Two novel nodal-related genes initiate early inductive events in *Xenopus*

Nieuwkoop center

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Accepted 4 October; published on WWW 14 November 2000

SUMMARY

In vertebrates, Nodal-related protein plays crucial roles in mesoderm and endoderm induction. Here we describe two novel *Xenopus* nodal-related genes, Xnr5 and Xnr6, which are first zygotically expressed at the mid-blastula transition, in the dorsal-vegetal region including the Nieuwkoop center. Xnr5 and Xnr6 were isolated by expression screening of a library enriched with immediate-early-type transcripts, and are strong inducers of both mesoderm and endoderm. They also induce the other nodal-related genes in the animal cap. In embryos, cerberus-short (nodal-specific inhibitor) can inhibit Xnr1 and Xnr2 express to the same extent goosecoid, but not Xnr5 and Xnr6 transcription. Xnr5 and Xnr6 are regulated completely cell autonomously, differently from other Xnrs in the cell-dissociated embryos. The expression of Xnr5 and Xnr6 is regulated by maternal VegT and b-catenin, but does not require TGF-β signaling. Therefore, expression of Xnr5 and Xnr6 is controlled by different mechanisms from other Xnr family genes.

Key words: *Xenopus*, Nodal, Xnr5, Xnr6, Nieuwkoop center, Endoderm, Mesoderm, VegT, b-catenin, Cer-S

INTRODUCTION

The dorsal lip region of the amphibian embryo was described as the organizer of body plan by Spemann and Mangold (1924). It was predicted that part of the mesoderm-inducing signal was released from the vegetal region beneath the Nieuwkoop center (Nieuwkoop, 1969). Following this work, the three-signal model was proposed, in which the first two kinds of signals are released from the vegetal region during the blastula stage (Slack et al., 1987). One is a pan or ventral mesoderm inducer, while the second induces dorsal mesoderm, including the Spemann’s organizer; the third signal is secreted from the anterior ectoderm, regulating the expression of some genes (Kimelman and Lemaire, 1999). It was predicted that part of the mesoderm-inducing signal was released from the vegetal region during the blastula transition, in the dorsal-vegetal region including the Nieuwkoop center. Xnr5 and Xnr6 are maternal determinants required for early embryonic events (Wylie et al., 1996; Zhang et al., 1998). The maternal transcripts of VegT, which encodes a transcription factor containing a T-box (Lustig et al., 1996b; Stennard et al., 1996; Zhang and King, 1996; Horb and Thomsen, 1997) are implicated in mesoderm induction by inducing Xnr1, Xnr2, Xnr4 and other TGF-β genes (Kofron et al., 1999). Kofron et al., showed that in VegT-depleted embryos the axis including head as well as trunk and tail, were completely rescued by Xnr1, Xnr2 and Xnr4, derrière also rescued the axis, but not the head. VegT is also required in endoderm determination, and

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part of the VegT signal is mediated by the Xnrs (Zhang et al., 1998; Kofron et al., 1999; Yasuo and Lemaire, 1999). Tbx-binding sites are present in the promotor region of Xnr1, and VegT can drive transcription via this region in the reporter assay (Kofron et al., 1999; Hyde and Old, 2000). Cycloheximide (CHX) treatment of embryo or cell dissociation experiments suggest that at least part of Xnr1 and Xnr2 expression is cell autonomous, under the control of a maternal factor (Yasuo and Lemaire, 1999). These observations could mean that the expression of Xnrs is initiated by VegT signal. Several studies have analyzed the regulatory elements of nodal-related genes (Adachi et al., 1999; Norris and Robertson, 1999; Osada et al., 2000). There are Fast-binding sites in intron 1 of Xnr1 and nodal, which are essential for their expression in the early inductive stage and the late asymmetrical stage. Inhibitor analysis using a dominant-negative form of activin receptor or cer-S (nodal specific inhibitor) has confirmed that nodal-related signaling is required to accumulate Xnr transcripts during the mesoderm and endoderm inductive events (Yasuo and Lemaire, 1999; Agius et al., 2000).

Here, we describe two novel nodal-related genes, Xnr5 and Xnr6, which, on the basis of their regulatory mechanisms, belong to a different group from the other Xnrs. Xnr5 and Xnr6 were isolated from a cDNA library constructed from embryos dorsalized using lithium chloride (LiCl) (Kao and Elision, 1988), in which immediate early-type transcripts were enriched at the mid-blastula transition (MBT) by inhibition of secondary transcription using the protein synthesis inhibitor, CHX. They were first detected at the MBT when zygotic transcription began, localized at the dorsal-vegetal region including the Nieuwkoop center. They quickly responded to the inductive signal, with transcription reaching a maximal level soon after the MBT, whereas Xnr1, Xnr2 and Xnr4 transcripts gradually accumulate around blastula and gastrula stages. Both Xnr5 and Xnr6 are strong inducers of mesoderm and endoderm, and can induce other Xnrs in animal caps. They are regulated cell autonomously by the maternal determinants VegT and β-catenin, but not by TGF-β signal. These results suggested that Xnr5 and Xnr6 may play a role in initiation of mesendoderm induction in Xenopus development.

MATERIALS AND METHODS

Embryos

*Xenopus laevis* embryos were obtained by artificial fertilization and were cultured in 10% Steinberg’s solution (SS) at 20°C. Embryos were staged according to Nieuwkoop and Faber (1956). Embryo dissection and animal cap assay were performed in 100% SS and were incubated until sampling.

Construction of LiCl-CHX cDNA library and screening

The cDNA library was obtained as follows. The 32-cell embryos were treated with 0.3 M LiCl for 8 minutes and then with 5 μg/ml CHX from stages 7 to 9; mRNA was then extracted from these embryos. A LiCl-CHX cDNA library was constructed using the AZAPII cDNA library system (Stratagene), and was screened by sib-selection. A library was plated with 1000 pfu/plate and the phage were suspended in SM buffer. The inserts were amplified by PCR with M13-20 and M13-RV primers using suspended phage. mRNA was synthesized from these PCR products by T3 mMESSAGE mMACHINE (Ambion), and 5-10 ng of mRNA was injected into vegetal ventral blastomers of eight-cell stage embryos. After culture for a day, the phenotypes of embryos were observed. Pools with the activity to induce a secondary axis were divided into 100 pfu/plate lots and the process of sib-selection was repeated until a single clone was isolated. Both strands of the clone were sequenced using the BigDye terminator system (PE Biosystems).

Microinjection

Microinjection was performed in 100% SS containing 4% Ficoll. mRNA was synthesized using SP6 or T7 mMESSAGE mMACHINE (Ambion) with templates from the following digested plasmids or PCR products: pCS2-XNR1; pCS2-XNR2 (Jones et al., 1995); pCS2- cer-L, pCS2-cer-S (Piccolo et al., 1999); pCS2-VegT (Zhang and King, 1996); pCS2-derrière (Sun et al., 1999); pdor (Xnr3) (Smith et al., 1995); pSP64T-Xnrnt-8 (Smith and Harland, 1991; Sokol et al., 1991; Christian et al., 1992); pSP64T-dvl1 (mouse) (Sussman et al., 1994); pRNA- Xsia (Lemaire et al., 1995); pXBC40 (β-catenin) (Yost et al., 1998); pSP64TEN-XMAD2 (Graff et al., 1996); pSP64TBVg1 (Thomsen and Melton, 1993); pSP64-DMN1/Stop (iAR) (Hemmatis- Brivanlou and Melton, 1992); pSP64-T- mTFRII-45 del21 (iBR, mouse) (Suzuki et al., 1994); pSP64TEN-XFS-319 (Hemmatis-Brivanlou et al., 1994); pXFDNxs (Amaya et al., 1993) and pNRRX-Xnr5; and pNRRX-Xnr6. pNRRX-Xnr5, pNRRX-Xnr6, NLS-lacZ and pCS2-cer-S were constructed by PCR. pNRRX-Xnr5 and pNRRX-Xnr6 contain only the Xnr5 and Xnr6 ORFs, respectively. pCS2-VegT and pCS2- derrière were constructed by optional screening. The pNRRX vector was constructed from pBluescriptII by first disrupting the Not1 and XhoI sites (Stratagene). Then the 5’ and 3’ globin UTRs from pSP64T were subcloned into the HindIII/PstI site of the vector. The additional cloning sites (NotI, EcoRI, EcoRV, XhoI) inserted into the BgIII site between the globin UTRs, and eight-base restriction enzyme sites were constructed in the 3’ end of the globin UTR to make a template for mRNA synthesis.

RT-PCR analysis

Total RNA isolation and RT-PCR were performed as described (Yokota et al., 1998). The PCR products were confirmed by Southern blotting and sequencing. Ornithine decarboxylase (ODC) (Osborne et al., 1991) and EF1-α were used as positive controls. Reverse transcriptase negative (RT-) reactions showed no evidence of genomic DNA contamination. The primers used were as follows: Xnr2 (F, ATCTGATGGCCCTTCAAGGC; R, GACCTTCTTCACACCTCACGCC); Xnr3 (F, AAGAAGACATCCTCACGTGG; R, TACGTGACCTGCCAACCTCA); Xnr4 (F, TTACAGATGG-TGCACACTC; R, AACCTGCTGATGACCTGG); Xnr5 (F, TCAACATCTTCTCACCTAGGCC; R, GGAACCTCTGAAAGGAAAGGAC); Xnr6 (F, TTCAGATATGACCTTGTTG; R, TTTCCTGTCCCCTGCTGCT); derrière (F, AGGCCAACAGTCTTCTG; R, ATGTGATGGTCATCCTGGCT); sia (F, CATCAGCACCTGACTGG); pNRRX-Xnr5, pNRRX-Xnr6, NLS-lacZ and pCS2-cer-S contain Xnr5 and Xnr6 ORFs, respectively. Reverse transcription using the protein synthesis inhibitor, CHX. They were first detected at the MBT when zygotic transcription began, localized at the dorsal-vegetal region including the Nieuwkoop center. They quickly responded to the inductive signal, with transcription reaching a maximal level soon after the MBT, whereas Xnr1, Xnr2 and Xnr4 transcripts gradually accumulate around blastula and gastrula stages. Both Xnr5 and Xnr6 are strong inducers of mesoderm and endoderm, and can induce other Xnrs in animal caps. They are regulated cell autonomously by the maternal determinants VegT and β-catenin, but not by TGF-β signal. These results suggested that Xnr5 and Xnr6 may play a role in initiation of mesendoderm induction in Xenopus development.

RESULTS

Expression cloning of Xnr5 and Xnr6

We constructed a cDNA library using embryos treated with CHX and LiCl. CHX treatment can lead to accumulation of immediate-early-type transcripts, and LiCl treatment leads to a hyperdorsalized phenotype in embryos. The clones were then
screened, based on their ability to induce a secondary axis. Consequently, we isolated two novel genes that could induce a partial secondary axis lacking the head structures when they were misexpressed ventrally. Analysis of the deduced amino acid sequences revealed that both were members of the TGF-β superfamily, and were similar to each other, and to the Xenopus nodal-related genes, Xnr1 and Xnr2 (Jones et al., 1995; Lustig et al., 1996a; Fig. 1A), so we named them Xnr5 and Xnr6 (DDBJ/EMBL/GenBank Accession Numbers AB038133 for Xnr5 and AB038134 for Xnr6). Xnr5 and Xnr6 encode proteins of 384 and 378 amino acids, respectively, and contain the RXXR cleavage sites. Seven cysteine residues (cysteine knot) are also present at the C-terminal region. This cleavage site and the cysteine knot are conserved among TGF-β superfamily proteins (Kingsley, 1994). The gene sequence of Xnr5 is 48%, 55% and 56% homologous to Xnr1, Xnr2 and Xnr6; Xnr6 is 52% and 58% homologous to Xnr1 and Xnr2, respectively (Fig. 1B). Levels of Xnr5 and Xnr6 transcripts were slightly increased when embryos were treated with LiCl under the same conditions used for library construction (data not shown). In this screening, a total of 30,000 clones were analyzed. Xnr5* and Xnr6* clones, very similar to Xnr5 and

**Fig. 1.** Sequence alignment and identities. (A) Alignment of the Nodal-related protein sequence from Xenopus Xnr5, Xnr6, Xnr1, Xnr2 and mouse Nodal. Identical or similar residues are highlighted by a dark or light background, respectively. Maturation cleavage sites (RXXR) are boxed. Asterisks indicate the seven cysteines within the mature region. (B) The percentage identities between full-length Xenopus Nodal-related proteins.
**Xnr5 and Xnr6 induce axial mesoderm and endoderm**

RT-PCR analysis was used to determine the temporal expression of Xnr5 and Xnr6 (Fig. 2A). No maternal transcripts were detected, and expression of both genes first appeared at stage 8-8.5, when zygotic transcription began (MBT). Their expression peaked during blastula and then Xnr6 expression gradually decreased after the onset of gastrulation, whereas Xnr5 was no longer detected in the mid-gastrula stage embryos. We also confirmed the later stage expression of Xnr5 and Xnr6 by RT-PCR. Xnr5 and Xnr6 was not detected from stage 12 to stage 30, unlike Xnr1 (data not shown). Xnr1, Xnr2 (Jones et al., 1995; Lustig et al., 1996a), Xnr4 (Joseph and Melton, 1997) and derrière (Sun et al., 1999) belong to the TGF-β superfamily, and are good candidate mesoderm inducers. Very low levels of Xnr1 and Xnr2 transcripts were also detected at stage 8.5, but these began to accumulate clearly after stage 9 (Fig. 2A). siamois (Lemaire et al., 1995), which is thought to be regulated by the dorsalizing signal, is also first expressed at the same stage as Xnr5 and Xnr6 (stage 8-8.5, Fig. 2A).

The spatial distribution of Xnr5 and Xnr6 expression was analyzed by whole-mount in situ hybridization as previously described (Harland, 1991). In the late blastula embryo, Xnr5 and Xnr6 mRNA was detected from the vegetal pole to the dorsal vegetal region, including the Nieuwkoop center (Fig. 2D-F). At early gastrula, an Xnr6 signal was seen at just beneath the dorsal lip (Fig. 2G), whereas no Xnr5 signal could be detected at this stage (data not shown). Unlike Xnr3 (Fig. 2B,C), Xnr5 and Xnr6 transcripts did not localize at the Spemann’s organizer. Since these probes could not penetrate into the deep endoderm region, we also examined expression using hemisectioned embryos. Both Xnr5 and Xnr6 were detected in dorsal endoderm at stage 8.5 (Fig. 2J,L), and then were detected through the deep endoderm region at stage 9 (Fig. 2K,M). All Xnr5 and Xnr6 signals were speckled in appearance. This pattern is often seen in the blastula and early gastrula embryo when the signal is detected at the marginal to vegetal regions (Jones et al., 1995). To support these observations, we also examined their patterns of expression using the RT-PCR method (Fig. 2O,P). Stage 9 embryos were divided into four parts (Fig. 2O), and then mRNA was prepared from each part. Xnr5 and Xnr6 are expressed at high levels in the dorsal-vegetal and lateral-vegetal regions (Fig. 2P). These results correlate well with those obtained from whole-mount in situ hybridization experiments.

**Xnr5 and Xnr6 are expressed at endoderm including the Nieuwkoop center**

**Xnr5 and Xnr6 induce axial mesoderm and endoderm**

To examine the activities of Xnr5 and Xnr6, we microinjected mRNA into ventral-vegetal blastomeres of eight-cell stage embryos (Fig. 3A-F). These injected embryos formed a secondary axis, and had no evident head structures (Fig. 3A,B). The secondary axes contained pharyngeal endoderm, notochord, muscle, neural tube and other axial structures (Fig. 3C,D). lacZ (NLS-β-Gal) mRNA was co-injected to trace the cell lineage (Fig. 3E,F). X-gal-stained cells were mainly seen in the endoderm, but some were also present in the axial mesoderm of the secondary axes (Fig. 3F). These cells seemed to have strong inductive activities for other structures. It was reported that Xnr1 could completely...
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Xnr5 and Xnr6 initiate embryonic induction and rescue UV-irradiated embryos, and that Xnr2 and Xnr4 could partially rescue this phenotype (Jones et al., 1995, Joseph and Melton, 1997). We performed the same kind of experiment, and found that both Xnr5 (1 pg) and Xnr6 (10 pg) could only partially rescue the phenotype (data not shown), which may reflect the fact that they induce different balance downstream genes to Xnr1. Animal cap assay further identified differences in activity between Xnr5 and Xnr6 (Fig. 4A-G). The explants injected with Xnr5 started to elongate at a lower concentration of mRNA (5 pg; Fig. 4B) than that of Xnr6 (20 pg; Fig. 4C). In contrast, explants seemed to be longer after injection of Xnr6. Sections of these explants showed that Xnr5 could effectively induce notochord and endodermal cell mass, whereas Xnr6 largely induced muscle (Fig. 4D-G). Xnr6 also induced small notochord and endodermal cells. We also performed RT-PCR analysis to check the expression of marker genes (Fig. 4H). Both Xnr5 and Xnr6 induced the pan-endodermal marker endodermin (edd) (Sasai et al., 1996) and the notochord-specific marker collagen type II (col II) (Amaya et al., 1993). The muscle-specific marker ms-actin (Stutz and Spohr, 1986) was upregulated by Xnr6 even at a low concentrations, but not by Xnr5. By contrast, the cardiac mesendodermal marker XNkx-2.5 (Tonissen et al., 1994) was induced by Xnr5, but not by Xnr6. In conclusion, both Xnr5 and Xnr6 can induce endoderm and axial mesoderm, but they have distinct inductive activities.

Xnr5 and Xnr6 can induce Xnr1 and Xnr2 expression

To further analyze Xnr5 and Xnr6 function, we examined the expression of early response genes. In addition to mesodermal (Xbrahcyury: Xbra) and endodermal (Xsox17β) markers Xnr5 and Xnr6 could also induce other TGF-β genes (Xnr1, Xnr2 and derrière) (Fig. 5A), but not siamois (data not shown). The next question was what mediated the Xnr5 and Xnr6 signals. Among a number of inhibitors co-injected with Xnr5 or Xnr6, tAR1, which is a dominant-negative form of activin receptor type II (Hemmati-Brivanlou and Melton, 1992), was able to suppress the expression of downstream genes (Fig. 5B). follistatin (FS) (Hemmati-Brivanlou et al., 1994) injection had no effect, suggesting that Xnr5 and Xnr6 signals were transduced via the
activin receptor without Activin. cerberus-long (cer-L) (Piccolo et al., 1999) also inhibited their inductive activities (Fig. 5B), and could rescue developmental abnormalities in Xnr5- and Xnr6-injected embryos (Fig. 5C-I). The rescued embryos had a normal axis including head, trunk and tail, and the ventroposterior tissues were also formed (Fig. 5G-I). The large cement gland and head may have been derived from the ventroposterior tissues (Fig. 5G-I). Wild type embryo (uninjected control); (H) Xnr5+cer-L-injected embryo; (I) Xnr6+cer-L-injected embryo. (J) cer-S inhibited Xnr5 and Xnr6 signaling in animal caps. Induction of Xnr1, Xnr2 and Xnr4 is suppressed by cer-S. Animal caps were dissected at stage 9, and cultured for 2 hours and were analyzed by RT-PCR. Expression of Xnr1 and Xnr2 is suppressed by tAR and cer-L. C-F, cer-L rescues Xnr5- or Xnr6-injected embryos. (C,E) Hyper-dorsalized embryos injected with Xnr5 or Xnr6 (20 pg) into both blastomeres at the 2-cell stage are shown, respectively. (D,F) cer-L (2 ng) co-injection rescues the phenotype. (G-I) Histological sections indicate that these rescued embryos have normal ventroposterior tissues; (G) Wild type embryo (uninjected control); (H) Xnr5+cer-L-injected embryo; (I) Xnr6+cer-L-injected embryo. (J) cer-S inhibited Xnr5 and Xnr6 expression. We next examined the effect of injection of cer-S into embryos (Fig. 5B-D). Injection of a low concentration of cer-S (100 pg/embryo) inhibited formation of head structures (Fig. 5C). At a high concentration (500 pg/embryo), in addition to that phenotype, cer-S also disturbed axial structures (Fig. 5D). Under these conditions, expression of Xnr1, Xnr2 and goosecoid (gsc) was greatly reduced in these embryos, whereas expression of Xnr5, Xnr6 and derrière was not changed (Fig. 5E). These results suggest that the expression of Xnr1 and Xnr2 but not Xnr5, Xnr6 and derrière are regulated by the Xnr family. Xnr5 and Xnr6 may be required for the expression of Xnr1 and Xnr2 in the embryo.

VegT and β-catenin regulate Xnr5 and Xnr6

The spatial and temporal patterns of expression of Xnr5 and Xnr6 suggest that these genes might be controlled by dorsal determinants. Previous studies have shown that if 2/3 of the vegetal hemisphere of an embryo is removed at 0.3 normalized time, dorsal axial structures are reduced, suggesting that dorsal determinants are localized to a specific region of the vegetal hemisphere at this stage (Kikkawa et al., 1996). Neither Xnr5 nor Xnr6 was expressed in these embryos (Fig. 7A), indicating that their expression is controlled by factors present in the deleted region before cortical rotation. To examine whether Xnr5 and Xnr6 expression requires cell-to-cell contact, we...
Fig. 6. The nodal-specific inhibitor cer-S inhibits the expression of Xnr1 and Xnr2 but not Xnr5 and Xnr6. (A) In embryos, among the inhibitors described in Fig. 5B, tAR1 and cer-L reduce the expression of Xnr1 and Xnr2, but do not affect Xnr5 and Xnr6. mRNA was injected radially around the vegetal pole at the four-cell stage. Injected embryos were cultured until stage 9.5 and were analyzed by RT-PCR. (B-D) cer-S inhibits axis formation. (B) Uninjected embryo; (C) 100 pg-injected embryo; (D) 500 pg-injected embryo. (E) cer-S greatly reduces the expression of Xnr1, Xnr2 and gsc. The expression of Xnr5, Xnr6 and derr is not affected. mRNAs were injected radially around the vegetal pole at the four-cell stage. The embryos were cultured until stage 9 (Xnr5 and Xnr6) or 10 (Xnr1, Xnr2, derr and gsc).

performed cell dissociation experiments (Fig. 7B). We used levels of Vg1 mRNA, which is maternally stored in the vegetal region, to verify that the ratio of animal to vegetal cells was not changed by embryo dissociation. Under these conditions, Xnr1 and Xnr2 transcripts were greatly reduced in number, whereas Xnr5 and Xnr6 transcripts were not affected (Fig. 7B). This result indicates that transcription of Xnr5 and Xnr6 is controlled cell autonomously. A portion of Xnr1 and Xnr2 expression may also be mediated cell autonomously.

Recent studies have proposed several candidate dorsal determinants and zygotic inducers (Heasman, 1997), so we next tested whether these factors could induce transcription of Xnr5 and Xnr6. Only VegT could induce Xnr5 and Xnr6 in a dose-dependent manner (Figs 7C, 8A). Xnr1 and Xnr2 were also induced under these conditions (data not shown).

To examine whether VegT could also regulate Xnr5 and Xnr6 expression in the embryo, we analyzed embryos depleted of VegT mRNA (Zhang et al., 1998). When 5 or 10 ng of antisense VegT oligos were injected into oocytes, the level of VegT mRNA was greatly reduced compared with uninjected embryos (Fig. 8B). In these embryos, expression of both Xnr5 and Xnr6 was reduced, suggesting that VegT may regulate Xnr5 and Xnr6 in vivo. Next, we injected Xnr5 and Xnr6 into VegT-depleted embryos to determine whether they can rescue the phenotype. VegT-depleted embryos do not form a blastopore at the gastrula stage, and have no axial structures in later stages (Zhang et al., 1998)(Fig. 8C, bottom). Injection of Xnr5 or Xnr6 could rescue the blastopore formation (data not shown), and dorsal axis formation including head, trunk and tail (Fig. 8C), in the same manner as Xnr1, Xnr2 and Xnr4 (Kofron et al., 1999). Several inhibitors were co-injected with VegT to test whether they could affect the induction of Xnr5 and Xnr6 (Fig. 8D). Activin, Nodal-related, BMP and FGF signal inhibitors

Fig. 7. Inducer of Xnr5 and Xnr6. (A) Deletion of dorsal determinants abolish Xnr5 and Xnr6 transcripts, like siamois. Two thirds of the vegetal hemisphere of the embryo was deleted before cortical rotation (Δ2/3V). The embryos were cultured until stage 9 and were analyzed by RT-PCR. 0.3 NT (normalized time) is 0.3 of the first cell cycle period. Normalized time is used in the experiments on the first cell cycle (Elinson, 1985; Render and Elinson, 1986). (B) Xnr5 and Xnr6 are regulated cell autonomously. Dissociated embryos were cultured in Ca2+- and Mg2+-free modified Barth’s solution (MBS) from the first cleavage and vitelline membranes were removed at the two-cell stage. They were cultured until stage 9.5 and were analyzed by RT-PCR. Xnr5 and Xnr6 are expressed in both embryos, whereas the expression of Xnr1 and Xnr2 is greatly reduced. (C) VegT induces Xnr5 and Xnr6 in animal caps, but Wnt and TGF-β signaling does not. Xnr6-8 (20 pg), Dvl1 (500 pg), β-catenin (500 pg), siamois (sia, 20 pg), Smad2 (2 ng), Bvg1 (100 pg), Xnr1 (100 pg), Xnr2 (100 pg), Xnr3 (1 ng), VegT (500 pg) and derr (1 ng) were injected at the two-cell stage. Animal caps were dissected at stage 9, and cultured for 2 hours, and were analyzed by RT-PCR.
DISCUSSION

We have isolated two novel Xenopus nodal-related genes, Xnr5 and Xnr6, and analyzed their function in early inductive events. Their deduced amino acids sequences were similar to each other, and to Xnr1 and Xnr2, especially in the mature region. cer-S and a dominant-negative form of activin receptor suppressed Xnr5- and Xnr6-mediated induction of downstream genes, suggesting that, like Xnr1 and Xnr2, Xnr5 and Xnr6 signals are also inactivated by direct binding with Cer-S, and their signals are mediated via the Activin receptor. These results indicate that the structures of Xnr5 and Xnr6 are quite similar to Xnr1 and Xnr2. All of these four factors can induce a secondary axis lacking the head structures when they are misexpressed ventrally. Xnr4 also has similar activity (S. T., C. Y., Y. O. and M. A., unpublished). On the other hand, it has been reported that Xnr1 and Xnr2 have different inductive activities. Xnr2 has stronger mesoderm inductive activity than Xnr1 (Jones et al., 1995), but the endodermal marker Gata4 is induced more effectively by Xnr1 (Yasu and Lemaire, 1999).

In the present study, it was not possible to compare Xnr5 and Xnr6 activities precisely with other inducers because they were cloned into different plasmids, leading to different translation efficiencies. However, Xnr5 clearly had stronger endoderm inductive activity than Xnr6. Injection of only 20 pg of Xnr5 mRNA induced animal caps to differentiate into an endodermal cell mass, whereas 20 pg of Xnr6 mRNA induced mesodermal tissue, such as muscle. These different activities of Xnrs in endoderm or mesoderm induction may depend on different binding affinities to the receptors, or to different co-activators, such as FRL1 (Kinoshita et al., 1995). FRL1 belongs to EGF-CFC family, was isolated as a novel FGF receptor ligand, and induced mesoderm when overexpressed. EGF-CFC family genes have been isolated in other animals, including oep (zebrafish; Zhang et al., 1998), cripto and cryptic (mouse; Ciccodicola et al., 1989; Dono et al., 1993; Shen et al., 1997). Embryos that have no maternal and zygotic oep transcripts display a similar phenotype to a double-mutant of Xnr1 and Xnr2 (Yasu and Lemaire, 1999). Rescue experiments of the oep mutant suggest that oep is an essential co-factor for sqt and cyc. The mechanism of interaction between Nodal and EGF-CFC family proteins remains unclear (reviewed by Shen and Schier, 2000).

The TGF-β signal is required for both mesoderm and endoderm induction in vivo (Asashima, 1994; Slack, 1994; Harland and Gerhart, 1997), and several factors have been identified that have mesoderm and endoderm inducing activities. Among them, Xnr1, Xnr2 and Xnr4 are considered zygotic inducers. Cer-S, which has been reported to be a specific antagonist of Xnrs (Piccolo et al., 1999), inhibits the expression of organizer genes and Xbra when injected into embryos (Agius et al., 2000). A dominant-negative cleavage mutant of Xnr2 (cmXnr2) specifically suppresses Xnrs activities, and injection of cmXnr2 into the embryo also suppressed mesodermal and endodermal marker expression (Osada and Wright, 1999). These results confirm that Xnrs signals are required for mesoderm induction and regulate dorsoventral patterning. Recent reports have suggested that Xnr1 and Xnr2 are regulated by VegT and β-catenin (Clements et al., 1999; Kofron et al., 1999; Yasuo and Lemaire, 1999;
Fig. 9. Model of endoderm and mesoderm induction by known nodal-related genes in Xenopus. We have modified the previous model based on our results with Xnr5 and Xnr6. At the MBT, when zygotic transcription starts, vegetally localized maternal VegT induces Xnr5 and Xnr6 in cooperation with β-catenin to make the Nieuwkoop center. However, Xnr5 and Xnr6 may also be regulated by other pathways. Quick accumulation of Xnr5 and Xnr6 extends throughout the deep endoderm. In this phase, only low levels of Xnr1, Xnr2 and Xnr4 expression are induced cell autonomously. Then endodermal expression of Xnr5 and Xnr6 leads to accumulation of Xnr1, Xnr2 and Xnr4 in the endodermal region, which in turn act in endoderm determination in cooperation with VegT, and induce the expressions of Xnr1, Xnr2, Xnr4, gsc and Xbra, among other genes, in the equatorial region (mesoderm induction, including formation of organizer). β-catenin directly regulates expression of siamois, Xtwin and Xnr3 in the dorsal side, and plays a role in determination of anterior endoderm and organizer.

Agius et al., 2000; Hyde and Old, 2000, both of which are regarded as maternal dorsal determinants (Larabell et al., 1997; Zhang et al., 1998; Wylie et al., 1996). These maternal signals are thought to act as initiators of Xnr expression. Xnr1, Xnr2 and Xnr4 transcripts are first detected at low levels at mid-blastula, and accumulate after late blastula in endoderm. Inhibitor experiments and promoter analysis suggest that this accumulation in endoderm requires a TGF-β signal, probably Xnr (Agius et al., 2000; Osada et al., 2000). Our results also suggest that Xnr1 and Xnr2 expression is largely regulated by an Xnr signal, since both Xnr1 and Xnr2 expression was greatly reduced in cer-S-injected or cell-dissociated embryos. Xnr5 and Xnr6 are good candidate endogenous regulators of other Xnrs for a number of reasons. Xnr5 and Xnr6 can induce Xnr1, Xnr2 and Xnr4 in animal cap assay, even at low doses. Xnr5 and Xnr6 transcripts quickly accumulate at the MBT in endoderm region. Of the various developmental regulators tested, only VegT could induce Xnr5 and Xnr6 in animal caps, and Xnr3 and Xnr5 transcripts were greatly reduced in VegT-depleted embryos, strongly suggesting that VegT is an endogenous regulator of Xnr5 and Xnr6. Exogenous antipodean also induced Xnr5 and Xnr6 in presumptive ectoderm (data not shown), although endogenous antipodean is expressed in distinct different regions to Xnr5 and Xnr6 in the embryo. antipodean may mimic VegT function in the animal cap. Moreover, β-catenin may also be involved in induction of Xnr5 and Xnr6 in cooperation with VegT. Xnr5 and Xnr6 differ from Xnr1 and Xnr2 in that in presumptive ectoderm, they cannot be induced by TGF-β signals, such as Bvg1, Xnr1, Xnr2 or smad2, all of which can induce Xnr1 and Xnr2. We also tested the effects of Activin protein treatment (100 ng/ml), injection of a range of Xnr1 mRNA doses (5-500 pg) and co-injection of Xnr1 with β-catenin mRNA (500 pg each), but no induction of Xnr5 and Xnr6 was detected when these explants were cultured for 2 hours (data not shown). However, very weak induction of Xnr5 and Xnr6 was occasionally observed in explants cultured for over 3 hours after injection of a large amount of Xnr1 or smad2 or Xnr1 plus β-catenin mRNA. Since antipodean expression was also detected in these explants, this induction of Xnr5 and Xnr6 must be due to antipodean, and not directly to the TGF-β signal. The endogenous mechanism of induction of Xnr5 and Xnr6 is also obviously different from that of Xnr1 and Xnr2. Unlike Xnr1 and Xnr2, Xnr5 and Xnr6 are regulated in a completely cell autonomous manner, and their endogenous expression is not affected by the injection of IAR or cer-S.

Xenopus models of mesoderm induction and endoderm determination have been described previously (Slack et al., 1987; Heasman, 1997; Kimelman and Griffin, 1998; Clements et al., 1999; Yasuo and Lemaire, 1999; Zorn et al., 1999; Agius et al., 2000). Results obtained in these models indicate that maternal VegT initiates a zygotic inducer, Xnr expression. On the dorsal side, β-catenin synergistically acts with VegT to determine a gradient of Xnr activity. Moreover, β-catenin also acts independently, directly inducing siamois, Xtwin and Xnr3 in the dorsal sides of the embryo. However, only very low expression of Xnr1, Xnr2 and Xnr4 is induced cell autonomously by maternal factors. Higher level expression of these genes requires cell-to-cell contact and is reduced by cer-S in embryos, suggesting that expression of Xnr1, Xnr2 and Xnr4 is largely regulated by an Xnr signal. In contrast, in the present study, we have shown that Xnr5 and Xnr6, different from other Xnrs, are regulated in a cell autonomous manner and are not controlled by an Xnrs-mediated signal. In the light of these results, we propose the following model of Xnr5 and Xnr6 function (Fig. 9). At the MBT, when zygotic transcription starts, maternal VegT induces Xnr5 and Xnr6 in cooperation with β-catenin to make the Nieuwkoop center. Xnr5 and Xnr6 may also be regulated by other pathways. Xnr5 and Xnr6 transcripts quickly accumulate and are widely distributed through the deep endoderm. In this phase, only a low level of Xnr1, Xnr2 and Xnr4 transcripts are also induced cell autonomously. Then endodermal expression of Xnr5 and Xnr6 induces further expression and accumulation of Xnr1, Xnr2 and Xnr4. These Xnrs, which accumulate in the endodermal region, determine endoderm formation in cooperation with VegT, and induce the expression of Xnr1, Xnr2, Xnr4, gsc, Xbra and other genes in the equatorial region (mesoderm induction). β-catenin directly regulates expression of siamois, Xtwin and Xnr3 in the dorsal side, and plays a role in determination of anterior endoderm and organizer.

nodal-related genes have also been isolated in other animals, but to date only one has been isolated in mouse, chick and ascidian each, and two have been reported in zebrafish. In
contrast, six nodal-related genes have already been identified in Xenopus. These six Xirs can be classified into several groups based on regulatory mechanisms and distinct patterns of expression. This implies that it is required to increase the number of Xirs in Xenopus embryo. Two nodal-related genes, squnt (sqt) and cyclops (cyc) have been reported in zebrafish. (Rebagliati et al., 1998a,b; Erter et al., 1998; Sampath et al., 1998; Feldman et al., 1998), and it seems that they share some kind of role in embryogenesis. Low levels of sqt mRNA are detected maternally, expression reaches a peak at the sphere stage and is greatly reduced after the shield stage. Conversely, maximum expression of cyc is detected at the shield stage (Erter et al., 1998; Rebagliati et al., 1998b). Although the spatial expression patterns of sqt and cyc coincide with each other in some regions, they have some distinct patterns of regional expression (Rebagliati et al., 1998b). For example, sqt but not cyc is also expressed in the extra-embryonic yolk syncytial layer (YSL) (Erter et al., 1998), which corresponds to the Nieuwkoop center in Xenopus. Thus, two zebrafish nodal-related genes are also regulated in different ways and seem to act in distinct processes. Further analysis is required to clarify the individual roles of the six Xirs in Xenopus embryogenesis.

We thank Drs E. Amaya, J. L. Christian, E. M. De Robertis, J. B. Gurdon, A. Hemmati-Brivanlou, M. Inobe, D. Kimelman, P. Lemaire, D. A. Melton, S. Nishimatsu, W. C. Smith, A. Suzuki, M. Taira, N. Ueno and C. V. E. Wright for providing materials. This work was supported by Grants-in-Aid for Scientific Research from Ministry of Education, Science, Sports and Culture of Japan, and by CREST (Core Research for Evolutional Science and Technology) of the Japan Science and Technology Corporation.

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