), although some, such as *Xbra*, showed only a slight, if any, delay in expression. This group includes genes expressed in the dorsoanterior mesoderm encoding factors with organizer-type activity, such as *chordin*. Together with the lack of effect on *Siamois*, the selective effects on gene expression and embryonic phenotype seem inconsistent with a simple global developmental delay caused by cmXnr2. The most affected genes were anterior endoderm markers associated with the head organizer function of this tissue (discussed below). In addition, however, the suppressed expression of putative endodermal determinant genes (*Mixer*, *XSox17 β*) suggests that Xnr signals are also involved in activating, or maintaining, gene expression programs responsible for general and region-specific endodermal determination, a process occurring at and around gastrulation. This conclusion would fit well with the initial 'pan-vegetal' expression of Xnrs, and at slightly later stages in the ventrolateral marginal zone (Jones et al., 1995).

The conclusion that nodal-related signaling is important for establishing dorsoanterior mesendodermal (including head organizer) fates, but not all mesodermal fates in the amphibian embryo differs somewhat from the more complete failure of mesendodermal induction in mouse *nodal*^{-/-} embryos (Conlon et al., 1994). One possibility is that this reflects differences in patterning processes between these species, although cmXnr2

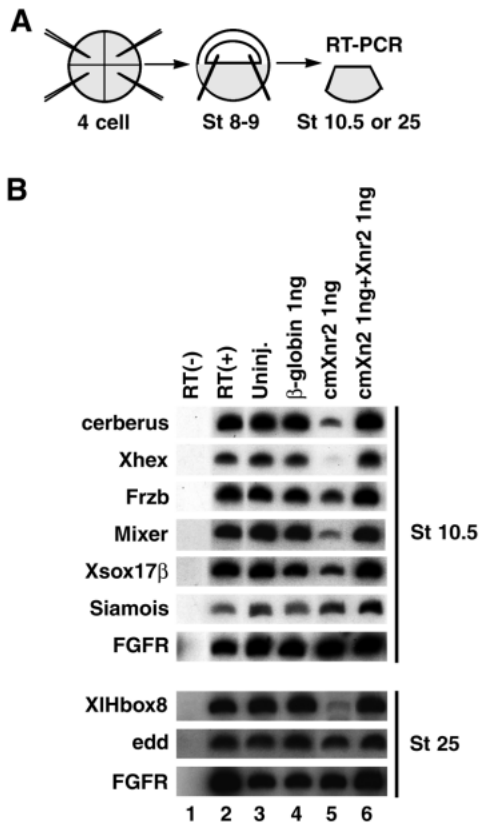


Fig. 7. cmXnr2 suppresses expression of anterior endodermal markers in vegetal explants. (A) Experimental design. RNAs were injected vegetally into 4-cell embryos, vegetal explants dissected at stage 9 and tested at stage 10.5 or 25 for endodermal marker expression. (B) RT-PCR analysis for early (*cerberus*, *Xhex1*, *Frzb*, *Mixer*, *Xsox17β* and *Siamois*), and late (*endodermin/edd* and *XIHbox8*) endodermal markers. RNA doses injected per blastomere are indicated. cmXnr2-injected VEs were staged by reference to undissected cmXnr2-injected embryos (see text). Suppression of anterior endodermal markers by cmXnr2 was abolished by coinjected *Xnr2* RNA. Note that *Siamois* expression was not affected by cmXnr2.

may have incompletely inactivated Xnr pathways under our experimental conditions. Alternatively, since Cerberus antagonizes nodal, BMP and XWnt signals (Piccolo et al., 1999), the suppression of Cerberus expression by cmXnr2 may allow residual inductions caused by BMP/Wnt ligands.

Relevant data has also come from studies in zebrafish, in which the *cyclops* and *squint* loci were recently identified as *nodal*-related genes (Erter et al., 1998; Feldman et al., 1998; Rebagliati et al., 1998; Sampath et al., 1998). Homozygous mutant *squint* or *cyclops* embryos have specific defects in dorsoanterior fates. For *squint*, these are correlated with delayed *gsc* expression within the organizer region. In contrast, double homozygous mutant *squint/cyclops* embryos exhibit a more complete failure of the induction of dorsal, but not ventrolateral, mesoderm, and embryonic endoderm does not form (Feldman et al., 1998). The dorsoanterior defects in frog embryos caused by interfering with nodal signals via cmXnr2 therefore seem similar to those caused by reducing *nodal*-related gene function in zebrafish. In relation to the posterior truncations also seen in a proportion of cmXnr2-injected

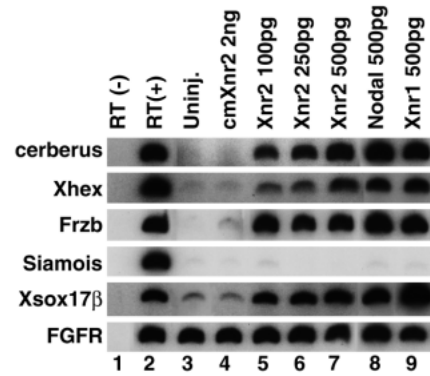


Fig. 8. Induction of anterior endodermal markers by mouse nodal and Xnrs. Animal caps injected with *nodal*, *Xnr1* or *Xnr2* RNA were analyzed at stage 10.5 by RT-PCR for the expression of anterior mesendodermal markers (*cerberus*, *Xhex1* and *Frzb*) and the general endodermal marker *Xsox17β*. *Siamois*, an early zygotic marker of dorsovegetal endoderm, was not induced.

embryos, we note that *sqt;cyc* double mutants also show defects throughout the embryonic axis, despite an initially localized effect on dorsal mesendoderm (Feldman et al., 1998). In both species, it is likely that the reduced specification and abnormal gastrulation in dorsal regions indirectly impairs morphogenesis and cell fate determination in ventrolateral regions.

Xnr interactions

One *nodal* gene is known in mouse and chick, while *Xenopus* and zebrafish contain multiple orthologs. It is possible that the activities of the mouse/chick gene are apportioned between several frog/fish genes (perhaps maintaining certain shared functions), or that different activities have arisen in the duplicated versions. We have not managed to make dominant negative reagents specific for each of the Xnrs to address this point. In fact, similar 'cleavage mutants' of Xnr1 and Xnr4 had mesoderm-inducing activities comparable to the unaltered ligands. Xnr1/Xnr4 both contain additional putative cleavage sites N-terminal of the expected site, and their inactivation may be required to block processing to the active ligand. The cmXnr2-mediated block to mesoderm induction by Xnr1, Xnr2 and Xnr4 raises the possibility of Xnr heterodimerization in vivo. Precedent for this suggestion comes from studies of baculovirus-produced BMP4/BMP7 heterodimers, which are more potent inducers of ventral mesoderm than either homodimer (Suzuki et al., 1997). Such heterodimerization could, in principle, allow flexibility in modulating Xnr signaling pathways in areas of coexpression. In relation to this point, although their expression patterns are not all clearly defined, overlap is seen in the early endoderm (*Xnr1* and *Xnr2*; Jones et al., 1995), and at least partially in the Spemann organizer region (*Xnr1*, *Xnr2* and *Xnr4*; Jones et al., 1995; Joseph and Melton, 1997).

Activin and Nodal/Xnr signaling

In control experiments, overexpression of an activin cleavage mutant did not alter embryonic development (Table 1). Cm-activin blocks the function of cotranslated activin, preventing tissue extension (Hawley et al., 1995) and mesoderm induction (S. O. and C. V. E. W., not shown) in animal caps. The lack of

effect of Cm-activin on embryos suggests that zygotically produced activin is not required during embryogenesis, consistent with similar studies in medaka fish (Wittbrodt and Rosa, 1994). Some experiments, however, suggest that early activin-related signals are essential for normal amphibian development. For example, dominant negative activin receptors that are relatively specific for activin (ActRIIBexd; Dyson and Gurdon, 1997), in addition to those with a more relaxed specificity (Hemmati-Brivanlou and Melton, 1992; Hemmati-Brivanlou and Thomsen, 1995; Schulte-Merker et al., 1994), cause anterior truncations and delay or inhibit mesoderm formation. These observations fit the hypothesis that zygotically expressed Xnrs relay earlier signals from activin/activin-like ligands, possibly including maternally deposited activin (Asashima et al., 1991), which would not be sensitive to cotranslational inhibition by Cm-activin.

Dorsal mesodermal fates are usually tightly associated with gastrulation-like morphogenetic movements. The delayed dorsoanterior marker expression in cmXnr2 RNA-injected embryos during gastrulation (Fig. 6) agrees with the decreased migratory activity of explanted head mesoderm (Fig. 5). In mouse *nodal*^{-/-} embryos, the failure of the few mesodermal cells that are generated to move from their site of origin (Conlon et al., 1994) may also connect dorsoanterior fates with morphogenetic behavior. Further connections between endoderm specification and gastrulation movements are suggested by the phenotypes caused by Mixer- and Xsox17-engrailed repressor domain chimeras (Henry and Melton, 1998; Hudson et al., 1997). An interesting case where the two processes can be uncoupled is presented by Xnr3, which can induce morphogenetic movements without altering cell fate (Smith et al., 1995). It is highly unlikely, however, that Xnr3 interference explains the cmXnr2-induced defects in vivo that we reported here, since we found that cmXnr2 does not block Xnr3-induced neuralization, and Xnr3 cannot block mesoderm induction by Xnr2 (Hansen et al., 1997). The atypical structure of Xnr3 (Smith et al., 1995) may prevent Xnr3 heterodimerization with other Xnrs.

Although cmXnr2 did not block mesoderm induction in animal caps mediated by coinjected *activin* RNA, cmXnr2 did suppress induction by purified activin protein. The lack of effect of cmXnr2 on *Xnr1* transcript induction by activin protein (Fig. 3) indicates interference at the level of Xnrs translated in response to activin treatment. The recent demonstration that activin-induced *Xbra* expression is suppressed by coinjected RNA encoding Cerberus (Hsu et al., 1998), a nodal antagonist (Piccolo et al., 1999), also suggests that Xnr effects represent a substantial fraction of the induction produced by activin. An apparent inconsistency comes from the lack of effect of preincubated activin and Cerberus proteins, which do not interact in vitro, on *Xbra* induction in animal caps (Piccolo et al., 1999). One possibility, however, is that Cerberus does not diffuse well through embryonic tissues, and cannot block Xnr-mediated induction once initiated by activin. This proposal is supported by the finding that neuralization by Cerberus protein (via its BMP antagonism) requires brief dissociation-aggregation of the animal cap (Piccolo et al., 1999).

We recently identified a strong activin-response element (ARE) within the *Xnr1* locus (A. Frisch, S. O. and C. V. E. W., unpublished). Together with our findings, reported above, we

propose that activin-induced *Xnr* expression in intact animal caps is a major factor contributing to the dose-dependent induction of mesoderm and, at the highest levels, endoderm. In contrast, when *activin* RNA is injected, its dispersal throughout the animal cap presumably produces a situation in which intercellular induction is immune to the effects of the Xnr-specific cmXnr2 reagent. A corollary of this is that the sharpened dose response curve of dispersed animal caps to activin protein (e.g. Green et al., 1992) may in part result from circumvention of Xnr-mediated relays.

Head induction and nodal signaling

The anterior deletions caused by cmXnr2 are reminiscent of the rostral defects observed in chimeric mouse embryos in which the visceral endoderm is composed of *nodal*-deficient cells (Varlet et al., 1997b). Similar anterior deficiencies are found in embryos homozygous null for transcription factor genes such as *Lim-1*, *Otx-2* and *Hesx1*, as well as some *HNF-3 β* mutants (Beddington and Robertson, 1998; Varlet et al., 1997a). The combined expression of these genes in the anterior visceral endoderm (AVE) of early gastrulation stage mouse embryos (Beddington and Robertson, 1998; Belo et al., 1997) is thought to localize a 'head organizer'-like activity within this extraembryonic tissue. Although our current knowledge of the interrelationships between the various AVE genes is rudimentary, the anterior defects in *nodal*^{+/-};*HNF-3 β* ^{+/-} embryos (Varlet et al., 1997a) presumably demonstrate at least one important interaction. Circumstantial evidence may place *lim-1* and *Otx2* downstream of nodal signaling. Mouse nodal induces *lim-1* in zebrafish embryos (Toyama et al., 1995), Xnr2 induces *Xlim-1* in *Xenopus* animal caps (S. O., unpublished observations), and we report here that nodal (Fig. 8) and Xnr (Fig. 7), can induce *cerberus*, a 'head-inducing' gene that can induce *XOtx2* expression (Bouwmeester et al., 1996). Together with the initial expression of *Xnr1* and *Xnr2* in endodermal cells just after zygotic transcription begins (Jones et al., 1995), the reduced expression of 'head organizer' genes (*cerberus*, *dkk-1*, *Xhex*, *Frzb*) caused by cmXnr2 suggests that an underlying reason for the anterior truncations is a reduced level of dorsoanterior endodermal specification. In principle, this suggests that nodal signaling plays similar roles in the anterior visceral endoderm of mouse and dorsal endoderm in *Xenopus*, and that these tissues may function equivalently in embryonic patterning (Beddington and Robertson, 1998).

According to a recently proposed model (Glinka et al., 1997), inhibition of both BMP4 and Wnt signaling pathways is a central step in head induction. The Xnr-mediated induction of the Wnt antagonists *cerberus*, *Frzb* and *dkk-1* and suppression of their expression by cmXnr2 (Figs 6 and 7) suggests a possible indirect, though positive, role for Xnr signaling in head specification. This model is supported by the finding that ventral misexpression of the BMP4 antagonist *noggin* induces headless secondary axes, while coexpression of *noggin* and *Xnr1* RNAs induces complete secondary axes (Lustig et al., 1996).

From work in amphibians and mammals, there is some evidence that activin and nodal-related factors are in the same pathway, or share signaling pathway components, leading to dorsoanterior mesoderm and head induction. In *Xenopus*, an activin-specific dominant negative receptor (ActIIBexd), or dominant negative versions of Smad2, also delay and/or

suppress dorsoanterior mesendodermal marker expression, producing anterior truncations similar to those reported here (Dyson and Gurdon, 1997; Hoodless et al., 1999). These results are confounded somewhat by the finding that genetic disruption of the activin type IIB receptor in mouse does not cause anterior truncations, but abnormal skeleton patterning and lateral asymmetry disturbances (Oh and Li, 1997). Nevertheless, mice that are homozygous mutant for *Smad2* or *nodal* show similar gastrulation defects, absence of mesoderm induction, and disruption of A-P axis polarity (Nomura and Li, 1998; Waldrip et al., 1998; Weinstein et al., 1998), suggesting that nodal signals through *Smad2*. Taken together, our results suggest that the requirement for an early acting nodal signaling pathway is conserved between mouse and *Xenopus* embryos.

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