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in Axial Patterning and Establishing Left–Right Asymmetry

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***Nodal-related 1 (ndr1)* and *nodal-related 2 (ndr2)* genes in zebrafish encode members of the nodal subgroup of the transforming growth factor-β superfamily. We report the expression patterns and functional characteristics of these factors, implicating them in the establishment of dorsal–ventral polarity and left–right asymmetry. *Ndr1* is expressed maternally, and *ndr1* and *ndr2* are expressed during blastula stage in the blastoderm margin. During gastrulation, *ndr* expression subdivides the shield into two domains: a small group of noninvoluting cells, the dorsal forerunner cells, express *ndr1*, while *ndr2* RNA is found in the hypoblast layer of the shield and later in notochord, prechordal plate, and overlying anterior neurectoderm. During somitogenesis, *ndr2* is expressed asymmetrically in the lateral plate as are nodal-related genes of other organisms, and in a small domain in the left diencephalon, providing the first observation of asymmetric gene expression in the embryonic forebrain. RNA injections into *Xenopus* animal caps showed that Ndr1 acts as a mesoderm inducer, whereas Ndr2 is an efficient neural but very inefficient mesoderm inducer. We suggest that Ndr1 has a role in mesoderm induction, while Ndr2 is involved in subsequent specification and patterning of the nervous system and establishment of laterality.**

***Key Words: Danio rerio;* zebrafish; nodal-related; forebrain; mesoderm; left–right asymmetry; organizer.**

INTRODUCTION

Pattern formation in embryogenesis is effected by the action of localized maternal determinants, by inductive cell interactions, and by the influence of organizing centers that integrate the generation of the body plan. The first and best-studied example of an organizing center in vertebrates is the Spemann organizer (Spemann and Mangold, 1924), which gives rise to axial mesoderm and endoderm, thereby defining the dorsal side of the gastrula. The dorsal organizer is a conserved feature of vertebrate embryogenesis, with analogous structures in mouse, chicken, and zebrafish embryos named the node, Hensen's node, and shield, respectively (reviewed in Tam and Quinlan, 1996; Lemaire and Kodjabachian, 1996).

In amphibians, the dorsal organizer arises during the process of germ layer formation. Signals from the underlying endoderm induce cells of the marginal zone to adopt mesodermal fates, and prospective mesoderm cells coincidentally acquire dorsal–ventral polarity. These inductive events are mediated at least in part by members of the FGF, TGF-β, and Wnt superfamilies and their downstream signaling cascades (reviewed in Kessler and Melton, 1994; Dawid, 1994; Miller and Moon, 1996; Heasman, 1997). During gastrulation, cells of the organizer and ventral mesodermal cells produce secreted signals that are mutually antagonistic, and their interactions help to establish lateral fates in the marginal zone and cause the neuralization and patterning of dorsal ectoderm (reviewed in Sasai and De Robertis, 1997; Moon *et al.*, 1997; Hemmati-Brivanlou and Melton, 1997). In zebrafish, molecular and genetic studies have provided evidence for generally similar roles for peptide growth factors in germ layer formation and dorsal–ventral patterning (Mizuno *et al.*, 1996; Hammerschmidt *et al.*, 1996a; Fisher *et al.*, 1997; Neave *et al.*, 1997; Nikaido *et al.*, 1997).

Insertional mutagenesis in the mouse has identified a new member of the TGF-β superfamily, nodal, that is

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involved in the initial steps of gastrulation and germ layer specification. Mutant embryos fail to gastrulate, the expression of T (brachyury) protein and other mesoderm markers is severly inhibited (Zhou *et al.*, 1993; Conlon *et al.*, 1994), and the development of rostral neural structures is impaired (Varlet *et al.*, 1997). Genes related to *nodal* were isolated from chicken and frogs with similar expression patterns in the organizer (Smith *et al.*, 1995; Ecochard *et al.*, 1995; Levin *et al.*, 1995; Jones *et al.*, 1995; Joseph and Melton, 1997). Injection experiments of mouse *nodal* RNA into zebrafish embryos and of *Xenopus nodal-related (Xnr)* RNAs into frog embryos indicated that these factors have dorsalizing and morphogenetic properties consistent with an important role in mesoderm induction and the specification or function of the vertebrate organizer (Smith *et al.*, 1995; Toyoma *et al.*, 1995; Jones *et al.*, 1995; Ecochard *et al.*, 1995; Lustig *et al.*, 1996; Glinka *et al.*, 1996; Joseph and Melton, 1997).

We report here the isolation and characterization of two *nodal-related* genes from the zebrafish named *ndr1* and *ndr2*, and studies on their role in early development. These results implicate nodal-related proteins in dorsal–ventral patterning and neural induction in zebrafish embryos, and further reveal left–right asymmetry of *ndr2* expression in the lateral plate and the developing forebrain.

MATERIALS AND METHODS

cDNA Library Construction

A gastrula stage cDNA library was made by random-priming of shield stage poly(A)$^+$ RNA according to Blumberg *et al.*, (1992) with minor modifications; cDNAs were cloned into the *Eco*RI site of Lambda ZAP II. We obtained about 35×10^6 recombinants per microgram of poly(A)$^+$ RNA.

Cloning of Zebrafish ndr1 *and* ndr2

First-strand cDNA from shield stage poly(A)$^+$ RNA was made by random-priming and used for PCR reactions with degenerate oligonucleotides to conserved regions of mouse nodal and *Xenopus* Xnr-1 and Xnr-2. Sense oligo F1: GGAATTCCC(AC)AC(AGCT)A-A(TC)CA(TC)GC(AGTC)TA; antisense oligo R1: GGAATTC(AG)-CAICC(AG)CA(AGCT)TC(AGCT)TC(AGCT)AC(ATG)AT. PCR products were subcloned, and homology to *nodal* was confirmed by sequencing. The original PCR product and a subclone were used to isolate cDNA plasmids, pNdr1 and pNdr2, from the shield library. Genomic sequences flanking the putative start and stop codons of the *ndr2* cDNA were cloned by PCR with the GenomeWalker kit (Clontech).

Construction of CS2$^+$ Expression Plasmids

Optimized Kozak translation initiation sequences were engineered into pNdr1 by PCR (sense primer CS2NDR1F; TAAGGATC-CACCATGTTTTCCTGCGGGCTCCT; and antisense primer CS2NDR1R; ATCGGATCCTAGAATTCTCAGTGGCAGC-CGCA). The product was cut with *Bam*HI and *Eco*RI and ligated into pCS2$^+$ (Turner *et al.*, 1994) to give pCS2$^+$Ndr1. The construct was confirmed by sequencing.

Embryo Injections and RNA Assays

Zebrafish embryos were obtained by natural matings (Westerfield, 1995) and staged according to Kimmel *et al.*, (1995). The following mutant alleles were used: the *cerebum*^{c4} allele (Fisher *et al.*, 1997) of *chordino* (formerly *dino*) (Schulte-Merker *et al.*, 1997); *swirl*^{ta72a} (Mullins *et al.*, 1996); *no tail*^{b160} (Halpern *et al.*, 1993); *floating head*^{n1} (Talbot *et al.*, 1995).

Embryos were injected at the 2- to 16-cell stage with 0.2 to 5 pg RNA as described previously (Toyama *et al.*, 1995). RNAs for injections were made with the Promega Megascript kit using a 10:1 molar excess of m^{7}G(5′)ppp(5′)Gm to GTP. RNA injection into *Xenopus* embryos, animal explant culture, and RNA extraction and blotting were carried out as described by Taira *et al.*, (1997); probes were *goosecoid* (Cho *et al.*, 1991), *chordin* (Sasai *et al.*, 1994), *Xbra* (Smith *et al.*, 1991), *nrp-1* (Richter *et al.*, 1990), *noggin* (Smith and Harland, 1992), *follistatin* (Hemmati-Brivanlou *et al.*, 1994), and EF-1αS (Krieg *et al.*, 1989).

In Situ *Hybridization and Immunohistochemistry*

In situ hybridization and immunohistochemistry were carried out as described (Toyama *et al.*, 1995). MF20, a mouse monoclonal antibody against a chicken myosin heavy chain (Gonzalez-Sanchez and Bader, 1984), was obtained from the Developmental Studies Hybridoma Bank and used at a 1:50 dilution; embryos were fixed in MEMFA (0.1 M Mops, pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde) for 4 h (25°C) or overnight (4°C). MZ15 (gift of F. Watt), a mouse monoclonal antibody against a keratan sulfate (Smith and Watt, 1985), was used at 1:300; embryos were fixed for 30 min (25°C) in MEMFA or for 2 h at 25°C in methanol:DMSO (8:2). Sensitivity was best with methanol:DMSO but morphology was poorer. Embryos were mounted in 70% glycerol in PBS, or cleared in benzyl alcohol:benzyl benzoate (1:2) prior to photography with a Zeiss Axiophot using Nomarski optics unless otherwise noted; images were scanned and contrast-enhanced using Adobe Photoshop 3.0.

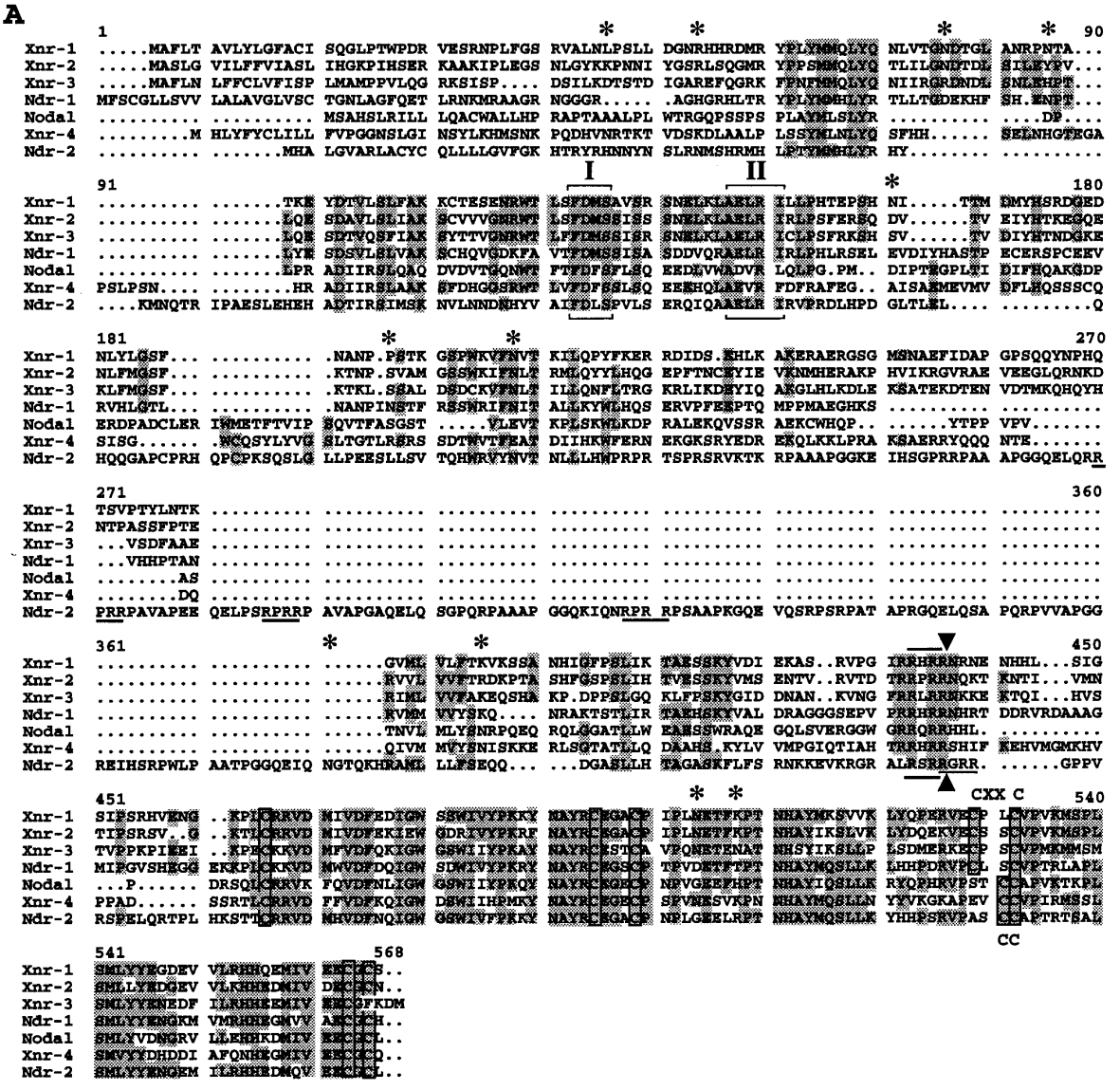
RESULTS

Cloning of Zebrafish nodal-related *Genes 1 and 2*

We used RT–PCR with degenerate primers followed by gastrula library screening to isolate cDNAs corresponding to two zebrafish genes that encode new members of the nodal subfamily of TGF-β growth/differentiation factors (Fig. 1). Sequence conservation between Ndr1 or Ndr2 and other nodal class proteins is highest in the carboxy-terminal 120–130 amino acids which is presumed to be cleaved from a dimeric precursor to give the mature form. Processing of the precursors at putative dibasic cleavage sites matching the consensus R-X-K/R-R (reviewed in Steiner *et al.*, 1992) would generate mature Ndr1 and Ndr2 ligands of 131 and 120/123 amino acids, respectively (Fig. 1A, arrowheads); the ambiguity for Ndr2 is due to the presence of two overlapping putative cleavage sites. In addition, three other sites matching the consensus occur upstream in Ndr2 (Fig. 1A).

The degree of amino acid similarity between nodal-like proteins is considerably higher than similarity to other members of the TGF-β family, among which BMP-8 is one

A



B

% IDENTITY IN THE MATURE REGION

	Ndr-2	Nodal	Xnr-1	Xnr-2	Xnr-3	Xnr-4	Cnr-1	Bmp-8
Ndr-1	68%	57%	67%	63%	59%	54%	66%	43%
Ndr-2		69%	67%	62%	56%	60%	70%	47%

FIG. 1. (A) Comparison of the sequences of zebrafish, mouse, and *Xenopus* nodal-related proteins. Residues conserved in at least four of the seven proteins are shaded. Asterisks show putative N-linked glycosylation sites (N-X-S/T). Arrowheads indicate putative cleavage sites (R-X-K/R-R) producing mature proteins; other sequences in Ndr2 matching the consensus are underlined. Regions I and II are conserved sequences in the precursor region. The conserved cysteines in the mature regions are boxed. *Xenopus* Xnr-1 and Xnr-2: Jones *et al.*, 1995; Xnr-3: Smith *et al.*, 1995; Xnr-4: Joseph and Melton, 1997; mouse Nodal: Zhou *et al.*, 1993. The Ndr1 and Ndr2 sequences have been deposited in GenBank under Accession Nos. AF002218 and AF002219. (B) Percentage amino acid identity in the mature region as determined by the BESTFIT alignment algorithm of the GCG programs.

of the most closely related examples (Fig. 1B), but does not allow assigment of orthology relationships within the nodal subfamily (see Discussion). The spacing of cysteines in the mature domain of Ndr1 is the same as in Xnr-1, Xnr-2, and cNR-1, whereas Ndr2, mouse nodal and Xnr-4 share the pattern common to most TGF-β factors (Fig. 1A); Xnr-3 has yet a third pattern of cysteine residues, lacking the terminal cysteine (Smith *et al.*, 1995).

There is much less sequence conservation in the precursor regions of nodal-related proteins, but certain sequences, for example, regions I and II (Fig. 1A), are conserved in some members of the TGF-β family including Univin, Radar, Dpp, 60A, BMP-2, and BMP-8. The precursor region of Ndr2 contains a proline–alanine-rich insert of about 100 amino acids that is not seen in other nodal class proteins. This insert appears to contain some internally repetitive structure which failed to reveal convincing similarities to other proteins in BLAST homology searches.

ndr1 *and* ndr2 *Are Expressed in the Blastoderm Margin and Divide the Shield into Two Regions*

Northern blots for *ndr1* reveal a 1.2-kb maternal RNA, and a 1.4-kb zygotic transcript which shows peak abundance around the sphere stage and declines sharply after the shield stage (Fig. 2A). The maternal expression of *ndr1* may indicate its participation in mesoderm formation which involves maternal factors in the zebrafish (Mizuno *et al.*, 1996). In contrast, *ndr2* RNA was not detected in the fertilized egg, reaches its maximal level at the shield stage, and then decreases (Fig. 2B) but remains detectable by *in situ* hybridization (see below, Fig. 4).

Whole mount *in situ* hybridization revealed that *ndr1* and *ndr2* are both expressed in restricted domains before gastrulation, and later subdivide the organizer into two distinct zones. *Ndr1* is expressed on one side of the blastoderm margin at the dome stage, the earliest stage examined (4.3 h postfertilization) (Figs. 3A and 3B), and shortly after the start of epiboly was detected throughout the margin (Fig. 3C). At the shield stage, *ndr1* RNA was restricted to a narrow strip of cells corresponding to the dorsal forerunner cells (Figs. 3D–3F, arrow), a subpopulation of approximately 20 cells that do not involute and eventually populate the dorsal roof of Kupfer's vesicle, a tail structure (Cooper and D'Amico, 1996; Melby *et al.*, 1996). In addition, very low levels of *ndr1* expression can also be seen in the dorsal hypoblast (Fig. 3E, arrowhead). We did not detect *ndr1* RNA by *in situ* hybridization after the bud stage, with 24-h embryos being the latest examined.

In contrast to *ndr1*, *ndr2* expression at the dome stage is radially symmetric at the margin (Fig. 4A), then concentrates on the dorsal side (Fig. 4B), and gradually becomes restricted to the dorsal hypoblast, the precursor of the notochord and prechordal plate (Kimmel *et al.*, 1990). Comparison of Figs. 3D and 4C illustrates that, at the start of gastrulation, the expression domains of *ndr1* and *ndr2* subdivide the shield into two distinct zones.

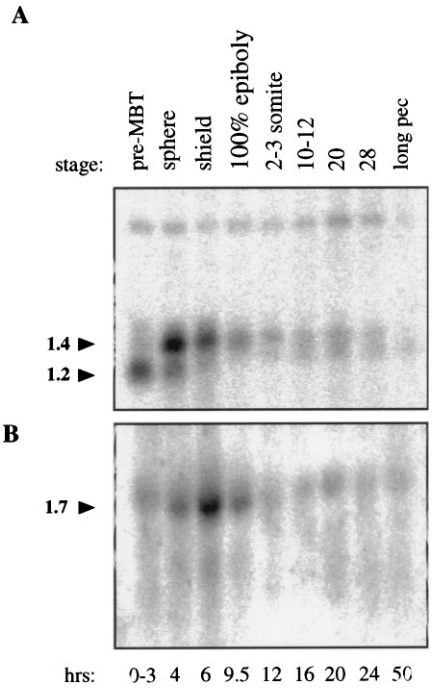


FIG. 2. Northern blot analysis of *ndr1* and *ndr2* expression. (A) *ndr1* has a maternal transcript of 1.2 kb; the abundance of the 1.4-kb zygotic RNA peaks at the sphere stage. Stages are listed above the lanes, hours at 28.5°C at the bottom. Pre-MBT (midblastula transition) denotes RNA pooled from embryos between the 1- and 1000-cell stage, before the onset of zygotic transcription. (B) *ndr2* expression peaks at the early gastrula stage (shield). Each lane contained 10 μg of total embryo RNA.

ndr2 continues to be expressed in the involuting dorsal hypoblast (Figs. 4D and 4E), and by the end of gastrulation (bud stage, 10 h), *ndr2* transcripts are present in much of the prechordal plate and anterior notochord (Figs. 4F–4H) and in a smaller posterior domain (Fig. 4G). In the anterior domain, *ndr2* expression has expanded to include the ventral portions of the neuroectoderm, but the highest staining intensity is associated with the mesodermal layer (Fig. 4H). A gap occurs in the anterior zone of *ndr2* expression (Figs. 4F and 4G, open triangle), likely to separate the polster (Figs. 4F and 4H, asterisk) from the posterior prechordal plate. The fates of the anterior and posterior parts of the prechordal plate are probably specified separately, since mutations exist which preferentially disrupt the development of each part (Brand *et al.*, 1996).

Left–Right Asymmetry of ndr2 *Expression*

During a period around the 20-somite stage, *ndr2* is expressed asymmetrically in two distinct regions of the embryo; this expression is transient, being absent at the 17-somite and the prim-5 (24 h) stage. *ndr2* RNA is present in lateral plate mesoderm along much of the length of the

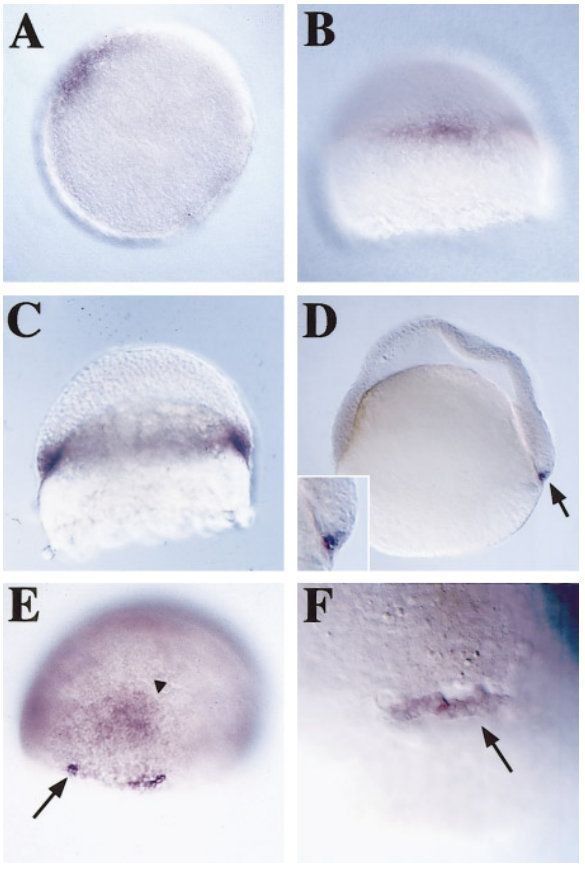


FIG. 3. Whole mount *in situ* hybridizations for *ndr1*. (A, B) Dome stage, animal pole (A) and lateral (B) views show asymmetric expression of *ndr1* at the blastoderm margin. (C) 30% epiboly, lateral view. (D–F) Shield stage. (D) lateral view (dorsal to the right). Arrow marks the site of *ndr1* expression in the shield, specifically within the area of the dorsal forerunner cells; the inset shows an enlarged view of this area. (E,F) dorsal view of *ndr1* expression in forerunner cells (arrow) at two magnifications. Arrowhead shows traces of *ndr1* RNA in more anterior aspects of the shield.

body on the left side, starting quite sharply next to the anterior hindbrain and extending at diminishing intensity towards the tail (Figs. 4K and 4L, triangle). This asymmetric expression is similar to the pattern seen for *nodal* and related genes in mouse, chicken, and *Xenopus* (Levin *et al.*, 1995; Lowe *et al.*, 1996; Collignon *et al.*, 1996; Sampath *et al.*, 1997). In addition, we observed *ndr2* expression to the left of the midline in a small domain of the posterior diencephalon (Figs. 4I–4L, arrow), just ventral to the epiphysis (marked ''e''). To our knowledge, this is the first observation of left–right asymmetry of gene expression in the embryonic forebrain. In a small fraction of embryos the asymmetry of *ndr2* expression was reversed or lost; in most cases both domains were affected in the same embryo; in rare cases the asymmetry of only the lateral plate or forebrain domain was changed. This alteration of handedness correlates with the fact that a low background fraction of embryos with reversed L–R asymmetry of the heart is generally found in wild-type strains of zebrafish (Chen *et al.*, 1997). Thus, nodal-related gene expression along the left–right axis displays both conserved and distinctive features in zebrafish compared to other vertebrates.

Expression of ndr1 *and* ndr2 *in Mutant Embryos*

Several mutations have been reported that affect dorsal–ventral polarity of the embryo and the formation and function of axial structures. We tested whether the expression of the *ndr* genes is affected in four such mutants. *swirl* embryos are partially dorsalized because of a mutation in the *BMP-2* gene (Mullins *et al.*, 1996; Kishimoto *et al.*, 1997), while *chordino* embryos are partially ventralized as a result of a mutation at the zebrafish homolog of the *chordin* locus (Hammerschmidt *et al.*, 1996b; Odenthal *et al.*, 1996; Fisher *et al.*, 1997; Schulte-Merker *et al.*, 1997). We examined embryos derived from crosses between heterozygous *swirl* or *chordino* parents and found that *ndr1* and *ndr2* expression at the shield stage was normal in all progeny.

Both the *no tail* (*ntl*) gene which encodes the homolog of the Brachyury protein and the *floating head* (*flh*) gene encoding an Xnot homolog are required for notochord formation (Halpern *et al.*, 1993; Talbot *et al.*, 1995). At the shield stage, *ndr1* and *ndr2* expression was normal in *ntl* and *flh* mutant embryos; at the bud stage, *ndr2* expression was also normal in *flh*, but altered in *ntl* embryos (*ndr1* is not expressed at this stage). Figures 5A, 5B, 5E, and 5F illustrates that *ntl* embryos have lost *ndr2* expression in the posterior axial region (arrow), but expression is seen in posterior–lateral domains (asterisk), possibly as a result of defective convergence–extension movements. At the anterior, the effect of *ntl* is more subtle, representing a widening of the *ndr2* expression domain (Figs. 5C and 5D). A broadening of midline structures such as floor plate and prospective notochord has been reported in *ntl* embryos (reviewed in Odenthal *et al.*, 1996).

Mutants defective in midline signaling such as *ntl* and *flh* affect the establishment of laterality (Danos and Yost, 1996; Chen *et al.*, 1997). Consistent with these observations we find that, at the 20-somite stage, the asymmetric expression of *ndr2* in *ntl* and *flh* mutant embryos is lost in the diencephalon (Figs. 5G, 5H, 5K, and 5L) and in the lateral plate (Figs. 5I and 5J, and not shown). These observations support the view that the *ndr2* gene might have a role in the establishment of laterality.

Differences in Inducing Capacities of Ndr1 and Ndr2 Are Revealed in Xenopus *Animal Explants*

Animal explants (caps) from *Xenopus* embryos are a widely used and valuable tool to test the function of inducing factors including those derived from heterologous sources. Therefore we compared the properties of *ndr1* and *ndr2* by injection of RNA into the animal region of *Xenopus*

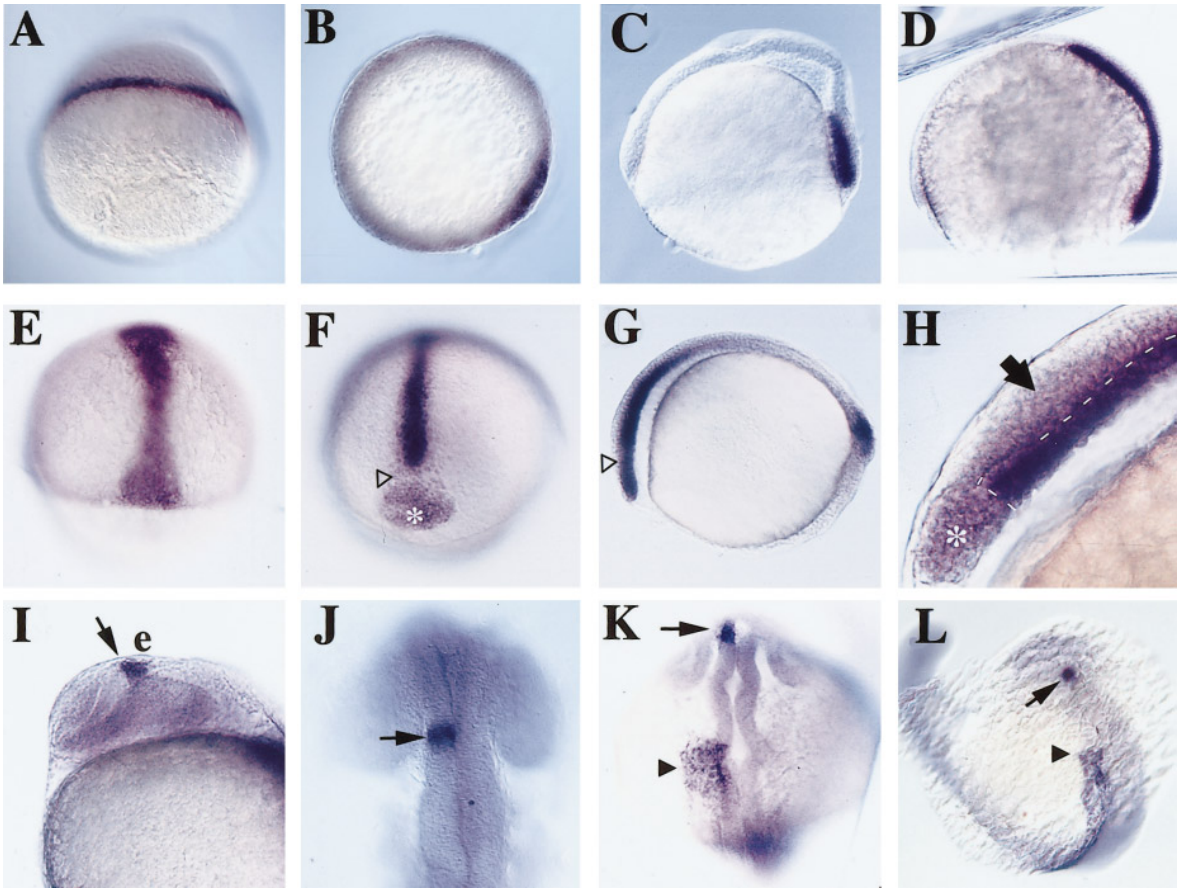
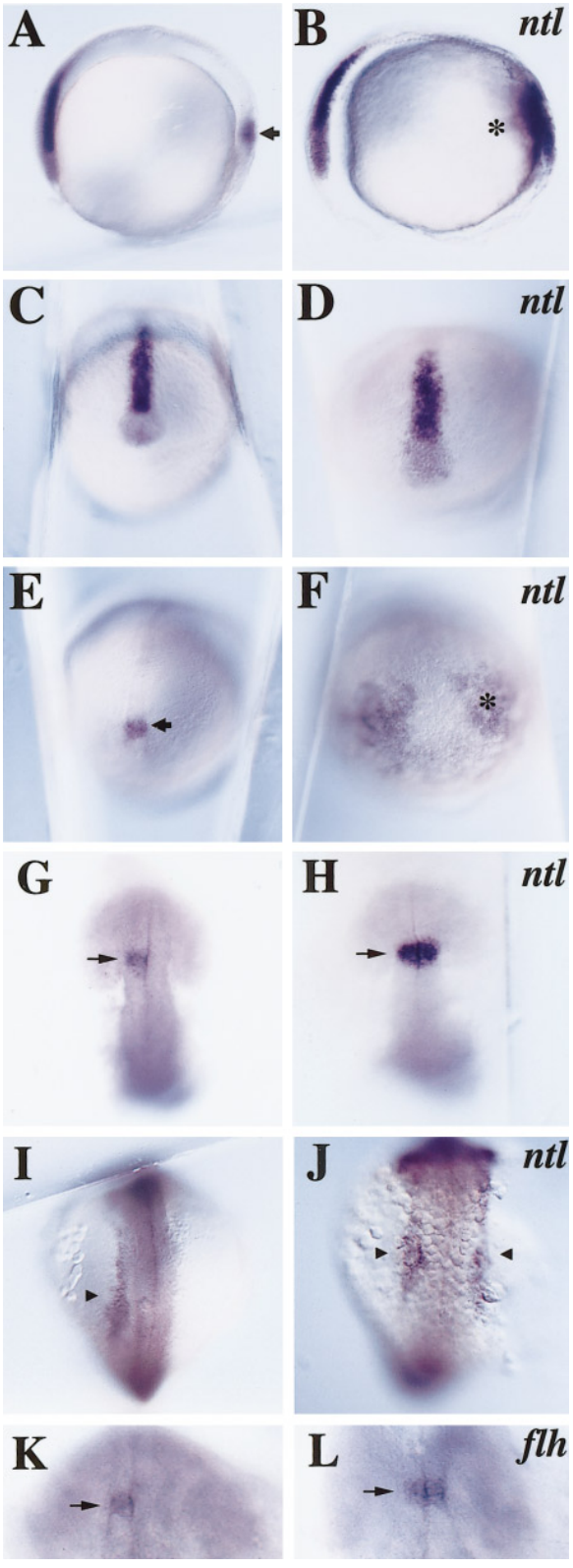


FIG. 4. Whole mount *in situ* hybridization of *ndr2* expression. (A) Dome stage, lateral view. (B) 30% epiboly, animal view; *ndr2* is expressed at the blastoderm margin. (C) Shield stage, lateral view (dorsal to the right); *ndr2* expression is restricted to the hypoblast layer of the shield. (D) 80% epiboly, lateral view, and (E) 70% epiboly, dorsal view; *ndr2* expression is seen in involuting hypoblast. (F–H) Bud stage; anterior view (F), lateral view with anterior to the left (G), higher magnification lateral view (H). *ndr2* is present in an anterior domain including the prechordal plate, anterior notochord, and ventral neuroectoderm (arrow); open triangles mark a gap in the anterior domain, asterisks indicate the polster, and a dotted line traces Brachet's cleft separating anterior mesoderm and neurectoderm. *ndr2* also is expressed in the tail bud (G). (I–L) 20-somite stage. Lateral view (I; anterior to the left), and dorsal view of the head (J; anterior to the top); *ndr2* is expressed to the left of the midline within the posterior diencephalon (arrow) just ventral to the epiphysis (e). Dorsal view (K), and dorsal–lateral view (L); *ndr2* is expressed in the left lateral plate (filled triangle), starting at a position next to the anterior hindbrain and extending caudally at decreased width and intensity of expression.

embryos, followed by dissection at the blastula stage, explant culture, and assay for the expression of several marker genes. Ndr1 induced the pan-mesodermal marker *Xbra*, the dorsal mesodermal markers *goosecoid* (*gsc*), *chordin* (*chd*), *noggin* (*nog*), and *follistatin* (*foll*), and at a later stage, the pan-neural marker *nrp-1* (Fig. 6); these inducing properties are quite similar to those of activin. Ndr2 behaved quite differently: while overexpression of this factor in animal caps strongly induced *nrp-1*, no induction of *gsc*, *chd*, *foll*, or *nog* was seen (Fig. 6); *Xbra* either was not induced (Fig. 6) or was induced very weakly as seen after long exposures in some experiments (not shown). Thus, the neural inducing effect of Ndr2 does not appear to be mediated by chordin, noggin, or follistatin (Sasai *et al.*, 1994; Lamb *et al.*, 1993; Hemmati-Brivanlou *et al.*, 1994), suggesting a distinct direct neuralizing pathway, possibly resembling that of Xnr-3 (Smith *et al.*, 1995; Hansen *et al.*, 1997) or of processing-enhanced Lefty-1 and Lefty-2 (Meno *et al.*, 1997).

Dorsalization by Ectopic Expression of ndr1 *in the Zebrafish Embryo*

The initial induction of mesoderm is likely to be mediated by maternal factors (see Dawid, 1994; Kessler and Melton, 1994; Heasman, 1997). Since *ndr1* is expressed maternally (Fig. 2) and can act as mesoderm inducer in *Xenopus* explants (Fig. 6), we tested whether this factor could induce dorsal mesoderm in zebrafish embryos. Injec-



tion of *ndr1* RNA induced a second organizer in up to 25% of the injected embryos (Figs. 7A–7D) as seen by expression of *gsc* and *lim1* (Stachel *et al.*, 1993; Toyama *et al.*, 1995), while in most embryos an enlarged dorsalized region was observed (not shown). This outcome might be expected from the fact that the position of the site of injection relative to the endogenous dorsal side of the embryo is unknown; a secondary dorsal center is likely to be generated if the RNA happens to be injected ventrally, while dorsolateral injection would lead to a single enlarged region of marker expression. In addition to activating dorsal genes, ventral gene expression is inhibited by the organizer (Hammerschmidt *et al.*, 1996a; see Sasai and De Robertis, 1997); as shown in Figs. 7E and 7F, the ventral marker *eve1* (Joly *et al.*, 1993) was inhibited by *ndr1* injection.

At later stages, the consequences of Ndr1 overexpression include dorsalization and partial axis duplication, as observed by immunostaining with the MZ15 antibody for notochord (Smith and Watt, 1985) and with MF20 for muscle myosin (Gonzalez-Sanchez and Bader, 1984). Injected embryos showed a gradation of phenotypes (Fig. 8) which is partly dose dependent. Between 14 and 45% of injected embryos in different experiments had expanded notochords (Figs. 8A–8D), associated with variable amounts of muscle tissue (Figs. 8B and 8D). Some of these embryos were shortened in the A–P dimension, possibly reflecting disturbance in convergence–extension movements.

A second notochord and adjacent muscle was seen in up to 30% of the *ndr1*-injected embryos (Fig. 8E); in some cases, distinct somites span the gap between the two notochords (arrow in Fig. 8F). Stronger and earlier expression of myosin on only one side, the ''inner'' side, of each of the duplicated notochords was consistently observed (Figs. 8E and 8F). Similar to the situation in embryos injected with mouse *nodal* RNA (Toyama *et al.*, 1995), no obvious duplication of head structures was seen. The most extreme phenotype consisted of embryos with large, disorganized areas of notochord and muscle, resembling the radialized

FIG. 5. *ndr2* expression in *ntl* and *flh* mutant embryos. The left column shows wild-type siblings, the right column shows *ntl* homozygotes (B, D, F, H, J) or a *flh* homozygote (L). (A–F) Bud stage. (A, B) Lateral view, anterior to the left. (C, D) Anterior view. *ndr2* is expressed in anterior notochord and prechordal plate. The embryonic stage shown here is slightly earlier than that shown in Figs. 4F and 4G, and therefore the gap within the anterior expression domain is not yet evident. *ndr2* expression is slightly wider in the *ntl* embryo. (E, F) Posterior view. *ndr2* staining is found in the tail bud of wild-type siblings (arrow), while in mutant embryos, tail bud staining is absent but a broader lateral and posterior region is positive (asterisk); 16 (24%) of 66 embryos in a cross between heterozygous *ntl* zebrafish showed the mutant pattern. (G–L) 20-somite stage. In wild-type embryos, *ndr2* is expressed asymmetrically in the lateral plate and diencephalon, but expression is present bilaterally in both *ntl* (H, J) and *flh* mutants (L).

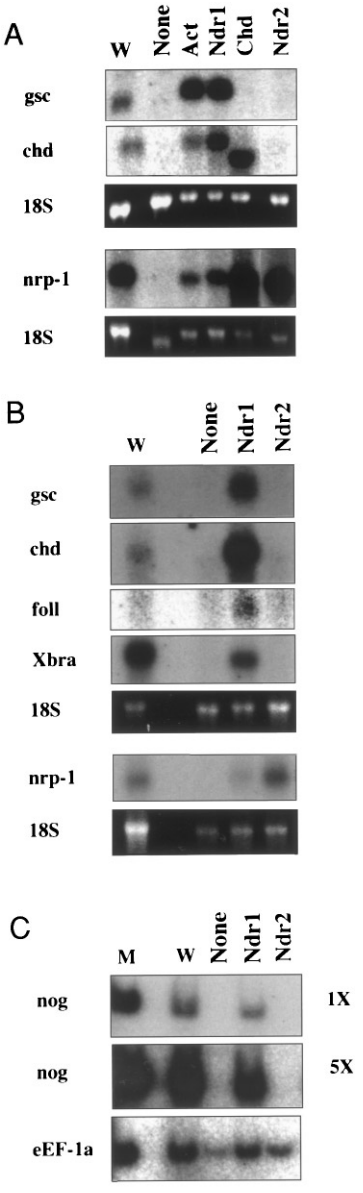


FIG. 6. Differential inducing activity of Ndr1 and Ndr2 in *Xenopus* animal caps. RNAs (listed on top) encoding Ndr1 (400 pg), Ndr2 (400 pg), activin (20 pg), or chordin (700 pg) were injected into the animal region, and explants were cultured until equivalent stage 11.5 for the assay of *gsc*, *chd*, *Xbra*, and *follistatin*, and to equivalent stages 25–27 for assay of *nrp-1* by Northern blotting. Stained 18S RNA is shown as a loading control. A and B show experiments with different batches of embryos. The faster-migrating *chordin* band in the *chordin*-injected sample in A represents the injected RNA which lacks UTRs. (C) *noggin* RNA was assayed at stage 11.5 by RT–PCR; a standard exposure (top row) and a fivefold longer exposure (second row) are shown to illustrate the absence of *noggin* RNA in the *ndr2*-injected caps. eEF-1α primers spanning an intron were used to control for genomic DNA contamination and for RNA levels. M, PCR amplification with noggin or eEF-1α plasmid as template; W, RT–PCR with whole embryo RNA; none, uninjected animal caps.

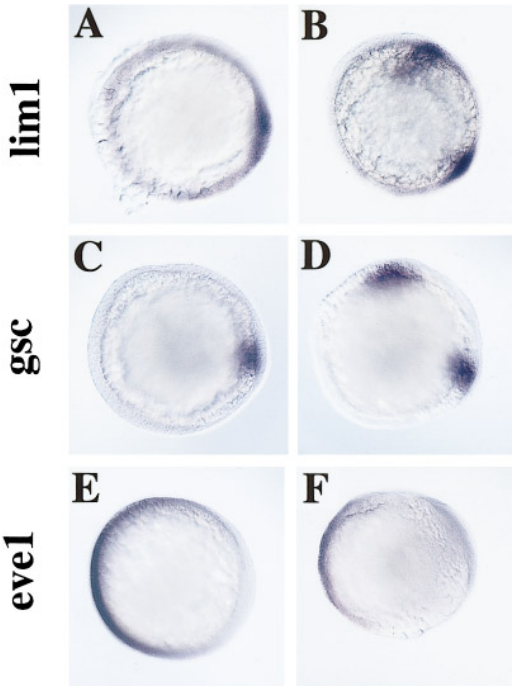


FIG. 7. Induction of marker genes by injection of *ndr1* RNA into zebrafish embryos. Animal pole views are shown of uninjected embryos (A,C,E), and embryos injected with *ndr1* RNA (B,D,F). The probes used are indicated to the left of each pair of embryos. Up to 25% of injected embryos generated a secondary organizer region as judged by an ectopic area of *lim1* or *gsc* expression (A–D). The intensity and extent of *eve* expression in the injected embryos (F) was reduced compared to the controls (E).

embryos obtained by lithium treatment (Stachel *et al.*, 1993). This phenotype was seen in a dose-dependent manner, ranging from none after injection of 0.2 pg RNA to an average of 27% of the embryos in five experiments in which 5 pg of *ndr1* RNA was injected.

DISCUSSION

Subgroups of Nodal-Related Proteins

We have cloned and characterized two new members of the TGF-β superfamily that are expressed specifically in dorsal structures of the zebrafish embryo. Because these two factors are most closely related to the proteins of the nodal subclass, we have named them Nodal-related 1 and 2 (Ndr1 and Ndr2). Spacing of cysteine residues within the mature region suggests three subgroups of nodal-related proteins. One group includes mouse nodal, Xnr-4 and Ndr2, a second includes Xnr-1, Xnr-2, cNR-1, and Ndr1, while Xnr-3 constitutes the third group. The heterogeneity in spacing between the first two groups involves the fourth and fifth cysteine residues (Fig. 1). The fourth cysteine forms the intermolecular disulfide bond be-

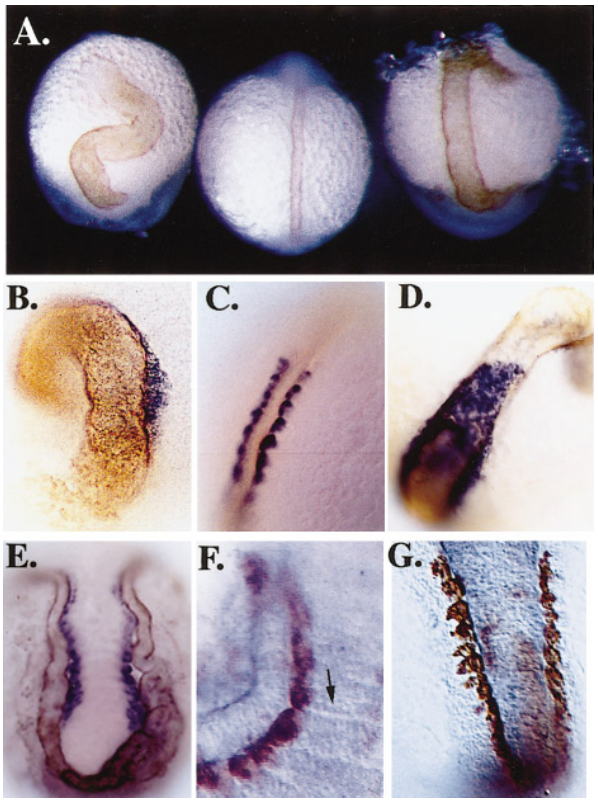


FIG. 8. *ndr1* RNA injections lead to expansion of dorsal cell fates or axis duplication in zebrafish embryos. MZ15 antibody which recognizes the notochord marker keratan sulfate is shown in brown (A–E) or blue (F,G); MF20 antibody, recognizing muscle myosin heavy chain, is shown in blue (B–E) or brown (F,G). Different phenotypes were observed: widened and curved notochord (A), control embryo in center; wide and foreshortened notochord associated with variable amounts of muscle (B,D; control in C); duplicated notochords fused to form a U-shape (E,F), where myosin staining is strongest on the inside edge and the somite boundaries span the gap between the two notochords (arrow in F); and widened notochord and neural plate flanked by relatively well-organized somites (G). Untreated siblings were at 14–15 somites (A–E), and 20–21 somites (F,G).

tween the two subunits of the mature protein, raising the possibility that such variations may affect intermolecular contacts between the two subunits (Schlunegger *et al.*, 1993; Griffith *et al.*, 1996).

Conserved and Distinct Functions of Nodal-Related Factors

In the mouse, nodal is required for the proper formation of the primitive streak and the mesoderm, and mutant embryos do not complete gastrulation (Zhou *et al.*, 1993; Conlon *et al.*, 1994). If only a single *nodal* gene exists in the mouse, its later functions would not be apparent in mutants because of this early lethality. Multiple nodal-related factors have been discovered in *Xenopus* (Jones *et al.*, 1995; Smith *et al.*, 1995; Ecochard *et al.*, 1995; Joseph and Melton, 1997), and now in zebrafish. It is possible that the multiple functions in which mouse nodal is implicated, mesoderm induction and streak maintenance (Zhou *et al.*, 1993; Conlon *et al.*, 1994), head formation (Varlet *et al.*, 1997), and establishment of left–right asymmetry (Collignon *et al.*, 1996), are divided among different nodal-related factors in frogs and fish. Of the two factors we have isolated, Ndr1 is a good candidate for the early, mesoderm-inducing function of nodal. *ndr1* mRNA can be detected in the egg, zygotic expression starts and peaks very early in the blastoderm margin, the region where prospective mesoderm cells involute (Fig. 3), and Ndr1 is effective as a mesoderm-inducing factor (Figs. 6–8); thus, Ndr1 may be involved in mesoderm induction during blastula stages. While the kinetics of synthesis and turnover of Ndr1 protein are unknown, the rapid disappearance of *ndr1* RNA suggests that its function may be limited to initial stages of mesoderm formation. As early as the shield stage the level of *ndr1* RNA has decreased drastically, and it is localized mainly in the dorsal forerunner cells (Fig. 3), possibly implicating it in the specification of Kupfer's vesicle (Cooper and D'Amico, 1996; Melby *et al.*, 1996).

Ndr2 differs from Ndr1 in that it has only very weak, if any, mesoderm-inducing activity but strong neural-inducing activity in the *Xenopus* animal cap system. Although it is maximally expressed during gastrulation, *ndr2* is also expressed later in development. These properties imply that *ndr2* may have both early and late functions in the embryo. Ndr2 expression in the involuting cells of the shield and subsequently in anterior axial tissues suggests that it may mediate some of the inductive roles of the prechordal plate and notochord, such as patterning of ventral brain and spinal cord, paraxial mesoderm, and the ventral cranium (reviewed in Nieuwkoop, 1989; Brand *et al.*, 1996; Li *et al.*, 1997). Many of these functions have been ascribed to members of the hedgehog family (reviewed in Hammerschmidt *et al.*, 1997), but ectopic expression of *shh* or dominant negative inhibitors of protein kinase A do not completely rescue all midline patterning defects in mutants like *cyclops* and *no tail* (Ungar and Moon, 1996; Hammerschmidt *et al.*, 1996c). It was shown recently that Shh is not required for the formation of the midline floor plate cells in zebrafish (H. Schauerte, F. van Eeden, C.F., and P.H., unpublished data), and experiments comparing *shh* misexpression phenotypes in normal and *spadetail* embryos also support the idea that additional midline signals are needed in parallel with Shh (Weinberg *et al.*, 1996).

Nrd2 Shares Left–Right Asymmetry of Expression with Other Nodal Class Genes

In mouse and chicken embryos, there is transient left–right asymmetry of *nodal* or *cNR-1* RNA around the node. This

early asymmetry is not evident for *Xnr-1, -2,* or *-3* in *Xenopus* (Smith *et al.,* 1995; Jones *et al.,* 1995), or for *ndr1* and *ndr2* in the zebrafish. These species-specific differences may signify a divergence in the earliest steps in the establishment of L–R asymmetry; consistent with this possibility, recent experiments suggest that Vg1, rather than Xnr-1, -2, or -3, acts as an early left–right patterning signal in amphibians (Hyatt *et al.,* 1996), although Xnr-1 can influence left–right asymmetry when expressed at later stages (Sampath *et al.,* 1997).

A conserved asymmetric pattern of expression of nodal class genes in the lateral plate suggests a role for these genes in establishing handedness (Levin *et al.,* 1995; Lowe *et al.,* 1996; Collignon *et al.,* 1996). We observed a transient expression of *ndr2* in the lateral plate predominantly to the left of the midline in a stripe that extends posteriorly above the yolk toward the tail (Figs. 4K and 4L). This expression is similar to that observed in mouse, chicken, and frog embryos and implicates Ndr2 in the establishment of L–R asymmetry of internal organs in the zebrafish.

While nodal-related factors have previously been observed in the left lateral plate, such asymmetry has not been reported in neural tissues; however, *lefty-1* and *lefty-2,* distinct members of the TGF-β superfamily, are expressed on the left side in the prospective floor plate and ventral mid- and hindbrain of the mouse (Meno *et al.,* 1996, 1997). Asynchrony along the L–R axis of *Krox-20* expression in the neural plate and neural crest in *Xenopus* (Bradley *et al.,* 1992) and of Lim3 protein expression in zebrafish pituitary anlage (Glasgow *et al.,* 1997) has been reported, but *ndr2* is the only gene to date that shows a strong L–R asymmetry of expression in the forebrain.

The functional significance of the asymmetric forebrain expression of *ndr2* is not known, but it may be related to the development of the habenular nuclei which are located just ventral to the epiphysis. Especially in amphibians, some fishes, and other animals but not in mice, these nuclei exhibit pronounced L–R asymmetry, with the left habenula divided into two lobes which together are larger than the single right lobe (Braitenberg and Kemali, 1970; Morgan *et al.,* 1973; reviewed by Harris *et al.,* 1996). Von Wollwaerth (1950) observed in newt embryos that reversal of handedness in the habenulae and visceral organs was correlated, implying common features in the left–right patterning mechanisms in different organs. The asymmetric expression of *ndr2* in both the forebrain and trunk of zebrafish embryos and the changes in these patterns in *flh* and *ntl* mutants provide support for this idea.

Relationships of Nodal Group Factors in Different Vertebrates

Neither Ndr1 nor Ndr2 can be identified as an ortholog of any nodal-related factor in another vertebrate. As mentioned above, sequence relationships and cysteine patterns show no consistent pattern. Considering expression and activity, Ndr1 is similar to nodal and Xnr1,2 in terms of mesoderm-inducing potential but differs in being expressed in a much more restricted region dorsally; also, unlike nodal and Xnr1, Ndr1 is not expressed, asymmetrically or otherwise, in the lateral plate. While Ndr2 is expressed in a pattern more similar to nodal and Xnr1, including asymmetric expression, its apparently direct neural-inducing ability is more closely related to the properties of Xnr3; however, it does not share the special cysteine pattern of the latter. This lack of linear orthology relationships is not exceptional among members of the TGF-β superfamily; for example, TGF-β itself has three known forms in mammals and two forms in *Xenopus,* of which only one, TGF-β2, is a clear ortholog (Rebbert *et al.,* 1990). Thus it apears that there has been functional reassortment among members of the TGF-β superfamily during vertebrate evolution.

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