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A *Xenopus* nodal-related gene that acts in synergy with noggin to induce complete secondary axis and notochord formation

K. D. Lustig, K. Kroll, E. Sun, R. Ramos, H. Elmendorf and M. W. Kirschner*

Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA

*Author for correspondence

SUMMARY

Using a paracrine assay to screen for signaling proteins that could respecify ectodermal tissue, we isolated a *Xenopus* gene related to the mouse gene nodal, a member of the TGFβ superfamily. The gene is expressed in three regions in the early *Xenopus* embryo: first in the gastrula organizer, then in two stripes of cells flanking the posterior notochord in late neurulae, and finally in lateral plate mesoderm restricted to the left side of tailbud-stage embryos. Ectopic expression of the gene induces muscle formation in ectodermal explants and partial secondary axes in whole embryos. Together with noggin, another secreted protein also present in the organizer, it induces notochord formation in ectodermal explants and complete secondary axes in whole embryos. These results suggest that the nodal-related gene may act together with noggin to induce axial pattern during gastrulation and also may play a role in left-right asymmetry generation in the post-gastrula embryo.

Key words: left-right asymmetry, notochord formation, axis formation, nodal, noggin, *Xenopus*, neural induction, Spemann organizer

INTRODUCTION

The vertebrate body axis is elaborated as a result of extensive changes in cell movement and tissue specification that occur during gastrulation. Of particular importance in directing morphogenesis and tissue formation during amphibian gastrulation is a region of dorsal mesodermal cells called Spemann's organizer. Identified based upon its ability to induce a complete secondary body axis when transplanted into the ventral region of a host embryo (Spemann and Mangold, 1924), the organizer is a tissue with complex signaling properties, possessing at least three activities. It induces adjacent mesoderm to develop into somites, heart and kidney; it contributes to early neural induction and patterning; and it initiates and directs the convergent extension movements that underlie morphogenesis in the early embryo (Gerhart et al., 1991). After gastrulation, organizer cells differentiate into prechordal plate or notochord, tissues that pattern the developing neural tube and somites.

Although the biological functions of the organizer have been intensively studied for over seventy years, only in the last five years have candidate secreted molecules that carry out these functions been identified. When this work was begun, only three secreted proteins had been shown to be specifically expressed in the organizer during gastrulation: noggin (Smith and Harland, 1992), chordin (Sasai et al., 1994) and follistatin (Hemmati-Brivanlou et al., 1994). Noggin was isolated in a functional screen for genes capable of rescuing dorsal axis development in ventralized *Xenopus* embryos (Smith and Harland, 1992). Chordin was identified by combining subtractive cDNA hybridization with in situ RNA hybridization to identify genes highly expressed in the *Xenopus* organizer (Sasai et al., 1994). Both noggin and chordin neuralize animal pole explants (presumptive ectoderm) that would otherwise form epidermal tissue. Neuralization of *Xenopus* ectodermal explants is also induced by follistatin (Hemmati-Brivanlou et al., 1994), an activin antagonist originally isolated based upon its ability to inhibit follicle-stimulating hormone release from the pituitary (Nakamura et al., 1990).

We have recently developed a paracrine assay that can be used to identify genes capable of acting in a non-cell-autonomous manner to respecify presumptive ectoderm (Lustig and Kirschner, 1995). We have used this assay to identify and functionally characterize a gene related to nodal, a secreted TGFβ family member required for mesoderm formation in the mouse (Conlon et al., 1994; Zhou et al., 1993). While this work was underway, Jones et al. (1995) described the homology-based cloning of a *Xenopus* nodal-related gene, designated Xnr1, that is identical to the gene we have cloned. It was reported that Xnr1 is only strongly expressed during late blastula and gastrula stages of development, with enrichment in the organizer. In our study we found that Xnr1 is also strongly expressed in two regions of the post-gastrula embryo: in two small stripes of cells flanking the posterior notochord in late neurula stage embryos and in dorsolateral mesoderm restricted to the left side of tailbud stage embryos. To examine the function of Xnr1 during gastrulation, we have expressed it alone or in conjunction with the secreted protein noggin. Although Xnr1 has mesoderm- and neural-inducing activity on its own, in conjunction with noggin it can mimic three other activities of the gastrula organizer including the induction of a complete secondary axis, the formation of notochord and the stimulation of tissue movements reminiscent of convergent

extension behavior. Thus Xnr1 may act together with noggin to induce axial pattern during gastrulation and may also be involved later in the establishment of left-right asymmetry.

MATERIALS AND METHODS

Oocytes and embryos

Ovarian tissue was isolated from previously unovulated 2- to 3-year-old *X. laevis* frogs (NASCO). Stage VI oocytes were manually defolliculated and cultured at 18°C in modified Barth's solution (high salt), pH 7.4, containing penicillin at 100 units/ml, streptomycin at 100 μg/ml, and bovine serum albumin at 0.1 mg/ml (MBSH; Peng, 1991). Banded or spotted oocytes or oocytes that had become blemished during the defolliculation process were discarded.

Pigmented and albino *X. laevis* embryos were obtained by in vitro fertilization, dejellied and cultured at 18°C in 0.1× Marc's modified Ringers (MMR) containing 50 μg/ml gentamicin (Peng, 1991). Embryos were staged according to the method of Nieuwkoop and Faber (1967).

Paracrine signaling assay

The paracrine signaling assay was carried out as previously described (Lustig and Kirschner, 1995), with minor modifications. Briefly, ~50 ng GpppG-capped library RNA (~1 mg/ml) was microinjected about 45° off the pigment border into the center of the vegetal pole of a defolliculated *X. laevis* oocyte. After a 12 to 18 hour incubation to allow protein expression in the oocyte, an ectodermal explant from a blastula (stage 8.5) or early gastrula (stage 9.5-10) stage embryo was grafted onto the vegetal pole of the oocyte. Explants (~0.16 μm^2 square) were cut from the apex of the animal pole using an eyelash scalpel or an electric cutting tool (Xenotek Engineering, Belleville, IL, USA). The oocyte-explant conjugates were cultured in 1× MBSH, pH 7.4, and changes in the developmental fate of the grafted tissue were evaluated by visual scoring or by analyzing marker gene expression by reverse transcriptase-PCR (RT-PCR) or immunostaining.

Axis duplication assay

The axis duplication assay was carried out as previously described (Lemaire et al., 1995). Between 10 pg and 1 ng of GpppG-capped library RNA was injected into the vegetal region of a single ventral blastomere of a four-cell embryo. Embryos were visually scored for the presence and completeness of a secondary axis when they reached stage 18 and then again at stage 31. Ventral blastomeres were distinguished from dorsal blastomeres based upon their larger size and darker pigmentation and the presence of a sperm pigment trail.

Library construction and Sib selection

A cDNA library was constructed from gastrula stage embryos (stage 10 to 10.5) that had been hyperdorsalized by LiCl treatment (Lustig and Kirschner, 1995). Aliquots from the unamplified library (containing 100-200 or 1,000-2,000 independent transformants) were plated on LB-agar plates containing 100 μg/ml carbenicillin. After an overnight incubation at 37°C or when bacterial colonies were approximately 1 mm in diameter, the colonies on a single plate were scraped with a rubber policeman and pooled. A sample of the pooled bacteria was stored as a 20% glycerol stock at −80°C. Plasmid DNA isolated from the remainder of the culture was linearized with *Not*I and used as template to synthesize GpppG-capped RNA in vitro (Wormington, 1991).

To segregate the active clones in the positive pool of 1,000-2,000 clones, the pool was subdivided into twenty pools of 96 clones. A sample of the original glycerol stock was plated on LB-agar plates containing 100 μg/ml carbenicillin. After an overnight incubation at 37°C, a small sample of each colony was transferred with a toothpick to a single well of a 96-well plate containing 200 μl of LB supplemented with 100 μg/ml carbenicillin. The top of each plate was sealed with an adhesive plastic film to reduce cross contamination between wells. After an overnight incubation at 37°C, 100 μl of culture was removed from each well of a single plate and pooled. Plasmid DNA was isolated from these pooled cultures, linearized with *Not*I and then used to synthesize RNA in vitro. LB (100 μl) containing 40% glycerol was added to the remaining 100 μl of culture in each well and the twenty plates were stored at −80°C.

The positive pool of 96 clones was subdivided into 12 pools of 8 clones, which were used to make RNA as above. A toothpick was used to scrape a small sample of frozen culture from each of the eight wells that constitute a single row in the positive 96-well plate. Once a positive row was identified, the single active cDNA was identified by individually testing each well in the positive row. The single active cDNA obtained in this manner was sequenced on both strands using an automated DNA sequencer.

RT-PCR analysis, in situ hybridization and immunostaining

Gene expression in grafted explants was analyzed by RT-PCR as described (Lustig and Kirschner, 1995; Rupp and Weintraub, 1991; Wilson and Melton, 1994). The PCR primer sequences used for analysis of neural cell adhesion molecule (NCAM), muscle actin, brachyury, goosecoid, noggin and elongation factor 1-α (EF1-α) were as described (Hemmati-Brivanlou et al., 1994; Hemmati-Brivanlou and Melton, 1994). For Xnr1 analysis, the sequence of the forward primer was 5′ GCA GTT AAT GAT TTT ACT GGC 3′ and the sequence of the reverse primer was 5′ CAA CAA AGC CAA GGC ATA AC 3′. For NCAM and muscle actin analysis, explants were detached and assayed when sibling embryos reached stage 28. For brachyury and goosecoid analysis, explants were detached and assayed when sibling embryos reached stage 12.

Whole mount in situ hybridization of albino or pigmented embryos was carried out as described (Harland, 1991) using digoxygenin-11-UTP-labeled probes (Boehringer). A mixture of 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt, (BCIP) was used as substrate for the color reaction. Antisense probe for Xnr1 was synthesized with T3 RNA polymerase using *Eco*RI-linearized plasmid as template. To localize the transcripts, overstained embryos were embedded in JB-4 plastic resin and cut into 7 μm sections.

The presence of notochord or muscle in explants or explant sections was detected by immunostaining with the Tor-70 monoclonal antibody (Bolce et al., 1992) or the 12/101 monoclonal antibody (Kintner and Brockes, 1984), respectively. For immunostaining, fixed embryos were embedded in Paraplast and cut into 10 μm sections. Binding of primary antibodies was detected using a Cy3-labeled donkey anti-mouse secondary antibody (Jackson Immunoresearch).

Most images were obtained using incident or transillumination or epifluorescence on a Zeiss axiophot using ×2.5 or ×5 objectives. Some images were acquired on a Wild M8 stereomicroscope. All images were captured by video using a 3-color video rate CCD camera controlled by Northern Exposure software (Phase 3 Imaging Systems). Digitized images were opened in Adobe Photoshop to create montages. Brightness, contrast and color balance correction were performed using Photoshop.

RESULTS

Expression cloning of a *Xenopus* nodal-related gene

We used a paracrine signaling assay to screen a *Xenopus* cDNA expression library for genes capable of inducing neural tissue in presumptive ectoderm (animal caps). The cDNA library was prepared from gastrula stage LiCl-dorsalized

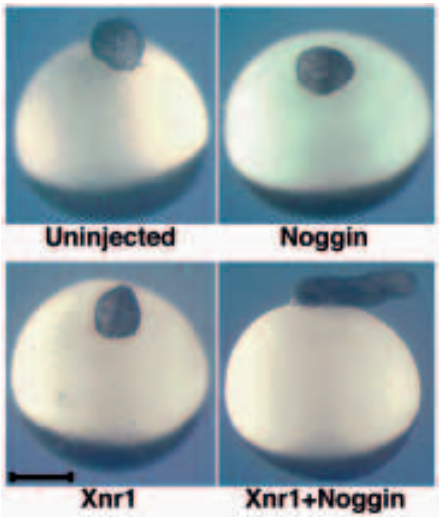

Fig. 1. Induction of tissue elongation by Xnr1 and noggin. Ectodermal explants from early gastrula stage embryos were grafted onto uninjected control oocytes or onto oocytes that had been injected with 100 pg of noggin RNA, 100 pg of Xnr1 RNA or 100 pg of both RNAs. The oocyte-explant conjugates were photographed when sibling embryos reached the early tailbud stage of development. Bar, 400 μm.

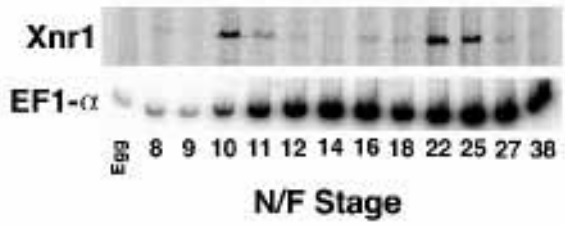

Fig. 2. Developmental expression of Xnr1. *X. laevis* embryos were harvested at the indicated stage and assayed by RT-PCR for expression of Xnr1. EF1-α was also assayed to show that comparable amounts of RNA were isolated from each sample. EF1-α is expressed at low levels prior to the mid blastula transition and at higher levels thereafter. Each PCR reaction contained the cDNA equivalent of one-tenth of an embryo.

embryos, which have larger organizer regions than wild-type embryos (Kao and Elinson, 1988). Pools of RNA (transcribed from pools of cDNA clones) were injected into *X. laevis* oocytes, which were then placed in direct contact with early gastrula stage ectodermal explants. Changes in explant fate were assessed visually or by RT-PCR analysis for NCAM, a pan-neural marker.

Of 370 pools tested, eight induced NCAM expression and the formation of cement gland, an anterior ectodermal derivative. Of these eight pools, three pools contained 100-200 clones (290 pools screened) and five pools contained 1,000-2,000 clones (80 pools screened). All active pools also induced elongation of the grafted tissue. One of the active pools (of 1,000-2,000 clones) induced substantially more tissue elongation than the other seven. PCR analysis showed that all eight active pools contained noggin (not shown), a secreted neural inducer previously isolated by Smith and Harland (1992).

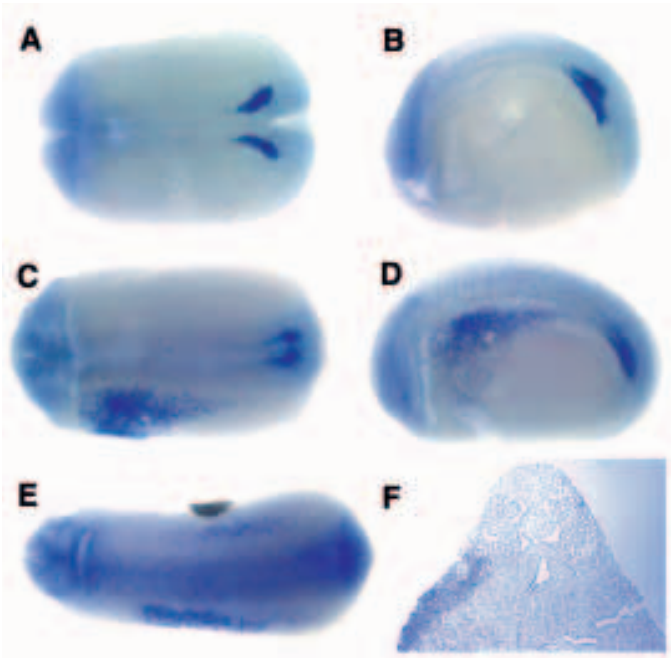

Fig. 3. Localization of Xnr1 transcripts by whole mount in situ hybridization. Embryos were hybridized with a digoxygenin-labeled Xnr1 antisense RNA probe. (A) Dorsal view of a stage 22 embryo. (B) Side view of embryo in A. (C) Dorsal view of a stage 24 embryo. (D) Side view of embryo in C. (E) Dorsal view of stage 28 embryo. Anterior is to the left in A-E. (F) Transverse section of a stage 24 embryo, showing Xnr1 expression in lateral plate mesoderm on the left side of the embryo. Expression was restricted to the left side in ~100 early tailbud stage embryos examined.

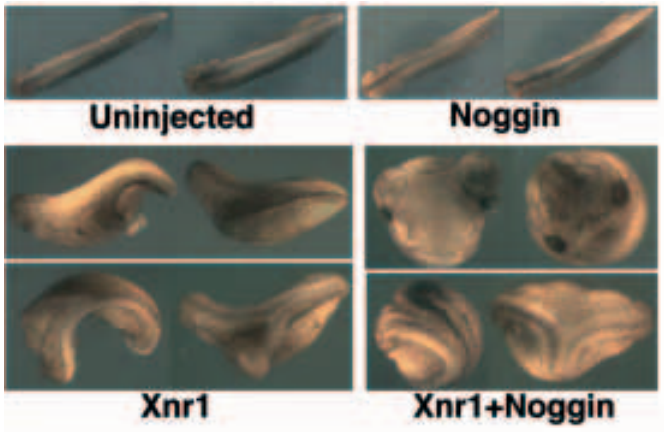

Fig. 4. Secondary axis formation by Xnr1 and noggin. A single ventral blastomere of a four-cell embryo was injected with 2 pg of noggin RNA, 200 pg of Xnr1 RNA or 2 pg of noggin RNA and 200 pg of Xnr1 RNA. Embryos were photographed when uninjected control embryos reached stage 31. All embryos are shown in dorsal view with anterior to the left except for the two upper embryos in the lower right panel, which are shown in anterior view.

Although induction of cement gland and neural tissue could be attributed to noggin, the extensive tissue elongation induced by one of the 1,000-2,000 clone pools could not be. The unusual behavior of this pool suggested the presence of a second secreted signaling protein, perhaps acting in concert

with noggin to promote cell movement. The five noggin-containing pools of 1,000-2,000 clones that were positive for NCAM expression were further tested in an axis duplication assay. When injected into a single ventral blastomere of a four-cell embryo, four of the five RNA pools had effects indistinguishable from those of 10 pg noggin alone, inducing partial secondary axes lacking anterior structures such as eyes and cement gland. In contrast, the noggin-containing pool that had elicited striking tissue elongation in the paracrine assay induced secondary axes with complete anterior structure in about one-third of injected embryos and partial secondary axes in the other two-thirds (not shown).

These results strongly suggested that this pool contained at least one protein with signaling properties different from those of noggin. To segregate this activity from noggin, we subdivided the active pool into pools of ~100 clones, which were analyzed by PCR for noggin. One pool that did not contain noggin still induced partial secondary axes in whole embryos (not shown). This pool was iteratively subdivided until a single active cDNA was identified. When co-expressed with noggin, the cloned gene reconstituted the tissue elongation behavior induced by the original positive library pool (Fig. 1).

Sequence analysis revealed that the 1.6 kb clone has an open reading frame of 406 amino acids encoding a protein with a predicted molecular mass of 46 kDa. When we began to characterize the clone, sequence comparisons indicated that it was a member of the TGFβ superfamily of secreted proteins, and was most closely related to nodal, a gene required for mesoderm induction in the mouse (Zhou et al., 1993). While this work was underway, reports of three *Xenopus* nodal-related genes have been published. Jones et al. (1995) identified two *Xenopus* nodal-related genes, Xnr1 and Xnr2, and Smith et al. (1995) and Ecochard et al. (1995) identified a third *Xenopus* nodal-related gene, Xnr3 (a.k.a. fugacin). The nodal-related gene we have cloned is identical to the Xnr1 gene described by Jones et al. (1995). It has characteristic features of most TGFβ family members (Kingsley, 1994): a hydrophobic signal sequence at the amino terminus, a pro region, and a mature C-terminal region containing seven cysteines that are involved in dimerization, secretion and receptor-binding. An unusual feature is the presence of two amino acid residues between the third and fourth conserved cysteine residues, which are directly adjacent in most TGFβ family members.

Expression of Xnr1 during post-gastrula stages of development

Using RNase protection and whole mount in situ hybridization analysis, Jones et al. (1995) showed that Xnr1 transcripts are expressed in the dorsal marginal zone of early gastrula stage *Xenopus* embryos, with enrichment in the organizer. We have confirmed Xnr1 expression in early gastrula stage embryos by RT-PCR analysis (Fig. 2). In addition, we have used RT-PCR and whole mount in situ hybridization to analyze the spatial and temporal expression of Xnr1 during post-gastrula stages of development. By RT-PCR, transcripts are detected at low levels throughout post-gastrula stages. By in situ hybridization, Xnr1 transcripts are detectable during late neural tube and early tailbud stages. Transcripts begin to accumulate during stages 20 to 22 in two small stripes of cells symmetrically flanking the posterior notochord (Fig. 3A) and just dorsal to the hindgut (Fig. 3B). Expression is maintained through the tailbud stages, except that the domains of expression appear to move slightly posterior relative to the hindgut, probably due to convergent extension movements that occur during this period.

By the early to mid tailbud stage of development (stage 24), Xnr1 transcripts also begin to accumulate asymmetrically on the left side of the embryo. Transcripts are detected in a broad region lateral and ventral to the developing neural tube and just posterior to the foregut (Fig. 3C,D). Transcripts also accumulate in a line along the trunk of the embryo, just lateral to the neural tube. Asymmetric expression of Xnr1 persists until about stage 28, when transcripts generally begin to accumulate laterally on the right side of the embryo as well (Fig. 3E). Expression on the left side of the embryo extends laterally throughout the trunk region. In contrast, the domain of Xnr1 expression on the right side is approximately one-half the size of that of the left side and is restricted to the posterior half of the trunk region. The posterior and lateral domains of Xnr1 expression persist until the early tadpole stages. Sections of embryos stained in whole mount show that Xnr1 transcripts are expressed in lateral plate mesoderm (Fig. 3F).

Xnr1 acts in synergy with noggin to induce complete secondary axes

The original active pool induced a higher frequency of complete secondary axes in intact embryos than Xnr1 or noggin alone. To test whether complete axis generation was due to their combined action, RNAs encoding Xnr1 and noggin were injected into single ventral blastomeres of four-cell embryos. The embryos were cultured until the late tailbud stage of development and then visually scored for the presence and completeness of a secondary axis.

Approximately one-third of embryos co-injected with 200 pg of Xnr1 RNA and 2 pg of noggin RNA formed complete secondary axes, about the same frequency of complete axes induced by the original library pool. The remainder exhibited a range of partial duplications with varying degrees of anterior completeness. Embryos injected with 200 pg of Xnr1 RNA formed partial secondary axes lacking anterior structures (Fig. 4) and never formed complete secondary axes. Embryos injected with 2 pg of noggin RNA only rarely formed partial secondary axes. Injection of higher doses of noggin (10-100 pg) induced a high frequency of partial secondary axes but only very rarely complete secondary axes (not shown; Lemaire et al., 1995). At the highest doses of noggin injected (>1 ng), no secondary axes were induced; rather, the entire embryo became extremely hyperdorsalized, forming mostly head structures (not shown). Partial or complete secondary axes were observed in less than 0.1% of uninjected embryos.

Xnr1 and noggin together induce notochord in ectodermal explants

When tested in the paracrine assay, the library pool containing Xnr1 and noggin induced greater tissue elongation than library pools containing noggin alone. One plausible explanation for these enhanced morphogenetic movements is that Xnr1 and noggin are acting together to induce notochord, a tissue that undergoes substantial extension in the developing embryo. To test this idea, ectodermal explants were grafted onto oocytes expressing both proteins, cultured until sibling embryos reached stage 31, and then immunostained using the notochord-specific Tor-70 monoclonal antibody.

Co-injection of equal amounts of Xnr1 RNA and noggin RNA (total amount injected = 0.2 ng, 2 ng or 10 ng) induced explants to elongate dramatically away from the oocyte and to form notochord along much of their length. Notochord tissue was distinguishable in oocyte-explant recombinants based upon its 'stacked coin' appearance (Fig. 5, upper left panel) and its immunoreactivity with the Tor-70 antibody (Fig. 5, upper right panel). Explants that had been detached from the oocyte prior to sectioning and staining also showed abundant Tor-70 staining (Fig. 5, lower right panel). Explants grafted onto oocytes co-injected with 1 ng of Xnr1 and 1 ng of noggin RNA formed notochord in 9 of 19 explants. In contrast, explants grafted onto oocytes injected with 1 ng Xnr1 RNA (n=16) or 1 ng noggin RNA (n=13 explants) never formed notochord. A fivefold higher dose of Xnr1 (i.e. 5 ng) was alone capable of occasionally inducing notochord. Noggin alone, at all doses tested (0.1, 1, 5 and 25 ng), never caused notochord formation, nor were notochords ever induced in control explants grafted onto uninjected oocytes.

Stage-specific induction of mesoderm and neural tissue by Xnr1

The ability of Xnr1 to induce secondary axes suggests that it is capable of inducing mesoderm and neural tissue. To determine whether Xnr1 acts non-cell autonomously, we grafted ectodermal explants onto Xnr1-expressing oocytes, and then later detached the explants and assayed them by RT-PCR for expression of the mesoderm marker muscle actin and the pan-neural marker NCAM. Explants grafted onto activin- or noggin-expressing oocytes served as positive controls for mesoderm and neural induction, respectively. Explants from either blastula or early gastrula embryos were grafted onto the oocytes to probe for stage-specific effects of Xnr1.

Explants grafted onto oocytes injected with ≥1 ng of Xnr1 RNA generally elongated as a single process away from the oocyte attachment point (Fig. 6, upper right panel). RT-PCR analysis showed that the dorsal mesodermal marker muscle actin was expressed in the induced tissue regardless of whether it had been initially explanted at the blastula or gastrula stage of development. In contrast, Xnr1 was a more effective inducer of NCAM in blastula explants than in gastrula explants (Fig. 6, lower panel). Activin had similar effects as Xnr1: it induced muscle actin equally well in blastula and gastrula explants but induced NCAM more strongly in blastula than gastrula explants. Noggin induced NCAM expression but not muscle actin expression in blastula or gastrula explants. Control explants grafted onto uninjected oocytes partially rounded up (Fig. 6, upper left panel), and failed to express either muscle actin or NCAM (Fig. 6, lower panel).

To examine the distribution of muscle in the Xnr1-treated explants, the explants were fixed, sectioned and stained with the muscle-specific 12/101 monoclonal antibody. Immunofluorescence analysis indicated that the Xnr1-treated explants differentiated primarily into large blocks of muscle, comprising 80% of most tissues, regardless of whether the tissue was explanted at the blastula or early gastrula stage (Fig. 7, right panels). Control explants grafted onto uninjected oocytes failed to express muscle markers (Fig. 7, left panels).

An early response to Xnr1 treatment was the induction of the DNA-binding proteins goosecoid and brachyury. Late blastula-stage explants were grafted onto Xnr1-expressing oocytes and then detached about five hours later, when sibling embryos reached the late gastrula stage. RT-PCR analysis showed that explants grafted onto oocytes injected with 5 ng of Xnr1 RNA expressed the dorso-anterior mesodermal marker goosecoid and the pan-mesodermal marker brachyury (Fig. 8). Explants grafted onto oocytes injected with 1 ng of Xnr1 RNA expressed brachyury but not goosecoid. Control explants failed to express either marker.

DISCUSSION

Role of Xnr1 in Spemann organizer function

When expressed alone or together with noggin, Xnr1 has activities that might be expected of a signaling protein involved in organizer function. Alone, Xnr1 acts non-cell autonomously to induce at least two different types of mesoderm in blastula- or gastrula-stage ectodermal explants. Explants treated with low doses of Xnr1 express brachyury, a general mesoderm marker, but not goosecoid, an organizer-specific marker, or NCAM, a pan-neural marker. At higher doses, Xnr1 induces brachyury, goosecoid and NCAM. The induction of neural tissue by Xnr1 is presumably a secondary consequence of the induction of dorsal mesoderm, which has strong neural-inducing properties. The competence of presumptive ectoderm to form neural tissue is almost completely lost by the late gastrula stage of development. Thus, Xnr1 was probably a more effective inducer of neural tissue in blastula explants than in gastrula explants because blastula explants were exposed to secondary neural-inducing signals for a longer period of time prior to losing neural competence.

When expressed alone ectopically on the ventral side of intact embryos, Xnr1 or noggin induce the formation of a partial secondary axis that lacks anterior structures such as eyes and cement gland. In contrast, their co-expression induces the formation of a complete secondary axis, mimicking the effect of transplanting an early gastrula organizer to the ventral side of a host embryo. Do Xnr1 and noggin together induce an early dorsalizing center, which then induces the gastrula organizer, or do they act at gastrula stages to directly mimic organizer function? It is difficult to distinguish between these possibilities in experiments that involve RNA injection into intact two- or four-cell embryos, since this leads to protein expression throughout blastula and gastrula stages. The paracrine assay used to identify Xnr1 does not suffer from this drawback, however, since changes in tissue competence can be assessed simply by explanting the responding tissue at different stages of development. We therefore used this approach to show that early gastrula-stage ectoderm could be induced by either Xnr1 or noggin to form mesoderm or neural tissue, respectively, or by both Xnr1 and noggin to form notochord. These findings are consistent with the idea that Xnr1 and noggin can act even as late as the early gastrula stage of development to induce mesoderm with organizer-like properties.

In further support of the idea that Xnr1 and noggin can completely reproduce the activity of factors normally involved in organizer formation, ectopic expression of either gene rescued complete axes in UV-ventralized embryos (Smith and Harland, 1992; Jones et al., 1995). It is not clear why Xnr1 and noggin rescue complete axes in UV-ventralized embryos but only

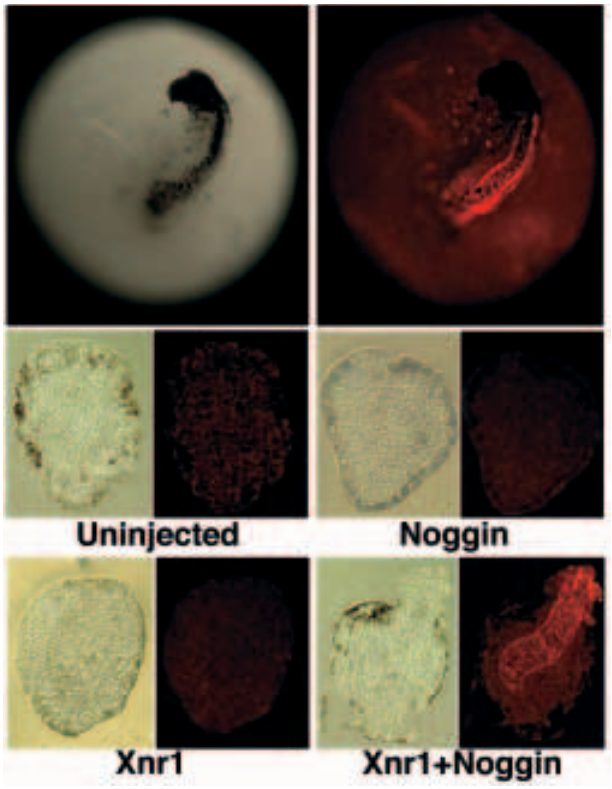

Fig. 5. Induction of notochord by co-expression of Xnr1 and noggin. Ectodermal explants from early gastrula stage embryos were grafted onto uninjected oocytes or onto oocytes injected with 1 ng noggin RNA, 1 ng Xnr1 RNA or 1 ng of both RNAs. Upper left panel: bright-field photograph of an explant that had been grafted onto an oocyte expressing both Xnr1 and noggin. Upper right panel: fluorescence micrograph of the same explant immunostained in whole mount with the Tor-70 notochord-specific antibody. Lower panels: differential interference contrast (DIC) and corresponding fluorescence micrographs of explant sections immunostained with Tor-70. Notochords up to 140 μm in length were detected within a single explant.

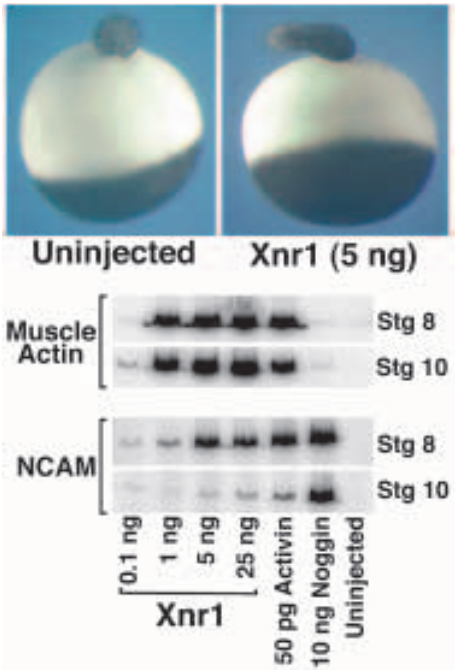

Fig. 6. Mesoderm induction is due to non-cell-autonomous signaling by Xnr1. Ectodermal explants from early blastula or early gastrula embryos were grafted onto uninjected oocytes or onto oocytes injected with the indicated amount of Xnr1, activin, or noggin RNA. Upper panel: oocyte-explant conjugates were photographed when sibling embryos reached the early tailbud stage. Lower panels: explants were detached from the oocytes when sibling embryos reached the early tailbud stage, and then assayed by RT-PCR for expression of the mesodermal marker muscle actin or the neural marker NCAM. Each sample consisted of a pooled set of six explants, and each PCR reaction contained the cDNA equivalent of one-tenth of an explant.

induce partial secondary axes in normal embryos. This may reflect the release of an inhibitory signal from the primary dorsal axis of a normal embryo, the depletion of dorsal factors in the normal embryo or the existence of a UV-sensitive factor required for regulation. It is possible to override this regulatory signal, if it does exist, since ectopic expression of both Xnr1 and noggin (this study) or the signaling protein Wnt (McMahon and Moon, 1989) induces complete secondary axis formation in normal embryos. A Wnt-like activity is believed to be one of the earliest signals in the embryo that ultimately leads to the formation of the gastrula organizer (Parr and McMahon, 1994). Thus the inability of Xnr1 or noggin by themselves to induce complete secondary axes in normal embryos is consistent with the idea that (when ectopically expressed) they exert their effects later in development than Wnt does.

Xnr1 is one of several *Xenopus* genes related to mouse nodal, a gene expressed in the node, the mouse equivalent of the *Xenopus* organizer. Mice homozygous for loss of nodal lack the primitive streak, fail to express certain mesodermal markers and exhibit migrational defects in the mesoderm that does form (Conlon et al., 1994; Zhou et al., 1993). Taken together, the results suggest that nodal is required for the induction and patterning of mesodermal tissue in the mouse. Since little is known of the signaling pathways upstream or downstream of Xnr1, and it is not yet possible to inhibit endogenous Xnr1 signaling in *Xenopus* embryos, it is unclear whether Xnr1 carries out a similar function in the frog. Nevertheless, Xnr1 is expressed in the correct place and time and has activities alone and in combination with noggin that are consistent with a possible role in the induction and patterning of axial mesoderm.

Left-right asymmetry of Xnr1

The expression pattern of Xnr1 resembles that of a nodal-related gene (cNR-1) implicated in left-right asymmetry generation in the early chick embryo (Levin et al., 1995). Like Xnr1 in *Xenopus*, cNR-1 is expressed in several different regions of the chick embryo during early development. In both chick and *Xenopus*, expression occurs in a relatively large region of lateral plate mesoderm on the left side of the embryo. cNR1 is asymmetrically expressed during late gastrula stages in the chick, however, whereas asymmetric expression of Xnr1 is not detected until the end of neurulation in *Xenopus*. Asymmetric expression of cNR-1 is initially confined to a small region just lateral and anterior to the left of Hensen's node, the

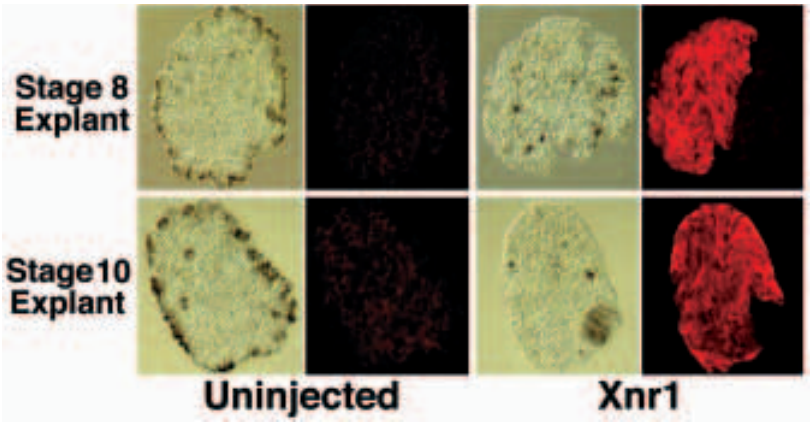

Fig. 7. Induction of muscle by Xnr1. Ectodermal explants from early blastula or early gastrula embryos were grafted onto uninjected oocytes or onto oocytes injected with 1 ng Xnr1 RNA. When sibling embryos reached the early tailbud stage, the explants were detached from the oocytes, sectioned and immunostained with the muscle-specific 12/101 antibody. Differential interference contrast (DIC) and corresponding fluorescence micrographs are shown.

chick equivalent of the *Xenopus* organizer. We did not detect a similar region of asymmetric expression in the *Xenopus* embryo. Instead, we found that Xnr1 is expressed in two small stripes of cells symmetrically flanking the posterior notochord just prior to and concomitant with its asymmetric expression in lateral plate mesoderm. cNR-1 is also expressed symmetrically during early chick development; however, this occurs at the outset of gastrulation within and lateral to the mesoderm-forming primitive streak.

During gastrulation, cNR-1 is expressed near but not within Hensen's node of the chick (Levin et al., 1995). In contrast, Xnr1 is expressed in the dorsal marginal zone during *Xenopus* gastrulation, with enrichment in the organizer (Jones et al., 1995). The apparent low abundance of Xnr1 transcripts during gastrula stages has made it difficult to use whole mount in situ hybridization to determine the spatial distribution of Xnr1 within the organizer (unpublished results). RT-PCR analysis of embryos cut into right or left halves showed that the overall level of Xnr1 transcripts is approximately the same on the left side as on the right side of the *Xenopus* embryo during gastrula stages (unpublished results). RT-PCR, though highly sensitive, may not be able to reliably detect small quantitative differences. Thus, at present it is unclear whether the strong asymmetric expression of Xnr1 observed during post-gastrula stages is due to a more subtle asymmetry in Xnr1 expression at gastrulation or whether the expression of Xnr1 during gastrulation is unrelated to its expression in the post-gastrula embryo.

cNR-1 is one of three asymmetrically expressed genes that may form a signaling cascade involved in left-right asymmetry generation in the chick (Levin et al., 1995). Whether Xnr1 participates in a similar signaling cascade in *Xenopus* remains to be determined. It is also not yet known in the chick or in *Xenopus* how left-right asymmetry in gene expression is first initiated or how asymmetric gene expression ultimately leads to changes in morphological structure. Nevertheless, the similarity between the expression patterns of Xnr1 and cNR-1 suggests that there may be conserved signaling pathways generating left-right asymmetry among different vertebrates.

Expression cloning by screening small pools

We identified Xnr1 by screening small library pools with a paracrine assay. Because the pool size is inversely related to the concentration of each clone in the pool, the use of small pool sizes was expected to increase the probability of a protein being detected based on its biological activity. This turned out

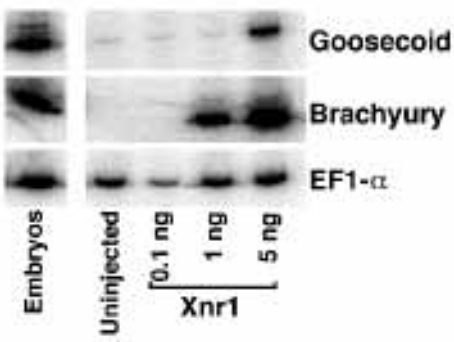

Fig. 8. Induction of brachyury and goosecoid by Xnr1. Ectodermal explants from late blastula stage embryos were grafted onto uninjected oocytes or onto oocytes injected with the indicated amount of Xnr1 RNA. Explants were detached from the oocytes when sibling embryos reached the late gastrula stage, and then assayed by RT-PCR for expression of brachyury or goosecoid. Each sample consisted of a pooled set of six explants, and each PCR reaction contained the cDNA equivalent of one-tenth of an explant. EF1-α was assayed to show that comparable amounts of RNA were isolated from each sample.

to be the case; even a tenfold difference in pool size substantially increased the frequency with which we detected noggin with a paracrine assay. On a per clone tested basis, noggin was identified over twice as frequently when pools of 100-200 were screened than when pools of 1,000-2,000 were screened. It was more labor intensive to prepare and screen small pools than to prepare and screen large pools. Nevertheless, this increased effort seems compensated by the increased frequency with which active cDNAs are functionally identified.

The increase in sensitivity obtained by screening small pools also potentially expands the type of assay that can be used in expression screens, to include those previously thought too insensitive for this use. These include assays that detect enzymatic activities, interactions with antibodies, nucleic acids or proteins, or practically any other easily measurable biological or physical property of a pooled cDNA, RNA or protein. Small pools are also less likely than large pools to contain multiple active cDNAs (which can confound the sib selection process) and they can be subdivided in fewer steps to a single active cDNA.

In the present study, Xnr1 and noggin by themselves were active in the paracrine and axis duplication assays, thereby

enabling us to follow each separately through the sib selection process and then ultimately recombine them to generate the original phenotype. These findings suggest the feasibility of carrying out expression screens in the presence of known proteins, as happened here fortuitously. Such a combinatorial approach might enable the identification of proteins that would otherwise be difficult to isolate because they lack detectable biological activity on their own.

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Note added in proof

Lowe et al. (1996) have also recently reported that Xnr1 expression is restricted to the left side of *Xenopus* embryos (*Nature* **381**, 158-161).