Nodal-related signals induce axial mesoderm and dorsalize mesoderm during gastrulation

C. Michael Jones, Michael R. Kuehn, Brigid L. M. Hogan, James C. Smith and Christopher V. E. Wright

1 Department of Cell Biology, Vanderbilt University Medical School, Nashville, TN 37232-2175, USA
2 Howard Hughes Medical Institute, Vanderbilt University Medical School, and Department of Cell Biology, Vanderbilt University Medical School, Nashville, TN 37232-2175, USA
3 Laboratory of Developmental Biology, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK
4 Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

SUMMARY

Mouse embryos homozygous for a null mutation in nodal arrest development at early gastrulation and contain little or no embryonic mesoderm. Here, two Xenopus nodal-related genes (Xnr-1 and Xnr-2) are identified and shown to be expressed transiently during embryogenesis, first within the vegetal region of late blastulae and later in the marginal zone during gastrulation, with enrichment in the dorsal lip. Xnr-1 and mouse nodal function as dose-dependent dorsalantier and ventral mesoderm inducers in whole embryos and explanted animal caps. Using a plasmid vector to produce Xnr proteins during gastrulation, we show that, in contrast to activin and other TGFβ-like molecules, Xnr-1 and Xnr-2 can dorsalize ventral marginal zone explants and induce muscle differentiation. Xnr signalling also rescues a complete embryonic axis in UV-ventralized embryos. The patterns of Xnr expression, the activities of the proteins and the phenotype of mouse nodal mutants, all argue strongly that a signaling pathway involving nodal, or nodal-related peptides, is an essential conserved element in mesoderm differentiation associated with vertebrate gastrulation and axial patterning.

Key words: nodal, axial mesoderm, dorsalization, gastrulation, TGF-β, Xenopus

INTRODUCTION

Formation and patterning of the mesoderm is fundamental to the establishment of the vertebrate body plan. In Xenopus, prospective mesoderm forms in the equatorial region (marginal zone) of the blastula as a result of signals originating in the vegetal hemisphere (reviewed in Smith, 1989a,b; Slack, 1994). The inducing signals from the presumptive dorsal and ventral vegetal regions appear to be quite distinct. This is based on the observation that dorsal marginal quadrants explanted shortly after induction differentiate notochord and muscle, while the remaining three-fourths of the marginal zone forms only ventral tissues (e.g. blood and loose mesenchyme; Boterenbrood and Nieuwkoop, 1973; Dale and Slack, 1987a). However, in the embryo, large amounts of muscle arise from lateral marginal zone tissue that was originally specified as ventral mesoderm (Dale and Slack, 1987b; Moody, 1987a,b). This is because the dorsal mesoderm (Spemann’s Organizer) produces a third signal(s) converting adjacent mesoderm from ventral into more dorsal mesoderm (see Slack, 1994 and references therein). This process, called dorsalization (Dale and Slack, 1987a), is well illustrated by the observation that ventral marginal zone explants can be dorsalized towards a muscle fate in contact with dorsal marginal zone explants.

Many years of study have led to the identification of factors with activities expected of each of the three signals discussed above. Activin and BVg1 exhibit dorsal mesoderm inducing activity, while bFGF and BMP4 act as ventral mesoderm inducers (for review, see Slack, 1994). However, this is complicated by data from dominant negative FGF receptor experiments suggesting that dorsal induction is superimposed upon a preexistent FGF signaling pathway (Cornell and Kimelman, 1994; Amaya et al., 1993). The recently isolated secreted proteins noggin and chordin are candidates for the Organizer-derived dorsalizing activity (Smith et al., 1993; Sasai et al., 1994). Many lines of evidence lead to the proposal that a fourth signal, mediated by BMP4 (and possibly Xwnt8), actively maintains the ventral mesodermal fate. The ventral-dominant BMP4/Xwnt8 signal may also titrate the strength of the dorsalization signal in the prospective mesoderm so that the full range of dorsal/lateral/ventral mesodermal tissues is produced (Jones et al., 1992a; Dale et al., 1992; Christian and Moon, 1993; Sive, 1993; Maeno et al., 1994; Suzuki et al., 1994; Graff et al., 1994; Schmidt et al., 1995; Fainsod et al., 1994). While the above molecules are implicated in early patterning, but our understanding of their precise role is still quite vague and incomplete, partly because of a lack of definitive genetic analysis.

The mechanisms controlling mesodermal patterning in mammalian embryos are just beginning to be dissected, with current ideas being based largely on gene expression patterns and fate mapping studies. However, with an increasing
frequency, insight has come from analyzing mutant phenotypes resulting from gene targeting and insertional mutagenesis in embryonic stem cells (see Faust and Magnuson, 1993; Beddington and Smith, 1993 and references therein). A gene essential for embryonic patterning, called nodal, was isolated based upon its disruption by a proviral insertion in the 413.d mutant mouse strain. Embryos homozygous for the disrupted allele contain little or no mesoderm, fail to form a recognizable primitive streak and are arrested in development during early gastrulation (Conlon et al., 1991, 1994; Iannaccone et al., 1992). Undisrupted nodal signalling is thus essential for the formation of mesodermal cell lineages and subsequent patterning events (Zhou et al., 1993; Conlon et al., 1994). nodal RNA is detected by RT-PCR in very early streak stage embryos and, at gastrulation, transcripts are localized to cells at the anterior tip of the primitive streak surrounding the node (Zhou et al., 1993; Conlon et al., 1994). The node is an organizing center similar to the dorsal lip of frog embryos (Beddington, 1994), and cells arising from it populate most of the axial mesoderm (Lawson and Pedersen, 1992; for review see Hogan et al., 1995).

The nodal gene encodes a TGFβ-type secreted molecule. The biologically active ligand derived from the C terminus contains seven conserved cysteine residues found in the DVR (decapentaplegic/Vg-related) group of the TGFβ superfamily. The nodal sequence is slightly more similar to the BMP superfamily. The nodal sequence is dose-dependent on the determining events (Zhou et al., 1993; Conlon et al., 1994). Xnr proteins dorsalize ventral, but at gastrulation become localized to the marginal zone, with enrichment at the dorsal lip. Both Xnr proteins dorsalize ventral marginal zone (VMZ) tissues during gastrulation, a function shared by noggin and chordin, but not activin. The asymmetric expression of Xnr-1 and Xnr-2 in the dorsal marginal zone during gastrulation puts the gene products in the correct place and time to be endogenous dorsalizing signals. Consistent with these patterning activities, Xnr signalling also rescues a complete embryonic axis in UV-ventralized embryos.

MATERIALS AND METHODS

Construction of Xβ-nodal

Genomic DNA encoding the nodal N terminus was digested with NotI just downstream of the signal sequence (Zhou et al., 1992), and annealed to a cDNA fragment encoding the rest of nodal cut with NotI at the 5’ end. PCR was used to generate a 1 kb fragment encoding the complete protein. For insertion into pSP64-Xβm, the 5’ primer (CCAAAACGGCCTCCATCGAGACTG) introduced a BspHI site at the initiation codon, and the 3’ primer (reverse complement of GCCTCTCAGAAGCCTCGGGGGAGTTCG) introduced Smal downstream of the stop codon. PCR fragment was digested with BspH-Smal, and exchanged for the β-globin insert of pSP64-Xβm (Wright et al., 1989), prepared by BstEI digestion, blunt-ending and NcoI digestion. This construct, pXβ-nodal, contains zero nucleotides of nodal 5’ UTR; the translation initiation context is TTGGCCCATGAG. Fidelities of PCR reactions and subcloning were checked by sequencing the entire insert. pXβ-nodal was EcoRI linearized and capped RNA synthesized with SP6 polymerase (Jones et al., 1992a).

Isolation of nodal-related cDNAs

PCR using degenerate oligonucleotides corresponding to mouse nodal sequence gave no nodal-related sequences from Xenopus genomic DNA. A 1 kb EcoRI-Sphi fragment of cDNA ENH1 (Zhou et al., 1992), containing nodal protein-coding sequences, was also used to screen gastrula cDNA and genomic libraries at low to moderate stringency. Approximately 15 million plaques were screened; all yielded either no positive clones or high nonspecific hybridization. Subsequently, a radiolabeled PCR fragment (nucleotides 1069 to 1411) of the nodal mature region was used. Because nodal transcripts localize to the node in mouse embryos (Zhou et al., 1992), a dorsal lip-specific λZAPI cDNA library (Blumberg et al., 1991) was screened at reduced stringency (Sambrook et al., 1989). Washes were 0.5x SSC/0.1% SDS/37°C, 250,000 plaques of the unamplified (UDL) library and 1.5 million plaques of amplified (ADL) library were screened. Eight clones were isolated, falling into 2 groups. UDL1 encoded Xnr-1, while UDL2,3,5,6 and ADL1,6,8 encoded Xnr-2. ADL library rescues with a 5’ UDL1 probe produced 16 additional Xnr-1 cDNAs. Complete sequence on both strands of the longest Xnr-1 and Xnr-2 cDNAs was obtained with a Sequenase II kit (USB), specific primers and overlapping sequences generated from HaeIII-HinfI and Rsal-HinfI subclones.

Whole-mount in situ hybridization

The protocol of Harland (1991) was used with minor modifications (R. Harland, personal communication) and the BM-purple substrate. Unhydrolyzed probe RNAs corresponded to the entire pBluescript cDNA inserts of Xnr-1 and Xnr-2.

Xnr injection constructs

cDNAs of Xnr-1 and Xnr-2 with the shortest 5′ UTRs (approx. 40 nt) were isolated with Smal-Xhol (Xnr-1; cDNA clone ADL-15) or EcoRI (Xnr-2; clone UDL-3), blunt-ended and inserted into blunt-ended BgII-digested pSP64T (Krieg and Melton, 1984). Both resulting constructs, pSP64T/Xnr-1 and pSP64T/Xnr-2, were linearized with Smal, and capped mRNA generated using SP6 polymerase. pCSKA plasmid constructs were generated by isolating coding sequences for mouse nodal, Xnr-1, Xnr-2, or activin from the pXβ or pSP64T plasmids described above. Mouse nodal was isolated from pXβ-nodal with HindIII-Smal, Xnr-1 and Xnr-2 were removed from their pSP64T constructs by HindIII-Smal, and activin from pSP64T-activin (gift from D. Melton) using HindIII-EcoRI. Inserts were blunt-ended and inserted into EcoRV-cut pCSKA vector (gift of R. Harland). Injected DNA was CsCl-purified supercoiled DNA.

RNase protection probes and RNA isolation

Xnr-1: a 5’ 450 bp EcoRI fragment was subcloned into pBluescript. The construct was linearized with HindIII, and antisense RNA probe generated with T7 polymerase.

Xnr-2: two probes (HE4 and HH5) were used interchangeably. HE4 is a 350 bp HindIII-EcoRI fragment from the 3′ end of the cDNA, subcloned into Smal cut cDNA fragment, linearized with BamHI, transcribed with T3 polymerase. HH5 is a 200 bp HindIII fragment
subcloned into Smal cut pBluescript, linearized with BamHI, transcripted with T3 polymerase.

Probes for goosecoid, Xbra, Xhox-3, globin and actin were as previously described (Jones et al., 1992a). RNAse digestions were with RNases A and T1 for Xnr-1, and RNase T1 alone for goosecoid, Xbra, actin and both Xnr-2 probes. Test RNA was isolated from embryonic tissues by SDS/proteinase K digestion and selective precipitation with lithium chloride (Jones et al., 1992a).

**Embryo manipulations**

*Xenopus* embryos were obtained by in vitro fertilization, reared in normal amphibian medium (NAM; Slack, 1984) and staged according to Nieuwkoop and Faber (1967). For animal cap assays, RNAs were injected into 1-cell embryos in 5% Ficoll/75% NAM. Animal caps were isolated from stage 8 blastulae, cultured in 75% NAM until appropriate stages, when tissues were either frozen (RNA isolation) or fixed (histological analysis). In VMZ dorsalization experiments, plasmids were injected at the 4-cell stage into both ventral blastomeres (50-70 pg total), close to the cleavage plane between them. Ventral was distinguished by the darker pigmentation (Nieuwkoop and Faber, 1967); correct assignment was greater than 97%. Embryos were incubated to stage 10-10.25, when the blastopore lip clearly marks the dorsal side. VMZs were then isolated, consisting of equatorial tissues spanning a 60° arc centered on the midline. Explants were cultured in 75% NAM until appropriate stages for molecular or histological analyses.

**Histological analysis**

Tissues were fixed overnight at 4°C in 3.7% formaldehyde, 50% ethanol, 2% acetic acid, 40% NAM. After further overnight fixation in 3.7% formaldehyde/phosphate-buffered saline, tissues were dehydrated, wax embedded, sectioned (7 μm) and stained with Feulgen/light green/orange G (Green et al., 1990).

**RESULTS**

**Nodal induces axial mesoderm in *Xenopus* embryos**

Mouse *nodal* was ectopically expressed in *Xenopus* embryos by injection of in vitro synthesized RNA (Materials and Methods). Whole embryos expressing *nodal* develop normally until early gastrula, but then exhibit grossly altered morphogenetic movements, and later fail to develop a neural tube. Injected embryos adopt a comma-shaped appearance, with no recognizable anteroposterior or dorsoventral axis, and a large ‘proboscis’ similar to LiCl-hyperdorsalized embryos. Histological analysis reveals formation of extensive notochord immediately under a very thin epidermis, adjacent to very large somitic arrays, and no visible neural tissue (data not shown). The formation of excess notochord and muscle is apparently at the expense of ventral mesodermal tissues, suggesting that the whole embryo is hyperdorsalized. Fig. 1 demonstrates that dorsal mesodermal markers, goosecoid and muscle Actin, are upregulated in *nodal*-injected embryos. We conclude that *nodal*-RNA injection strongly dorsoanteriorizes *Xenopus* embryos.

The mesoderm inducing activity of mouse *nodal* was assayed in animal caps explanted from blastulae injected at the 1-cell stage with *nodal* mRNA (Fig. 2). Such explants elongate extensively compared to controls (Fig. 2B), a behavior mimicking the convergent extension of axial mesodermal cells in normal development (Keller and Tibbetts, 1989). Histological examination of *nodal*-injected explants confirms induction of extensive axial mesoderm, characterized by massive amounts of notochord and muscle (Fig. 2D).

Animal caps from injected embryos were then assayed for expression of mesodermal markers: *Xbra*, a general mesoderm marker at early gastrula stages, and *goosecoid* and muscle *actin*, markers of dorsoanterior and paraxial mesoderm, respectively (Fig. 2E). *nodal*-injected animal caps express *Xbra*, showing that mesoderm is induced. Furthermore, *goosecoid* and muscle-specific *ms* actin expression is upregulated in *nodal*-injected embryos. Higher levels of *ms*-actin mRNA are detected precociously in embryos expressing *nodal*. Cytoskeletal actin serves as a control for RNA integrity in the lanes. C, control; N, mouse *nodal* RNA-injected.

**Two *Xenopus* nodal-related genes**

The results of mouse *nodal* RNA injections suggested that *Xenopus* embryos contain an endogenous signalling pathway recognizing *nodal* signals. We therefore undertook a search for frog *nodal*-related genes. cDNAs encoding two separate *nodal*-related proteins were isolated from a dorsal blastopore lip-specific cDNA library (see Materials and Methods). Extensive RT-PCR, cDNA and genomic library searches strongly suggest that these genes are the *Xenopus* homologs of *nodal* (see Discussion).

The two *Xenopus* nodal-related genes have been called *Xnr-1* and *Xnr-2* (for *Xenopus nodal*-related-1 and -2). Within the two groups of cDNAs, closely related A and B copies were found for both *Xnr-1* and *Xnr-2*, representing the pseudotetraploid nature of the *Xenopus* genome. Fig. 3 shows the...
marker), muscle-specific actin and Xbra. Decreased doses (100, 10, or 1 pg/embryo) do not induce goosecoid, but intermediate concentrations induce muscle-specific actin and Xbra. All samples are from the same injection experiment. goosecoid and Xbra were assayed at sibling stage 10.5, and muscle actin at stage 20. ODC and cytoskeletal actin serve as controls for RNA integrity and loading in samples.

**Fig. 2.** Characterization of animal caps induced by injection of mouse nodal RNA. Morphological (A,B), and histological (C,D) analysis of control and nodal-loaded animal caps. (A,C) Control caps form atypical epidermis, but caps expressing mouse nodal (B,D) elongate and differentiate dorsal mesodermal tissues, often consisting almost entirely of notochord and small patches of muscle. Abbreviations: epi, atypical epidermis; ms, muscle; no, notochord.

(E) Analysis of gene expression induced in animal explants by different nodal RNA doses. High doses (1 ng RNA/embryo) induce goosecoid (a dorsanterior mesoderm marker), muscle-specific actin and Xbra. Decreased doses (100, 10, or 1 pg/embryo) do not induce goosecoid, but intermediate concentrations induce muscle-specific actin and Xbra. All samples are from the same injection experiment. goosecoid and Xbra were assayed at sibling stage 10.5, and muscle actin at stage 20. ODC and cytoskeletal actin serve as controls for RNA integrity and loading in samples.

**Fig. 3.** Alignment of amino acid sequences of mouse nodal with Xnr-1, Xnr-2 and a chicken nodal-related peptide. (A) Deduced amino acid sequences of Xnr-1 and Xnr-2. A hydrophobic region (underlined) at the N terminus of each Xnr resembles a secretory signal sequence, with cleavage predicted according to the algorithm of von Heijne (1986). Four potential N-linked glycosylation sites (consensus N-X-T/S) are present in each protein. Three are positionally conserved between Xnr-1/Xnr-2. Putative basic proteolytic processing sites (RRxRR, underlined) begin at residues 277 (Xnr-1) and 278 (Xnr-2). Asterisks indicate identities, double dots represent conservative changes.

(B) Alignment of C-terminal mature regions of Xnr-1, Xnr-2, mouse nodal and a newly isolated chick nodal-related sequence. Alignments begin at the putative basic processing site of each molecule. The region of cysteine spacing unique to the Xnr factors (C-X-X-C) is underlined. Vertical lines represent identities in all four proteins. Dashes represent spaces introduced to optimize alignments.
Protein sequences of Xnr-1 and Xnr-2, an alignment of their predicted mature ligand regions with that of mouse nodal and a novel chick nodal-related sequence. Both Xnrs have a stretch of hydrophobic amino acids at the N terminus characteristic of a signal leader sequence found in secreted proteins. Xnr-1 and Xnr-2 cDNAs encode proteins of 406 and 405 amino acids, respectively, with predicted unprocessed relative molecular masses of approx. 46×10^3. Both Xnrs contain four potential N-linked glycosylation sites, and a characteristic cleavage sequence (RRxRR) which, based on studies with related proteins, is a site of specific proteolysis releasing the biologically active carboxyl-terminal ligand. Within the predicted ligand sequence, Xnr-1 and Xnr-2 are 87% similar to each other, and 78% and 73% similar to mouse nodal.

A key feature of proteins in the BMP/activin subgroup of the TGFβ superfamily is the conservation of seven cysteine...
residues in the mature region (Kingsley, 1994) that appear critical for dimerization, secretion, receptor-binding and biological activity (Mason, 1994). The Xnr-1 and Xnr-2 ligands also contain seven cysteines in similar positions, but the spacing in one region is altered compared to nodal and other TGFβ-like factors. In nodal, two adjacent cysteine residues are found approximately 35 amino acids from the C terminus, while in the Xenopus proteins, two cysteine residues are present, but separated by two amino acids (Fig. 3B). We note that the chick nodal-related sequence reported here is also characterized by this ‘split-cysteine’ arrangement.

**Expression of Xnr-1 and Xnr-2 during Xenopus embryogenesis**

The temporal expression of Xnr-1 and Xnr-2 was analyzed by RNase protection. Both Xnrs are detected first at late blastula (stage 9), and expression peaks at early gastrula (Fig. 4A). After a decline to undetectability, a low signal for Xnr-1 is detected in late neurulae (stage 17; Fig. 4A), but Xnr-2 transcripts are not detected by this method after gastrula stages (e.g. stage 13). Undetectable RNA levels for both genes at stage 8 suggest the absence of long-lived maternally stored transcripts. Northern blot analysis shows that both genes produce single 1.6 kb transcripts (data not shown), consistent with the size of the longest Xnr-1 and Xnr-2 cDNAs.

Whole-mount in situ hybridization was used to analyze the spatial expression pattern of Xnr-1 and Xnr-2 RNA during development (Fig. 4B–H). For unknown reasons, detection of Xnr-1 and Xnr-2 by this method requires greatly extended color development (24-36 hours). Even then, weak signals are generated, with the weaker, Xnr-1, being at the limits of detectability. Xnr-1 and Xnr-2 signals are first detected as punctate perinuclear staining in vegetal cells over the bottom third of the late blastula (stage 9; see Fig. 4B for Xnr-1), but this disappears as development proceeds. In the stage 10.5 gastrula, Xnr-1 signal becomes localized to a 60° arc in the dorsal quadrant of the marginal zone (Fig. 4C). More detail is discernable regarding Xnr-2 expression. Just before gastrulation (stage 10), signal is observed above and slightly below the position of the future dorsal lip, in an approx. 120-180° arc spanning the dorsal midline (Fig. 4D). Not all cells display Xnr-2 signal, but positive cells are more abundant at the dorsal midpoint. In the stage 10.5 gastrula (Fig. 4E), Xnr-2 expression almost encircles the embryo, being most intense and uniform at the pucker of the dorsal lip, in a domain encompassing the Organizer region. Moving around the lateral and ventral marginal regions, labeled cells become progressively less abundant; only very few positive cells are detected at the ventralmost extreme. Xnr-2 is also expressed at lower levels in a thin band of pre-endodermal cells below the dorsal lip (Fig. 4F). The arc of Xnr-2 expression at stage 10.5 is wider than the expression domain of ‘Organizer-specific’ markers such as noggin (compare Fig. 4D, E to Fig. 4G). Because the in situ hybridization signals for Xnr-1 and Xnr-2 RNA were very weak, RNase protection analysis of RNA from dissected wild-type embryos was performed to check the validity of these data. Fig. 4I shows that Xnr-1 and Xnr-2 RNAs are primarily found in marginal tissue of stage 10.25 gastrulae, are low or undetectable in animal and vegetal tissues, and that both are enriched in dorsal halves of the gastrula compared to ventral halves.

**Xnrs are induced in response to dorsal mesoderm inducing signals**

Because Xnr-1 and Xnr-2 expression is detected in the gastrula marginal zone, we tested for their induction by mesoderm-inducing factors. Animal caps were explanted from embryos at stage 8, cultured in the presence of activin or bFGF, and Xnr RNA assayed at sibling stage 10, corresponding to the peak of Xnr-1 and Xnr-2 expression in normal embryogenesis. Examination of sibling caps at tailbud stages confirmed that each growth factor gave the appropriate mesoderm induction. Fig. 5A shows that both Xnrs are induced by activin, a dorsoanterior mesoderm inducer, but not by bFGF, a ventrolateral mesoderm inducer. Consistent with this, transcript levels of both Xnrs increase in embryos hyperdorsalized by LiCl treatment during cleavage stages, and are greatly downregulated in radially ventralized UV-treated embryos (Fig. 5B). We conclude that expression of Xnr-1 and Xnr-2 is enhanced by treatments leading to differentiation of dorsoanterior mesoderm.

**Xnr-1 and Xnr-2 induce axial mesoderm**

Injection of mRNA encoding each Xnr into Xenopus embryos produced massive hyperdorsalization, similar to the effects of mouse nodal, as characterized by external morphology and greatly increased expression of goosecoid and muscle-specific actin (data not shown). To test the mesoderm induction properties of each Xnr, RNA was injected into the 1-cell embryo and animal caps explanted (Fig. 6). Xnr-2 RNA induces extensive morphogenetic movement, similar to that caused by mouse nodal mRNA (compare Figs 6C and 2A). Animal caps

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**Fig. 5.** RNase protection analysis of Xnr-1 and Xnr-2 expression in animal caps treated with growth factors and LiCl- and UV-treated embryos. (A) Xnr-1 and Xnr-2 expression is induced in animal caps by activin, but not FGF, indicating activation by dorsal mesoderm-inducing signals. Control protections show Xnr-1 and Xnr-2 RNA in stage 10.5 sibling embryos. EF1-α assesses RNA loading in the samples. (B) Expression of Xnr-1 and Xnr-2 at stage 10 in UV-ventralized or LiCl-dorsalized embryos. UV-ventralization greatly reduces Xnr-1 and Xnr-2 transcript levels. In contrast, LiCl dorsalization results in a 2-4 fold increase (evaluated densitometrically) in Xnr-1 and Xnr-2 RNA compared to untreated siblings. EF1-α is used as a loading control. DAI, dorso-anterior index score of sibling embryos (Kao and Elision, 1988).
receiving similar amounts of Xnr-1 RNA, while undergoing significant morphological changes compared to controls (Fig. 6B), extend much less than Xnr-2 caps.

Histological analysis confirms the differential activities of Xnr-1 and Xnr-2 RNA. Xnr-2-loaded caps differentiate dorsal mesodermal tissues, including notochord and muscle (Fig. 6F). Xnr-1-loaded caps form notochord less often (data not shown), but consistently differentiate large blocks of striated muscle surrounded by loose mesenchyme (Fig. 6E). In this experiment, we note that it cannot be determined if equal amounts of active Xnr-1 and Xnr-2 ligand are produced per unit of RNA. Purified Xnr-1 and Xnr-2 mature protein will help distinguish if the apparent differences in mesoderm induction strength between Xnr-1 and Xnr-2 RNAs truly reflect intrinsically different signaling activities.

Xnr signaling induces dorsal and ventral mesodermal markers in animal caps in a dose-dependent manner. Fig. 6G shows that high doses of Xnr-2 RNA (100 pg/embryo) induce expression of goosecoid, cardiac actin and the pan-mesodermal marker Xbra. Lower doses (1-10 pg/embryo) induce the dorsolateral mesodermal marker, muscle-specific actin, but no longer induce goosecoid-expressing dorsal/ventral mesoderm. Xnr-1 RNA induces a similar gene expression profile in animal caps (data not shown). Fig. 6H shows that, at intermediate Xnr-2 doses (10 pg/embryo), the ventral markers Xhox-3 and globin are induced in place of the goosecoid induction at higher doses.

**Xnr signals dorsalize ventral marginal zone during gastrula stages**

Xnr-1 and Xnr-2 expression peaks during late blastula/early gastrula stages and is maximal in the dorsal marginal zone. We next tested whether Xnr expression at gastrula stages could modify the type of mesoderm induced earlier in the ventral marginal zone (Dale and Slack, 1987a; see Introduction). To overexpress Xnr-1/Xnr-2 during gastrula stages, we used plasmids containing Xnr-1 or Xnr-2 sequences driven by a cytoskeletal actin promoter (pCSKA:Xnr-1 and pCSKA:Xnr-2). pCSKA constructs begin to express high levels of RNA during early gastrula stages (Christian and Moon, 1992; Smith et al., 1993). pCSKA:Xnr-1 and pCSKA:Xnr-2 plasmids were injected into a ventral marginal location at the 4-cell stage. A similar construct designed to express activin, pCSKA:activin, in dorsalizing VMZs towards dorsal (muscle) fates. Xnr-1 RNA (100 pg/embryo) induces a strong phenotype similar to that caused by injecting Xnr-2 RNA into non-UV-treated embryos. When less Xnr-2 RNA (1-5 pg per blastomere) is injected into UV-treated embryos, recognizable partial anteroposterior and dorsoventral axes form in a small percentage of embryos but, most consistently, these groups of embryos still develop a hyperdorsalized phenotype (data not shown). This differential result could be considered consistent with the apparently different mesoderm induction activities of Xnr-1 and Xnr-2 RNAs in animal cap assays described above. Nevertheless, we conclude that Xnr signaling is sufficient to rescue complete embryonic development (Xnr-1), or notochord induction (Xnr-2), in radially ventralized embryos.

**DISCUSSION**

While the phenotype of embryos homozygous for the 413.d insertional mutation established that nodal is essential for mesoderm formation and embryonic patterning in mammals, it provided no direct clue as to the role that it played in these processes. We have shown here that nodal and Xnr-1/Xnr-2 are potent dose-dependent mesoderm inducers in Xenopus embryonic cells and, furthermore, that they can act during gastrulation as dorsalizers of ventral mesoderm. Nodal-related signaling can also rescue a complete embryonic axis in UV-ventralized embryos. While these experiments were being completed, we reported that injection of nodal mRNA into zebrafish embryos resulted in embryonic axis duplications preceded by ectopic activation of Organizer-specific markers such as goosecoid and lim-1 (Toyama et al., 1995). These functional studies thus suggest that nodal and nodal-related signaling pathways play an extensive role in mesodermal induction and patterning, and provide critical information in relation to models for the biological activity of nodal in vertebrate gastrulation.

**Xnr-1 and Xnr-2 are new members of the TGFβ superfamily**

The mature C-terminal ligands of TGFβ-like secreted signaling
molecules are released by proteolytic cleavage from larger precursors. Most members contain seven conserved cysteines within the mature region and are presumed to function as dimers (Kingsley, 1994). Crystallographic studies on TGFβ2 suggest that the dimeric ligand forms a 'cystine knot' (Daopin et al., 1992; Schlunegger and Grutter, 1992; McDonald and Hendrickson, 1993), in which the conserved cysteine residues function to stabilize the structure. Mouse nodal has the canonical cysteine spacing, but the *Xenopus* and chick nodal-related sequences described here, together with Xnr-3 (see below), are unique in that a pair of cysteines that are usually adjacent exhibit a split-cysteine spacing (CysXXCys; Fig. 3B).
The displaced cysteine corresponds to the residue normally forming an intermolecular disulfide bond in the dimer (Daopin et al., 1992; Schlunegger and Grutter, 1992), and mutation of this cysteine in activin preventsimerization, abrogating biological activity (Mason, 1994). Two other TGFβ-like molecules, Vgr-2/GDF-3 and GDF-9, encode peptides in which this cysteine is not displaced, but replaced by valine (Jones et al., 1992b; McPherson and Lee, 1993). It is possible that the displaced cysteine in Xnr peptides is still appropriately positioned to make the intermonomer disulfide bond, but we hypothesize that the associated structural changes could substantially affect the specificity of Xnr ligand/receptor interactions.

Xnr-1 and Xnr-2 as Xenopus cognates of mouse nodal
Several pieces of information imply that Xnr-1/Xnr-2 represent the Xenopus genes most closely related to mouse nodal. First, Southern blot analysis of Xenopus genomic DNA with mature region nodal probes detects no positive signals at medium to high stringency and only Xnr-1 or Xnr-2 signals at reduced stringency (C. V. E. W., unpublished observations). Second, PCR and low stringency screening with an Xnr-2 mature region probe on pre- and postgastrulation mouse embryo libraries results only in reisolating nodal cDNAs, suggesting that mouse Xnr-like sequences do not exist (C. M. J., unpublished observations). Third, chick nodal-related sequences (Fig. 3) also encode peptides with the Xnr-like ‘split-cysteine’ pattern. It appears that nodal-related peptides exhibit more divergence than usual during vertebrate evolution, because molecules like activin and BMP4 are almost identical even in evolutionarily distant vertebrate species. Thus, nodal-related peptides in lower vertebrates may be more likely to resemble frog Xnr than mammalian nodal. Fourth, functional data presented in this manuscript demonstrate that mouse nodal and Xnr genes are essentially interchangeable, each inducing similar patterns of gene expression and mesoderm differentiation.

Another recently isolated Xenopus gene, Xnr-3, also encodes a nodal-related molecule with a split-cysteine motif (Smith et al., 1995). Xnr-1, Xnr-2 and Xnr-3 therefore represent frog prototypes of a new group of the TGFβ superfamily. However, Xnr-3 differs substantially from Xnr-1/Xnr-2 in other parts of the mature ligand region, including an extended C terminus. While Xnr-3 exhibits partial dorsalizing activity in UV rescue experiments, unlike Xnr-1/Xnr-2, it cannot induce axial mesoderm in animal caps (Smith et al., 1995). Xnr-3 is expressed in a pattern overlapping Xnr-1/Xnr-2 such that, together with the different activities of the three proteins, the possibility is raised of their functional interplay during mesodermal patterning processes.

Xnr-1 and Xnr-2 and axial mesoderm patterning in Xenopus
Mesoderm induction begins around the 32-64 cell stage of Xenopus development (Jones and Woodland, 1987). This is before zygotic transcription begins (Newport and Kirschner, 1982) and induction therefore depends upon factors stored as RNA or protein in the oocyte. Xnr-1 and Xnr-2 transcripts are first detected after zygotic transcription begins, in the vegetal hemisphere of late blastulae, suggesting that neither Xnr functions in the initial mesoderm induction process. No maternal transcripts for either Xnr-1 or Xnr-2 are detected (data...
not shown), but it is not known if Xnr s are available to the early embryo as maternally supplied protein, as is found for activin (Fukui et al., 1994). Studies with Xnr antibodies will be needed to address this possibility.

Alternatively, the finding that Xnr RNA is first expressed in the vegetal region of the blastula raises the possibility that Xnr signaling at this stage may act as a relay factor, maintaining or intensifying the initial mesoderm induction signals to allow continued formation and differentiation of the mesoderm. A similar idea has been proposed for FGF and activin (Slack, 1994). In contrast, the vegetal Xnr expression could be involved in the specification of the endodermal fate. In either case, it is important to consider that differential processing of Xnr precursors, as has been proposed for Vg1 (Dale et al., 1993; Thomsen and Melton, 1993), could cause a dorsal-ventral skewing in the production of active Xnr ligand.

**Xnrs dorsalize ventral marginal zone mesoderm during gastrulation**

Diversification of ventrolateral mesoderm into graded domains that turn into striated muscle, nephric tubules and lateral plate mesoderm occurs during gastrulation through dorsalization – a process dependent upon signals from the Spemann Organizer (Dale and Slack, 1987a; Lettice and Slack, 1993). Potent mesoderm inducers such as activin cannot dorsalize ventral mesoderm during gastrulation (Smith et al., 1993; Lettice and Slack, 1993; this study). However, two unrelated non-mesoderm inducers, noggin and chordin, induce muscle differentiation in VMZ explants which otherwise would not form muscle (Smith et al., 1993; Sasai et al., 1994). Results presented here demonstrate that Xnr-1 and Xnr-2, produced during gastrulation, can also divert VMZ explants from a ventral program to a muscle fate. Thus, Xnr-1 and Xnr-2 are new candidates for mediators of the dorsalization process in vivo, but differ from previously characterized signaling factors (noggin, chordin or activin) in being both mesoderm-inducers and dorsalizers of ventral mesoderm during gastrulation.

The expression patterns of Xnr-1 and Xnr-2 at the gastrula stage are compatible with their role as mediators of dorsalization. Xnr-1 and Xnr-2 expression is highest in the marginal zone and, as illustrated particularly well for Xnr-2, appears to form a gradient of expression with a dorsal maximum and a ventral minimum (Fig. 4D,E). We speculate that the specification of different fates within the spectrum of dorsal-ventral mesoderm depends upon the local level of Xnr signaling. It will be important to understand the link between Xnr expression and earlier patterning events in the embryo; for example, if the level of Xnr-1 and Xnr-2 expression in the marginal zone is a direct response to the relative levels of dorsoanterior, rather than ventroposterior, mesoderm inducers.

**Nodal-related signalling and the vertebrate body plan**

Mouse nodal and *Xenopus* nodal-related molecules are dose-dependent mesoderm inducers, a finding consistent with the failure to form mesoderm in mouse embryos homozygous null for *nodal*. In addition, the detection of mouse *nodal* transcripts in the early primitive streak and then around the node is very similar to the pattern described here for *Xenopus* Xnr-1 and Xnr-2. It is unclear why *Xenopus* has three *nodal*-related genes, while mouse carries out similarly complex developmental processes with only a single *nodal* gene. Mouse nodal may represent an evolutionary convergence in the same molecule of the different activities of separate ancestral *nodal*-like genes. Alternatively, the number and type of *nodal*-like genes in different vertebrate classes may depend upon the different strategies developed for germ layer specification and gastrula-
tion (including the degree to which separate ways of regulating nodal activities must be achieved). Whether all three *Xenopus* nodal-related peptides (Xnr-1, Xnr-2 and Xnr-3) are required for normal *Xenopus* development, and if interplay between them is an important aspect of their function, is under analysis.

Is nodal or activin more important in vertebrate embryonic development? The Xnr activities mimic well the effects of activin as a dose-dependent mesoderm inducer in animal caps (e.g. Green et al., 1992). In fish embryos, the effect of injecting dominant negative activin ligands implies that activin is required for mesoderm induction in vivo (Wittbrodt and Rosa, 1994). Consistent with this, Hemmati-Brivanlou and Melton (1992) showed that a truncated dominant negative activin receptor blocked mesodermal induction in *Xenopus* embryos. However, evidence against this point of view also exists. The dominant negative activin receptor interferes with the signaling pathways of several other TGFβ-like ligands, and experiments with follistatin, an activin antagonist, lead to the conclusion that activin is not required for mesoderm induction (Schnet-Merker et al., 1994). Moreover, early embryogenesis is perfectly normal in mouse embryos carrying null mutations for either, or both, activin subunits (Matzuk et al., 1995; Vassalli et al., 1994; Schreve et al., 1994), although rescue by maternal activin protein might obscure an early mesoderm induction role (Matzuk et al., 1995). In contrast, the severe mesodermal defect in mouse embryos homozygous null for nodal clearly places this gene in a separate category. Complementary studies with mouse, zebrafish, and now *Xenopus* and chick, using embryological, molecular and genetic approaches, strongly argue that nodal and nodal-related signaling molecules play a central role in axial mesodermal patterning.

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