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

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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 *β-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*, *β-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 mesoderm (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 organizer to dorsal-lateral mesoderm causing dorsalization. A number of dorsalizing factors have been identified previously, including *noggin*, *chordin* and *follistatin*, all of which inhibit BMP signaling by direct binding to BMP (Zimmerman et al., 1996; Piccolo et al., 1996; Fainsod et al., 1997). Several candidates for the first two signals have also been reported, and it has been suggested that a TGF-β signal is required in both mesoderm and endoderm induction (Asashima, 1994; Slack, 1994; Harland and Gerhart, 1997).

Mutant analysis in other vertebrates, such as mouse and zebrafish, indicates that *nodal*-related genes belonging to the TGF-β superfamily mediate mesoderm induction signaling (Schier and Shen, 2000). Four *nodal*-related genes have been isolated in *Xenopus*. *Xnr1*, *Xnr2* and *Xnr4* have mesoderm induction activity (Jones et al., 1995; Lustig et al., 1996a; Joseph and Melton, 1997). *Xnr3* is different from the other Xnr family members in that it does not have the conserved cysteine residue in its C-terminal region that is present in other TGF-β superfamily genes; it cannot induce mesoderm and has neural induction activity (Smith et al., 1995). Using the *nodal*-specific inhibitor *cer-S*, a recent report has shown that zygotic Xnrs act as both dorsal and ventral mesoderm inducers in vivo (Piccolo et al., 1999; Agius et al., 2000). *Xnr1* and *Xnr2* can also induce endodermal genes when they are expressed ectopically (prospective ectoderm; animal cap), and are involved in the determination of endodermal cell fate in the embryo by regulating the expression of some genes (Kimelman and Griffin, 1998; Clements et al., 1999; Osada and Wright, 1999; Yasuo and Lemaire, 1999).

In *Xenopus*, it was shown in embryos depleted of maternal mRNA using antisense oligodeoxynucleotides that *VegT* and *β-catenin* 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

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 promoter 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 Elinson, 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 λZAPII 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 blastomeres 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-terminater 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-Xwnt-8* (Smith and Harland, 1991; Sokol et al., 1991; Christian et al., 1992); *pSP64T-dvl1* (mouse) (Sussman et al., 1994); *pRN3-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* (*tAR*) (Hemmati-Brivanlou and Melton, 1992); *pSP64T-mTFRII-45 del21* (*tBR*, mouse) (Suzuki et al., 1994); *pSP64TEN-XFS-319* (Hemmati-Brivanlou et al., 1994); *pXFD/Xss* (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 obtained by optional screening. The *pNRRX* vector was constructed from *pBluescriptII* by first disrupting the *Not*I and *Xho*I sites (Stratagene). Then the 5′ and 3′ *globin* UTRs from *pSP64T* were subcloned into the *Hin*dIII/*Pst*I site of the vector. Additional cloning sites (*Not*I, *Eco*RI, *Eco*RV, *Xho*I) inserted into the *Bgl*II 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, ATCTGATGCCGTTCTAAGCC; R, GACCTTCTTCAACC-TCAGCC); *Xnr3* (F, AAGAAGCATCTCCTCAGTTGG; R, TAC-GTAGCTCAGCCAACTTCA); *Xnr4* (F, TTACAAGATGC-TGCACACTCC; R, AACTCTGCATGTATGCGTGG); *Xnr5* (F, TCACAATCCTTTCACTAGGGC; R, GGAACCTCTGAAAGG-AAGGC); *Xnr6* (F, TCCAGTATGATCCATCTGTTGC; R, TTCTCGTTCCTCTTGTGCCTT); *derrière* (F, AGCCACAAGG-ATCTCTGTGC; R, ATTGATCGATTGCCTCCTGC); *sia* (F, CTACCGCACTGACTCTGCAA; R, GGCAGATGTCTGGCTC-TTCT); and *Col II* (F, ATTCAGTTGACCTTCCTGCG; R, TCCATAGGTGCAATGTCTACG).

goosecoid (*gsc*), *Xbrachyury* (*Xbra*) and *ODC* are described in Xenopus Molecular Marker Resource (http://vize222.zo.utexas.edu/). *Xnr1* (Jones et al., 1995), *ms-actin*, *EF1-α* (Takahashi et al., 1998), *edd* and *XNkx-2.5* (Sasai et al., 1996) and *Xsox17β* (Hudson et al., 1997) were as previously described. Histological analysis was carried out as previously described (Yokota et al., 1998).

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

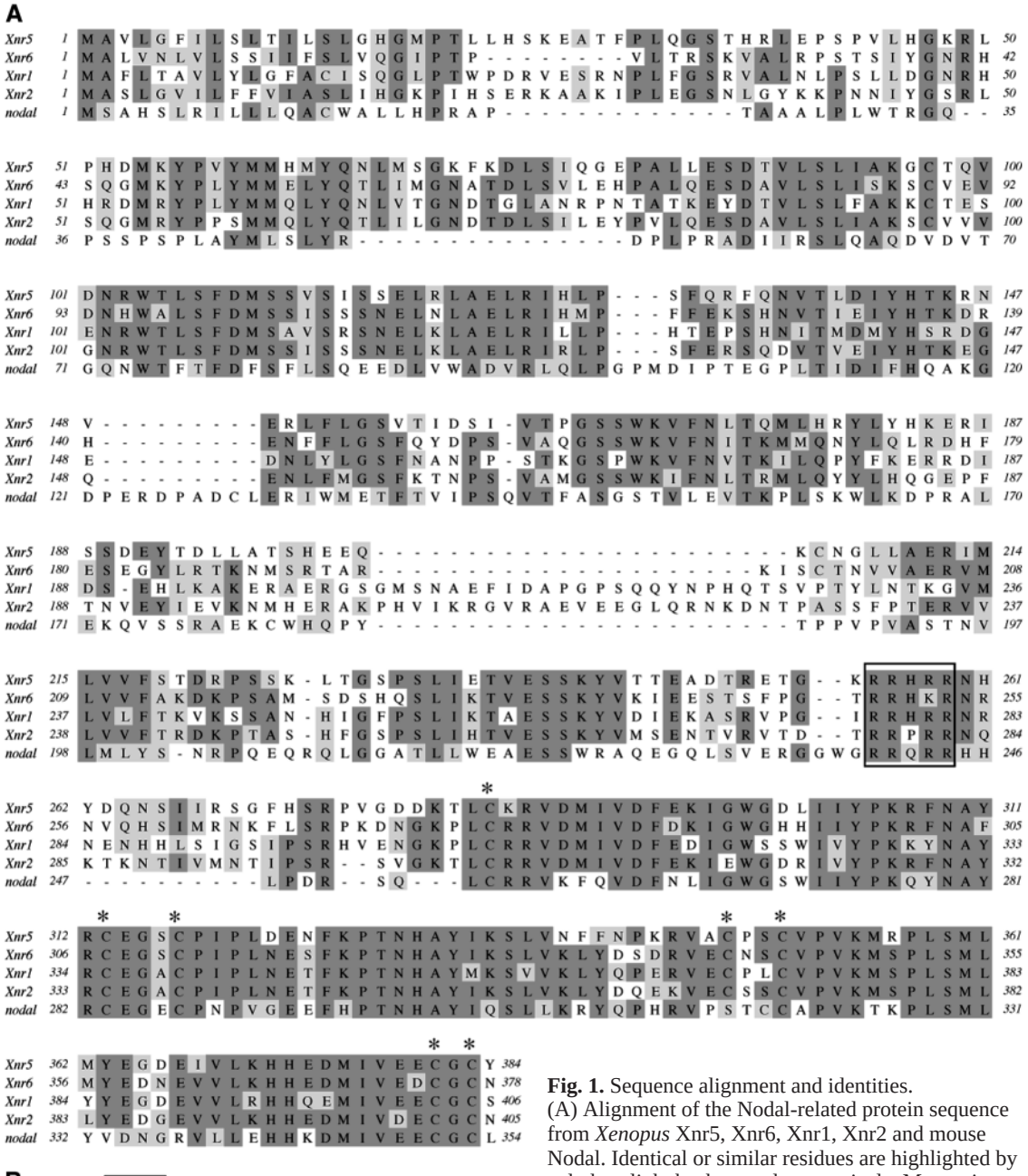

Fig. 1. Sequence alignment and identities.
(A) Alignment of the Nodal-related protein sequence from *Xenopus* Xnr5, Xnr6, Xnr1, Xnr2 and mouse Nodal. Identical or similar residues are highlighted by a dark or light background, respectively. Maturation cleavage sites (RXXR) are boxed. Asterisks indicate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

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

Xnr6, respectively, were also obtained, whereas *Xnr1*, *Xnr2* and *Xnr4* were not. Therefore, *Xnr5* and *Xnr6* are not 'A/B copies' in the genome of the pseudotetraploid frog *Xenopus laevis*.

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

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* 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

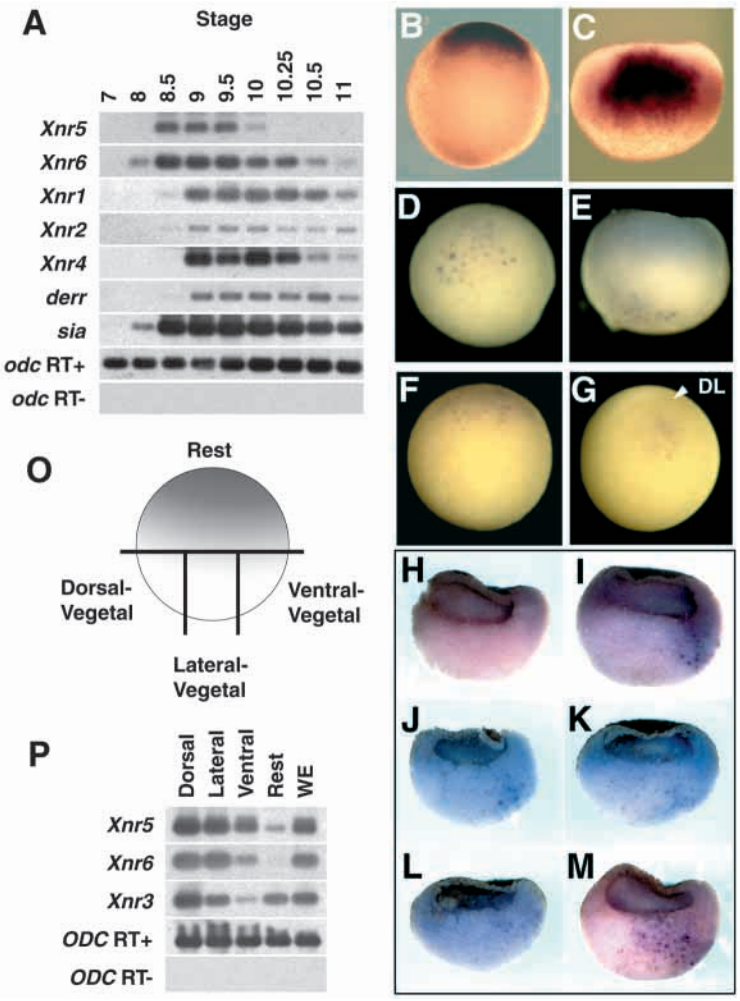

Fig. 2. Temporal and spatial expression patterns of *Xnr5* and *Xnr6*. (A) Temporal expression analyzed by RT-PCR. Zygotic expression of *Xnr5* and *Xnr6* starts at stage 8-8.5, at the mid-blastula transition (MBT). *Xnr1*, *Xnr2*, *Xnr4* and *derrière* (*derr*) are only weakly expressed at this stage. (B-M) Spatial expression patterns of *Xnr3* (B,C,I), *Xnr5* (D,E,J,K), *Xnr6* (F,G,L,M) were analyzed by whole-mount in situ hybridization. Sense probe was used as a control (H). *Xnr3* is detected at the dorsal marginal region of the embryo, not at the vegetal region. By contrast, *Xnr5* and *Xnr6* are first detected at the dorsal vegetal region, and subsequently throughout the deep endoderm. *Xnr6* is detected under the dorsal lip (DL, indicated by arrowhead) at stage 10. B,D,F,G are vegetal views; C,E are dorsal views; H-M, wild-type embryos were hemisectioned before hybridization (dorsal is towards the right); J,L are stage 8.5 embryos; B-F,H,I,K,M are stage 9 embryos; G is a stage 10 embryo. (O,P) Spatial expression patterns were analyzed by RT-PCR. Embryos were dissected into four parts (dorsal-vegetal, lateral-vegetal, ventral-vegetal and rest of the embryo; O). *Xnr5* and *Xnr6* are strongly expressed in the dorsal- and lateral-vegetal regions (P). WE indicates whole embryo at the same stage. *Xnr3* is highly expressed in the dorsal region.

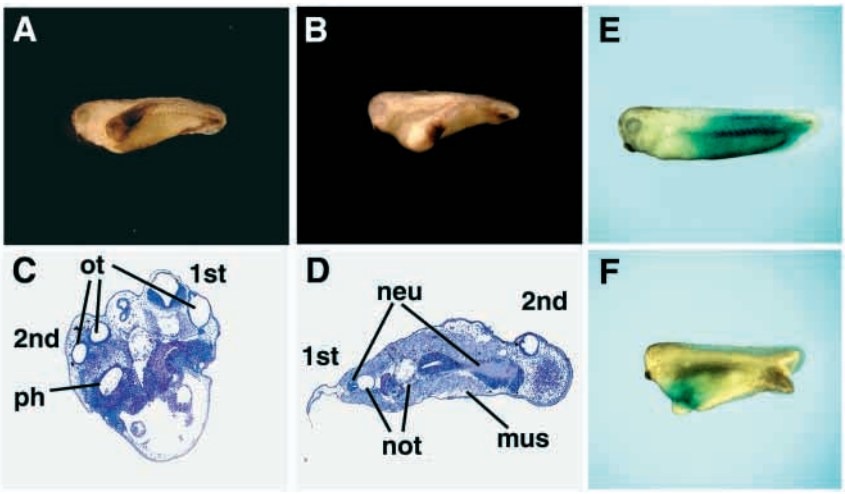

Fig. 3. Secondary axis induction by *Xnr5* and *Xnr6*. (A) *Xnr5* (2 pg/embryo) and (B) *Xnr6* (5 pg/embryo) mRNA induces a secondary axis. mRNA was injected into both ventral-vegetal blastomeres at the eight-cell stage and embryos were cultured until stage 30. (C,D) Transverse section of *Xnr5*- and *Xnr6*-injected embryos. The secondary axes have pharynx (ph), otic vesicle (ot), neural tube (neu), muscle (mus) and notochord (not). *lacZ* mRNA (250 pg/embryo) was injected with (E) or without (F) *Xnr5* (2 pg/embryo), and was detected by X-gal staining. *Xnr5*-expressing cells, indicated by the blue staining, differentiate into endoderm. *Xnr6* has a similar activity (data not shown).

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 (*Xbrachyury*: *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

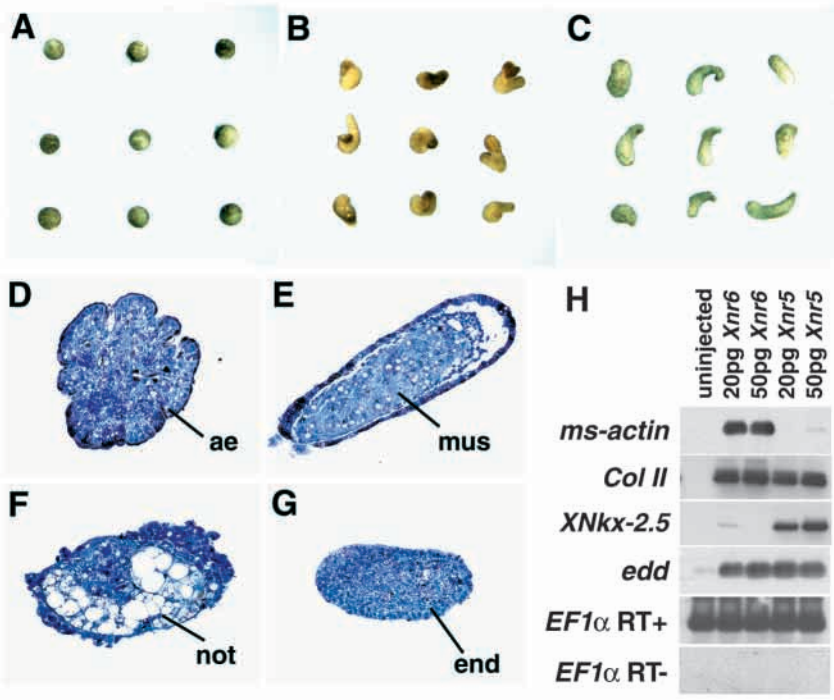

Fig. 4. *Xnr5* and *Xnr6* induce mesoderm and endoderm in animal caps (AC). (A-C) Elongation of *Xnr5*- and *Xnr6*-injected AC after a day: (A) control AC; (B) 5 pg *Xnr5*-injected AC; (C) 20 pg *Xnr6*-injected AC. (D-G) Histological sections of these animal caps: (D) control AC; (E) 20 pg *Xnr6*-injected AC; (F) 5 pg *Xnr5*-injected AC; (G) 20 pg *Xnr5*-injected AC. These AC differentiate into atypical epidermis (ae), muscle (mus), notochord (not) and endodermal cells (end), respectively. (H) RT-PCR analysis shows *Xnr5* and *Xnr6* induce mesodermal markers (notochord specific *collagen type II*; *col II*) and endodermal markers (*endodermin*; *edd*) in AC. *Xnr5* also induces *XNkx-2.5* but *Xnr6* does not. By contrast, *Xnr6* induces *muscle specific-actin* (*ms-actin*). For RT-PCR and histological analysis, embryos and AC were cultured for 3 days.

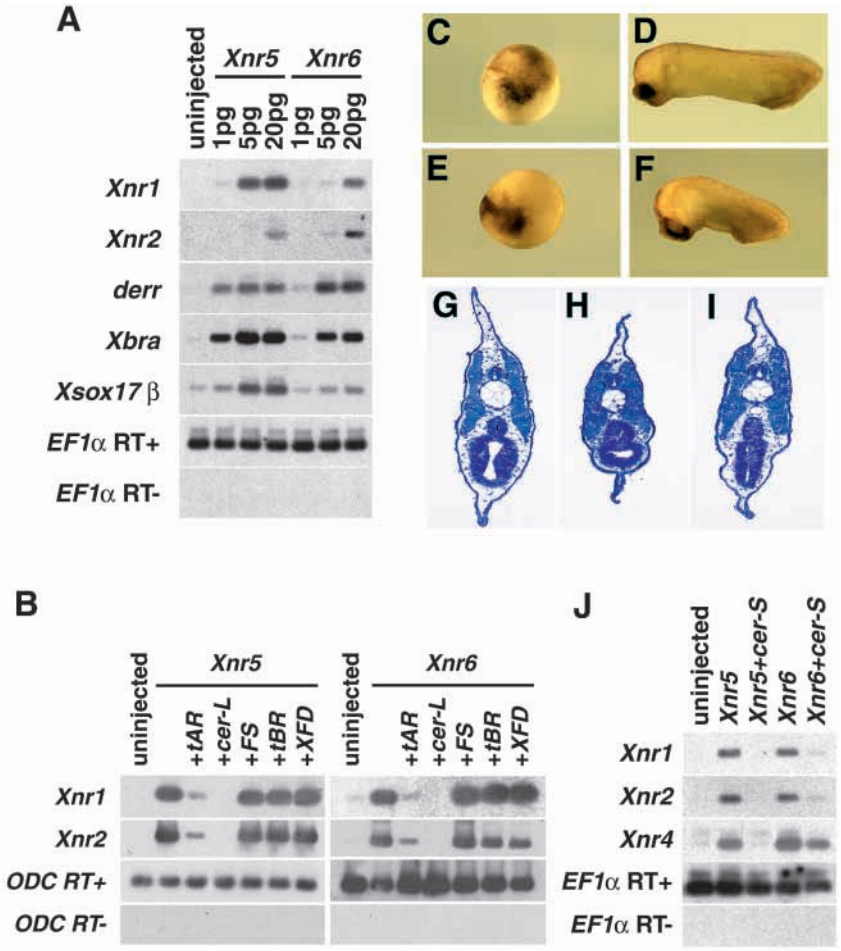

Fig. 5. *Xnr5* and *Xnr6* induce other early mesendoderm inducers and are the earliest nodal signaling molecules in embryogenesis. (A) *Xnr5* and *Xnr6* induce *Xbra*, *Xnr1*, *Xnr2*, *derr* and *Xsox17β* in animal caps. 0, 1, 5 and 20 pg of *Xnr5* or *Xnr6* were injected. Animal caps were dissected at stage 9, and cultured for 2 hours and were analyzed by RT-PCR. (B) *Xnr5* and *Xnr6* signaling are mediated via the activin receptor and are inhibited by *cerberus*. 2 ng of mRNA of inhibitors including *dominant-negative activin receptor* (*tAR*), *cerberus-long* (*cer-L*), *follistatin* (*FS*), *dominant-negative BMP receptor* (*tBR*) or *dominant-negative FGF receptor* (*XFD*) were co-injected with *Xnr5* or *Xnr6* (20 pg) at the two-cell stage. 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* 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.

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 anti-Wnt effect of *cer-L*.

These results indicate that Cer-L inhibits Xnr5 and Xnr6 activities, probably by binding to them, as is the case with Xnr1, Xwnt-8 and BMP4. To clarify this, further analysis was carried out using *cerberus-short* (*cer-S*), which encodes a C-terminal fragment of Cerberus, which lacks the Wnt- and BMP- binding regions and acts as a specific inhibitor of Nodal-related proteins (Piccolo et al., 1999). When *Xnr5* and *Xnr6* were co-injected with *cer-S*, the induction of *Xnr1*, *Xnr2* and *Xnr4* were markedly repressed (Fig. 5J). This result supports the idea that Cer-S acts as a multiple inhibitor of Xnrs via direct binding. Based on their temporal and spatial expression patterns, *Xnr5* and *Xnr6* may also regulate the expression of *Xnr1*, *Xnr2*, *Xnr4* and *derrière* in embryos. This hypothesis is supported by previous studies which have reported that the expression of *Xnr1*, *Xnr2* and *Xnr4* was suppressed when *cer-S* was injected into the embryo (Agius et al., 2000). To clarify the regulatory mechanisms of *Xnrs*, we also tested the effects of the injection of various inhibitors into embryos (Fig. 6A). Gene expression was monitored by RT-PCR. The *Xnr1* and *Xnr2* transcripts were down-regulated when *tAR1* or *cer-L* was injected (Fig. 6A), whereas no inhibitor could block *Xnr5* or *Xnr6* expression. We next examined the effect of injection of *cer-S* into embryos (Fig. 6B-D). Injection of a low concentration of *cer-S* (100 pg/embryo) inhibited formation of head structures (Fig. 6C). At a high concentration (500 pg/embryo), in addition to that phenotype, *cer-S* also disturbed axial structures (Fig. 6D). 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. 6E). 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

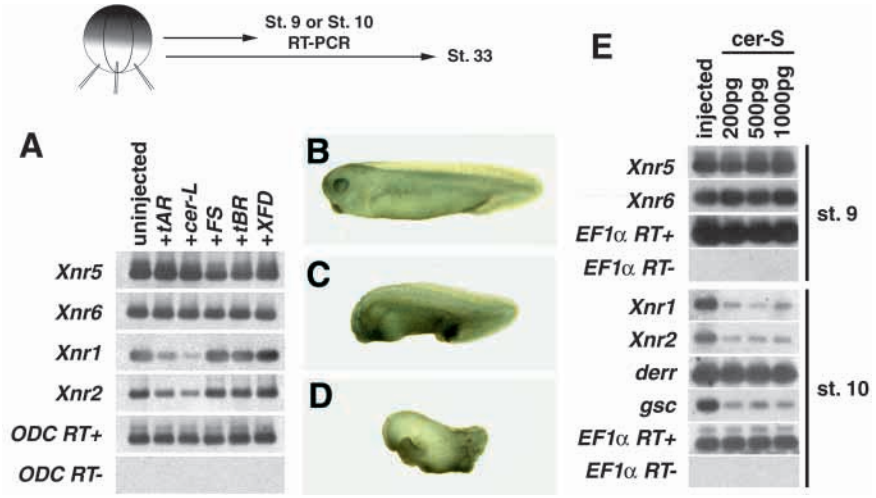

Fig. 6. The *nodal*-specific inhibitor *cer-S* inhibits the expression of *Xnr1* and *Xnr2* but not *Xnr5* and *Xnr6*. (A) In embryos, among the inhibitors described in Fig. 5B, *tAR1* and *cer-L* reduce the expression of *Xnr1* and *Xnr2*, but do not affect *Xnr5* and *Xnr6*. mRNA was injected radially around the vegetal pole at the four-cell stage. Injected embryos were cultured until stage 9 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

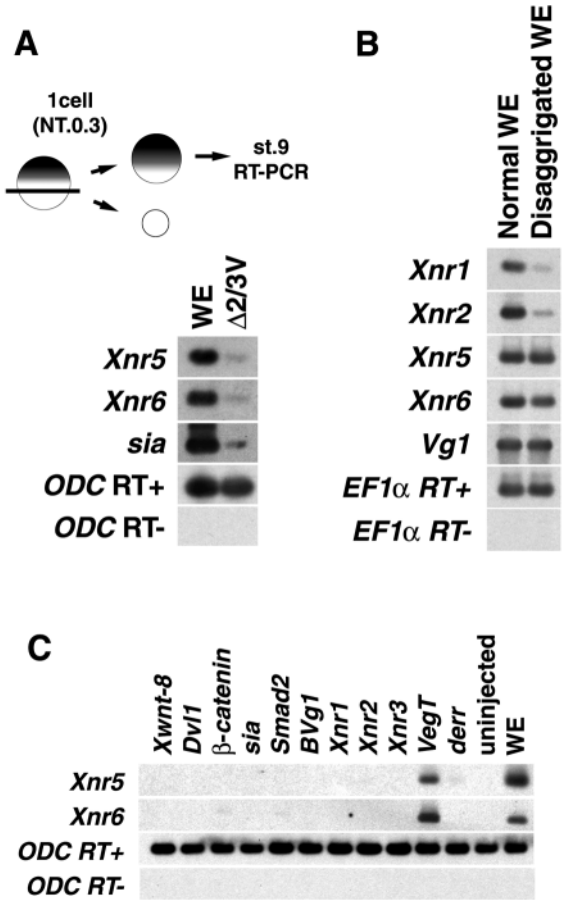

Fig. 7. Inducer of *Xnr5* and *Xnr6*. (A) Deletion of dorsal determinants abolish *Xnr5* and *Xnr6* transcripts, like *siamois*. Two thirds of the vegetal hemisphere of the embryo was deleted before cortical rotation (Δ2/3V). The embryos were cultured until stage 9 and were analyzed by RT-PCR. 0.3 NT (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 Ca^{2+}- and Mg^{2+}-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. *Xwnt-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.

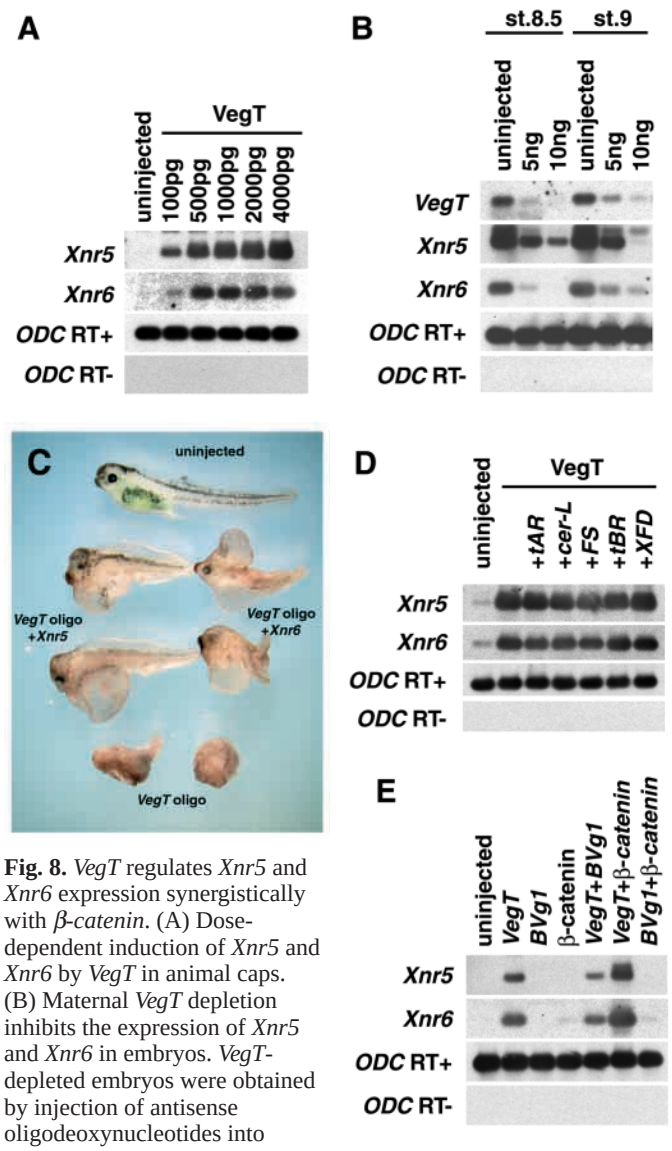

Fig. 8. *VegT* regulates *Xnr5* and *Xnr6* expression synergistically with *β-catenin*. (A) Dose-dependent induction of *Xnr5* and *Xnr6* by *VegT* in animal caps. (B) Maternal *VegT* depletion inhibits the expression of *Xnr5* and *Xnr6* in embryos. *VegT*-depleted embryos were obtained by injection of antisense oligodeoxynucleotides into oocytes using the host-transfer technique. *VegT* antisense oligo was used as previously described (Zhang et al., 1998). These embryos were cultured until stage 8.5 or stage 9 and were analyzed by RT-PCR. (C) *Xnr5* and *Xnr6* rescue the *VegT*-depleted embryos. *Xnr5* (2 pg) or *Xnr6* (10 pg) mRNAs was injected into two dorsal-vegetal blastomeres of the *VegT*-depleted embryo at the eight-cell stage and cultured for 4 days. Control uninjected embryo is shown in top row; the middle left two embryos are depleted of *VegT* and rescued by *Xnr5*, and the right two embryos are depleted of *VegT* and rescued by *Xnr6*. *VegT*-depleted embryos are shown in bottom row. (D) *Xnr5* and *Xnr6* induction by *VegT* in the animal cap is not inhibited by any of these inhibitors (described in Fig. 5B). (E) *β-catenin* enhances *Xnr5* and *Xnr6* induction by *VegT* in animal cap. *β-catenin* (500 pg), *VegT* (500 pg) and *BVg1* (200 pg) mRNA was injected into animal poles. (A,D,E) Animal caps were dissected at stage 9, and cultured for 2 hours, and analyzed by RT-PCR.

were tested, but none affected the expression of *Xnr5* and *Xnr6*. Although *β-catenin* alone could not induce transcription of these genes, in combination with *VegT* it induced marked upregulation of expression of both *Xnr5* and *Xnr6* (Fig. 8E).

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* (Yasuo 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 *squint* (*sqt*) and *cyclops* (*cyc*), both of which are *nodal*-related genes in zebrafish (Gritsman et al, 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;

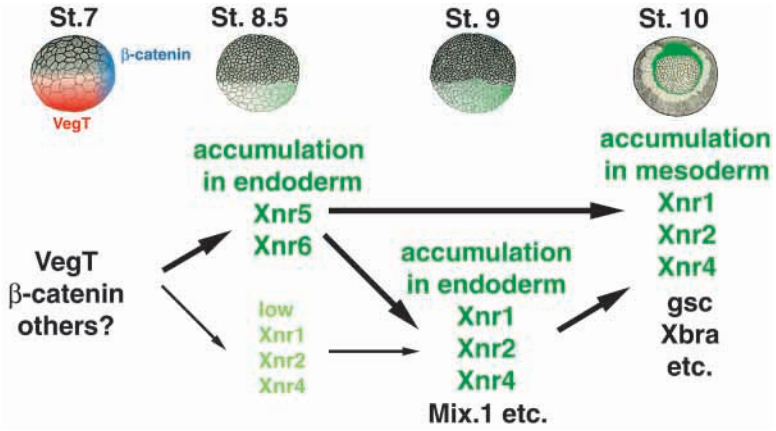

Fig. 9. Model of endoderm and mesoderm induction by known *nodal*-related genes in *Xenopus*. We have modified the previous model based on our results with *Xnr5* and *Xnr6*. At the MBT, when zygotic transcription starts, vegetally localized maternal *VegT* induces *Xnr5* and *Xnr6* in cooperation with *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 *Xnr5* and *Xnr6* 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 *tAR* 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 *Xnrs* 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 *Xnrs* in *Xenopus* embryo. Two *nodal*-related genes, *squint* (*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 *Xnrs* in *Xenopus* embryogenesis.

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