Development 124, 1465-1472 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV6272

Left-right asymmetry of a *nodal*-related gene is regulated by dorsoanterior midline structures during *Xenopus* development

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SUMMARY

Development of asymmetry along the left-right axis is a critical step in the formation of the vertebrate body plan. Disruptions of normal left-right patterning are associated with abnormalities of multiple organ systems, including significant congenital heart disease. The mouse *nodal* gene, and its homologues in chick and *Xenopus*, are among the first genes known to be asymmetrically expressed along the left-right axis before the development of organ asymmetry. Alterations in the expression pattern of mouse *nodal* and the chick homologue (*cNR-1*) have been associated with defects in the development of left-right asymmetry and cardiac looping (Levin, M., Johnson, R. L., Stern, C. D., Kuehn, M. and Tabin, C. (1995) *Cell* 82, 803-814; Collignon, J., Varlet, I. and Robertson, E. J. (1996) *Nature* 381, 155-158; Lowe, L. A., Supp, D. M., Sampath, K., Yokoyama, T., Wright, C. V. E., Potter, S. S., Overbeek, P. and Kuehn, M. R. (1996) *Nature* 381, 158-161). Here, we show that the normal expression patterns of the *Xenopus nodal*-related gene (*Xnr-1*) are variable in a large population of embryos and that *Xnr-1* expression is altered by treatments that perturb normal left-right development. The incidence of abnormal *Xnr-1* expression patterns correlates well with cardiac reversal rates in both control and experimentally treated *Xenopus* embryos. Furthermore, dorsal midline structures, including notochord and/or hypochord and neural floorplate, regulate *Xnr-1* expression prior to the specification of cardiac left-right orientation by repression of *Xnr-1* expression in the right lateral plate mesoderm during closure of the neural tube. The correlation of *Xnr-1* expression and orientation of cardiac looping suggests that *Xnr-1* is a component of the left-right signaling pathway required for the specification of cardiac orientation in *Xenopus*, and that dorsal midline structures normally act to repress the signaling pathway on the right side of the embryo.

Key words: left-right asymmetry, heart situs, notochord, *nodal*, *Xnr-1*, *Xenopus*

INTRODUCTION

The development of left-right asymmetry is highly conserved throughout vertebrate species. One of the earliest morphologic indicators of left-right asymmetry is cardiac looping. Cardiac looping is the process by which the straight heart tube develops a rightward and dorsal bend, forming the anatomic relationships necessary for normal cardiac chamber and outflow tract development. Disruptions of normal left-right asymmetry are associated with cardiac defects in many species, and are a cause of significant morbidity and mortality in the human neonate. Despite the late morphologic appearance of left-right asymmetry, establishment of the left-right axis has been linked to the establishment of dorsal-ventral and anterior-posterior axes in the early embryo (Danos and Yost, 1995). The early establishment of the left-right axis and the later morphologic expression of this asymmetry suggests that there is a signaling pathway that communicates left-right axis information to the developing organ systems.

A molecular pathway potentially involved in the regulation of left-right asymmetry has recently been described in the chick (Levin et al., 1995). This pathway involves the sequential expression of several signaling molecules, including activin, sonic hedgehog (Shh) and the chick nodal-related molecule cNR-1, in an asymmetric pattern along the left-right axis. Disruption of this signaling pathway in chick by alterations in activin or *Shh* expression prior to *cNR-1* expression is associated with an approximately 50% incidence of reversed cardiac orientation, or cardiac randomization. However, a number of the early molecules in this proposed pathway, which include activin, activin receptors and sonic hedgehog, are not expressed asymmetrically along the left-right axis in other vertebrate species (Dohrmann et al., 1993; Hemmati-Brivanlou et al., 1992; Kondo et al., 1996; Ekker et al., 1995; Collignon et al., 1996; personal observation). In contrast, homologues of the chick *nodal*-related gene *cNR-1*, which is expressed asymmetrically in the left lateral plate mesoderm later in development, are also expressed asymmetrically in other vertebrate species.

Asymmetric, left-sided expression of *nodal*-related genes has been described in mice and *Xenopus* (Lowe et al., 1996; Collignon et al., 1996; Lustig et al., 1996). In the mouse,

asymmetric left-sided expression of *nodal* is apparent in the left lateral mesoderm by early somite stages, which precede cardiac looping. Altered expression patterns of *nodal* have been found in mice with mutations that result in abnormalities of cardiac situs. Mice homozygous for *inv*, a recessive mutation that alters embryonic turning and organ situs, exhibit abnormal patterns of *nodal* staining, ranging from right-sided staining associated with situs inversus (Lowe et al., 1996; Collignon et al., 1996), to a rostral truncation of staining associated with random or ambiguous cardiac looping (Collignon et al., 1996). Mouse embryos homozygous for the *iv* mutation have randomized left-right orientation (Seo et al., 1992; McGrath et al., 1992) and a variable pattern of *nodal* expression, with normal, right, bilateral and absent expression of *nodal* being represented in this population (Lowe et al., 1996). The normal expression pattern of the *Xenopus* homologue of *nodal*, *Xnr-1*, is similar to that described in chick and mouse, with asymmetric expression in the left lateral mesoderm during tailbud stages (Lowe et al., 1996; Lustig et al., 1996). These studies suggest conserved roles for the *nodal* gene and its homologues in vertebrate left-right patterning and in the determination of cardiac orientation.

The conserved left-right asymmetric expression of *nodal*-related genes and the association of altered expression of these genes with abnormalities in cardiac orientation in vertebrates implicates *nodal*-related genes in the establishment of normal left-right asymmetry. As described earlier, some candidate molecules for early regulation of *nodal* expression are asymmetrically expressed in the chick model; however, asymmetric left-right expression of these candidates has not been seen in other vertebrates. Thus, the mechanisms that regulate asymmetric *nodal*-related gene expression are unknown. In *Xenopus*, the establishment of normal left-right cardiac asymmetry is linked to normal dorsoanterior development (Danos and Yost, 1995). The work presented here examines the role of dorsoanterior structures in the regulation of the *Xenopus nodal*-related gene, *Xnr-1*. Specifically, we describe the expression pattern and variability of *Xnr-1* expression in a control population of *Xenopus* embryos, show that the left-right asymmetry of *Xnr-1* expression is altered by disruption of dorsoanterior development, identify embryonic structures required for the normal pattern of asymmetric *Xnr-1* expression and determine the developmental period during which asymmetric expression of *Xnr-1* is specified by repression in the right lateral plate mesoderm, both in embryos and in explants. The results indicate that asymmetric *Xnr-1* expression is correlated with cardiac orientation and suggest that expression in the lateral plate mesoderm is an intermediary between a negative regulatory signal from the midline anterior structures and the cardiac primordia.

MATERIALS AND METHODS

Xenopus embryos

Albino *X. laevis* females were induced to ovulate by injection of 50 units of pregnant mare's serum gonadotropin (Sigma) into the dorsal lymph sac followed by injection of 800 units of human chorionic gonadotropin (Sigma) 24 hours later. Eggs were expressed from the ovulating females and fertilized with minced testis from pigmented males suspended in a modified Ringer's solution at one-third strength (R/3). The fertilized eggs were dejellied in 2% cysteine, pH 8.0. and incubated in R/3 at either 15°C or 21°C. Embryos were staged for use in experiments according to Nieuwkoop and Faber (1967). Heart orientation was scored in embryos anesthetized with 0.05% benzocaine, all other interventions were performed in R/3. Embryos were fixed for in situ hybridization in MEMFA (Danos and Yost, 1995; Harland, 1991) for 1-2 hours and stored in methanol at −20°C.

Whole-mount in situ hybridization

Antisense RNA probes for *Xnr-1* were synthesized from a cDNA insert of *Xnr-1* in pBS, (Jones et al., 1995; provided by C. Wright), linearized with *Pst*I and transcribed with T7 RNA polymerase. Probes were labeled with digoxigenin-rUTP (Boehringer Mannheim Biochemical). RNA probe synthesis and whole-mount in situ hybridization were performed using a standard protocol for *Xenopus* embryos (Sive et al., 1995). Embryos were scored for *Xnr-1* expression and photographed. Selected embryos were embedded in paraffin and sectioned at 20-25 μm in the transverse plane, mounted on glass slides and photographed using light microscopy.

UV irradiation

Fertilized eggs were dejellied, placed on quartz slides in R/3 and irradiated for 14-16 seconds with an ultraviolet light at 254 nm within 30 minutes of fertilization (Scharf and Gerhart, 1980). Embryos were either fixed at stage 24-26 for in situ hybridization or scored at stage 42-46 for dorsoanterior development and direction of heart looping. Dorsoanterior development was scored by a standard Dorsoanterior Index (DAI) as follows: DAI 5 - normal appearing embryos; DAI 4 - reduced forehead, close set eyes; DAI 3 - shortened embryo, cyclopic; DAI 2 - severely microcephalic, no retinal pigment, DAI 0-1 - acephalic (Kao and Elinson, 1988; Danos and Yost, 1995).

Dorsal tissue and notochord extirpation

Two dorsal tissue extirpation methods were used. In broad dorsal tissue extirpations, anterior notochord and some anterolateral tissues were removed from embryos at stage 15 (early neural fold) and stage 20 (neural tube stage), as previously described (Danos and Yost, 1996). Narrow extirpations were performed at stage 15 by removing only anterior notochord, hypochord and directly overlying prospective neural floorplate. Because of neural tube closure, comparable narrow extirpations could not be done at stage 20. Embryos were either fixed at stage 24-26 for in situ hybridization or scored for cardiac orientation at stage 42-46.

Lateral mesoderm explants

Right and left lateral plate mesoderm, overlying ectoderm and underlying endoderm were explanted from embryos at stage 15-16 and 20-21, using a tungsten wire or eyebrow knife. In a subset of embryos, endoderm was removed from the explants and discarded. Explants were cultured in R/3 at 15° C, fixed when sibling embryos were at stage 24 and assayed for *Xnr-1* expression by whole-mount in situ hybridization.

Data analysis

Whole-mount in situ hybridzations were scored for pattern of *Xnr-1* expression and categorized as left (normal expression pattern), right (mirror-image expression pattern), bilateral (expression in lateral plate mesoderm on both right and left sides), or none (no staining in the lateral plate mesoderm). Explants were scored for presence of *Xnr-1* expression. Appropriate sibling controls were processed in parallel to ascertain normal assay function. Statistical significance between groups was tested using a standard z-test of independent proportions, accepting $P<0.05$ or smaller as significant difference.

RESULTS

Xnr-1 expression patterns and spontaneous cardiac reversals in control populations

Xnr-1 expression in a control population of embryos was determined by in situ hybridization with an antisense RNA probe (Jones et al., 1995). Embryos at varied developmental stages, ranging from stage 7 (blastula) to stage 38 (late tailbud) were analyzed for expression pattern. The earliest left-right asymmetric expression was seen at stages 17-19 (late neural fold to neural tube stages) in the left posterior region of the embryo, just lateral to midline structures. By stages 21-23, the expression of *Xnr-1* spread anteriorly just lateral to the midline (Fig. 1A,B) and, by stages 23-26, was prominent in the anterior portion of the left lateral mesoderm (Fig. 1C-E). In embryos with heavier staining, *Xnr-1* expression was occasionally seen on the right posterior aspect lateral to the notochord, but did not extend anteriorly or laterally into the lateral plate mesoderm on the right side. By stage 26-27, *Xnr-1* expression in the posterior region of the embryo decreased (Fig. 1F). Anterior expression of *Xnr-1* decreased by stage 28-30. Asymmetric staining patterns were not apparent later in development (through stage 38).

The variability in normal *Xnr-1* expression in a control population was assessed by whole-mount in situ hybridization in 324 embryos at stages 21 through 26. *Xnr-1* expression was left-sided and asymmetric, or 'normal' in 88% of the embryos. Variations of *Xnr-1* expression in the control population at these stages included right-sided expression only in approximately 1% of embryos, bilateral expression in 8% of embryos and no *Xnr-1* expression in 3% of the embryos assayed. As discussed later, this rate of abnormal *Xnr-1* expression patterns correlates well with a 3-8% rate of spontaneous cardiac reversals in untreated batches of *Xenopus* embryos (Danos and Yost, 1995; unpublished observations).

Altered *Xnr-1* expression patterns are correlated with diminished dorsoanterior development and randomization of cardiac orientation

In order to determine if left-right cardiac orientation is correlated with *Xnr-1* expression patterns in *Xenopus*, embryos were treated with UV irradiation. UV irradiation in the first cell cycle disrupts both dorsoanterior and left-right axis formation and has been shown to randomize cardiac orientation at a DAI score of 3 (Danos and Yost, 1995). UV-treated, partially ventralized embryos at stage 24-26 were scored for DAI and assayed for *Xnr-1* expression by in situ hybridization (Figs 2, 3). DAI 5, or treated embryos with normal dorsoanterior development, were found to have normal, asymmetric left-sided expression of *Xnr-1* in 86% of the cases, which is not significantly different from the control population (Figs 2, 3A). DAI 4 embryos had an increased incidence of abnormal *Xnr-1* expression, with only 50% of embryos exhibiting normal left-sided asymmetric expression, while 31% of embryos exhibited bilateral *Xnr-1* expression (Figs 2, 3B). At DAI 3, no normal *Xnr-1* expression was seen (Figs 2, 3C). 49% of DAI 3 embryos exhibited bilateral *Xnr-1* expression, and 51% had no detectable Xnr-*1* expression. DAI 2 embryos also had a high percentage of abnormal *Xnr-1* expression (Figs 2, 3D) and embryos at less than DAI 1 were predominately without *Xnr-1* expression at stage 24 (Fig. 2).

A normal, asymmetric *Xnr-1* expression pattern was characteristic of embryos with normal dorsoanterior development (DAI 5), while there was an increased incidence of abnormal and often symmetric expression patterns in embryos with lower DAI scores (Fig. 3A-D). The percentage of embryos exhibiting abnormal *Xnr-1* expression at DAI 3-5 correlated well with cardiac reversal rates obtained from sibling embryos scored for DAI and cardiac orientation at stage 42-46. In this group of embryos, cardiac reversal rates were 3% in an untreated control group (n=148); 2% in treated embryos at DAI 5 (n=63); 9% in DAI 4 embryos (n=22); and 48% in DAI 3 embryos (n=33). Cardiac orientation could not be scored in the majority of DAI 2 embryos and no cardiac structures were visible in DAI 0-1 embryos. Thus, diminished dorsoanterior development was correlated with an altered *Xnr-1* expression pattern, as well as altered cardiac orientation. Of note, at DAI 3, where *Xnr-1* expression was either bilateral or absent, cardiac orientation was randomized.

Normal *Xnr-1* expression is dependent on the presence of midline dorsal cells during neurulation

Extirpation of dorsoanterior structures during early neurulation results in cardiac randomization, while extirpation of these structures after completion of neurulation does not alter cardiac orientation (Danos and Yost, 1996). This suggests a developmental stage-dependent regulatory function of dorsoanterior structures in the establishment of cardiac orientation prior to heart formation and looping. In order to determine whether asymmetric *Xnr-1* expression is dependent on dorsal midline cells in a stage-specific manner, three series of dorsoanterior midline extirpations were performed.

Broad extirpations of anterior notochord and dorsoanterior structures were performed on embryos at stage 15 or stage 20 as previously described (Danos and Yost, 1996). Stage 15 extirpations resulted in predominately bilateral *Xnr-1* expression (Fig. 4A,C). In contrast, stage 20 extirpations of the same tissues did not alter normal asymmetric *Xnr-1* expression (Fig. 4B,C). In previous studies, broad extirpation of dorsal midline structures at stage 15 is associated with randomization of cardiac orientation, while extirpation at stage 20 does not perturb cardiac situs (Danos and Yost, 1996). To further define the essential dorsal structures required for left-right development, narrow extirpations of only notochord, overlying neural floor plate and hypochord were performed at stage 15, leaving somites and other more lateral structures intact. This technique of more limited extirpation is not possible at stage 20 because of the overlying neural tube. A 36% incidence of cardiac reversals was obtained when narrow extirpations were performed at early neural fold stages (n=59), consistent with an increased incidence of bilateral *Xnr-1* expression using this technique (Fig. 4C).

These experiments demonstrate that removal of dorsoanterior structures, including notochord, has a developmental stage-dependent effect on *Xnr-1* expression as well as cardiac orientation. Removal of the dorsoanterior midline structures at stage 15, whether through a broad extirpation of tissue or a narrow extirpation of just notochord and overlying neural floor plate, is associated with bilateral expression of *Xnr-1* (Fig. 4C) and an increased incidence of cardiac reversals. Removal of analogous structures at stage 20 has no effect on normal asymmetric *Xnr-1* expression (Fig. 4B,C) or cardiac orientation.

Asymmetric *Xnr-1* expression is specified by repression in the right lateral plate mesoderm

The results of dorsal midline extirpation experiments support the hypothesis that notochord and/or neural floorplate and hypochord are involved in the regulation of *Xnr-1* expression. In order to investigate the dependence of *Xnr-1* expression in both the right and left lateral mesoderm on midline dorsal structures, lateral plate mesoderm was explanted from both right and left sides of the embryo, cultured and assayed for *Xnr-1* expression. Since endoderm has been shown to have a role in induction of cardiac primordia in *Xenopus* embryos (Nascone and Mercola, 1995), these experiments were performed with and without endoderm to investigate any additional regulatory effect of endoderm on *Xnr-1* expression. In contrast to the asymmetric expression pattern in untreated embryos, *Xnr-1* was expressed in both left and right lateral plate mesoderm explants obtained at stage 15 (Fig. 5A,B; Table 1), with no statistically significant difference in expression between the two groups. At stage 15, *Xnr-1* expression in left lateral plate mesoderm was slightly enhanced by the presence of endoderm (P<0.10) and *Xnr-1* expression in right lateral plate mesoderm was unaffected by the presence of endoderm. These results suggest that the presence of dorsoanterior midline structures inhibits the expression of *Xnr-1* in the right lateral plate mesoderm at later stages in development and that endoderm is not required after stage 15 for asymmetric *Xnr-1* expression.

To assess the timing of specification of *Xnr-1* expression in the lateral plate mesoderm, left and right lateral plates were

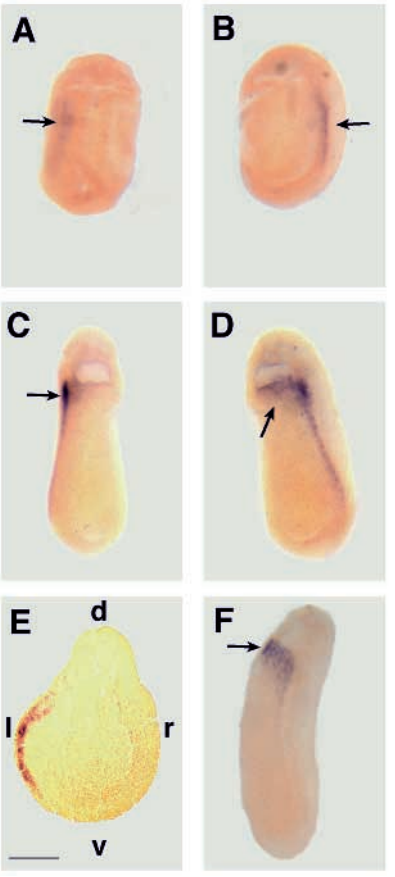

Fig. 1. *Xnr-1* expression in control embryos, assayed by whole-mount RNA in situ hybridization. Purple color indicates expression of *Xnr-1* RNA. (A) Expression in the left lateral middle and posterior region of stage 21 embryo (dorsal view). (B) Left lateral view of *Xnr-1* expression pattern in the same stage 21 embryo. (C) Dorsal view of a stage 24 embryo, and (D) left lateral view of the same embryo. *Xnr-1* is expressed in the left anterior lateral region, sweeping from the somites to the cardiac primordia along the dorsoventral axis. Posteriorly, *Xnr-1* is expressed just to the left of somites. (E) *Xnr-1* expression is apparent in the left lateral plate mesoderm, in a transverse section through stage 24 embryo. Scale bar, 0.2 mm. (F) *Xnr-1* expression becomes limited to the left lateral plate mesoderm, fading posteriorly, in a stage 26 embryo. Arrows indicate *Xnr-1* expression. Anterior at top of page except in E, where d, dorsal; v, ventral; l, left; r, right.

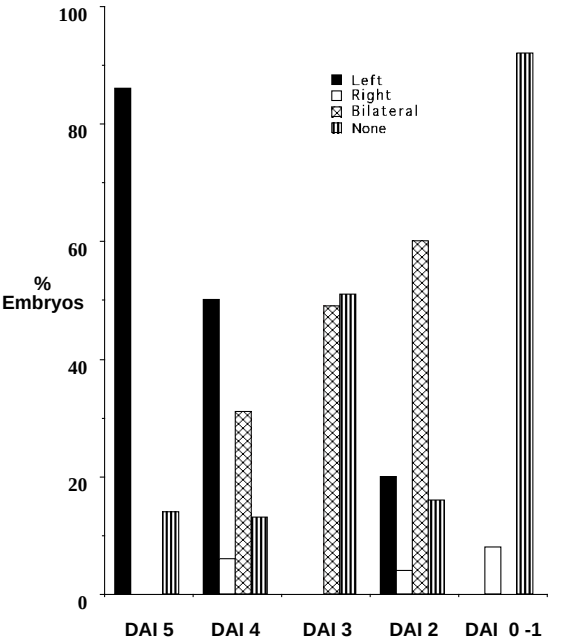

Fig. 2. *Xnr-1* expression patterns in UV-treated embryos is dependent on dorsoanterior development as scored by the Dorsoanterior Index (Kao and Elinson, 1988; Danos and Yost, 1995). As dorsoanterior development was diminished, the frequency of normal (left-sided, black bars) *Xnr-1* expression patterns decreased. The predominant classes of aberrant *Xnr-1* expression were bilateral expression (hatched bars) and no expression (vertical lined bars). For each DAI, DAI 5 (n=21), DAI 4 (n=16), DAI 3 (n=35), DAI 2 (n=25), DAI 0-1 (n=12), embryos were fixed and analyzed for *Xnr-1* RNA expression patterns by whole-mount in situ hybridization.

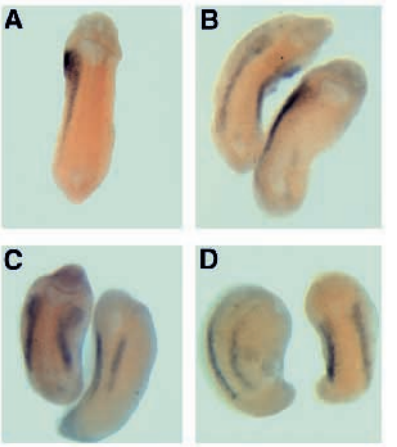

Fig. 3. Representative *Xnr-1* expression patterns in *Xenopus* embryos ventralized by UV irradiation. (A) Normal left expression of *Xnr-1* in a DAI 5 embryo. (B) Bilateral expression in the top embryo and left expression in the lower embryo, both at DAI 4. (C) Bilateral expression in two embryos at DAI 3. (D) Bilateral expression in embryos at DAI 2. Dorsal views, anterior at the top in all panels.

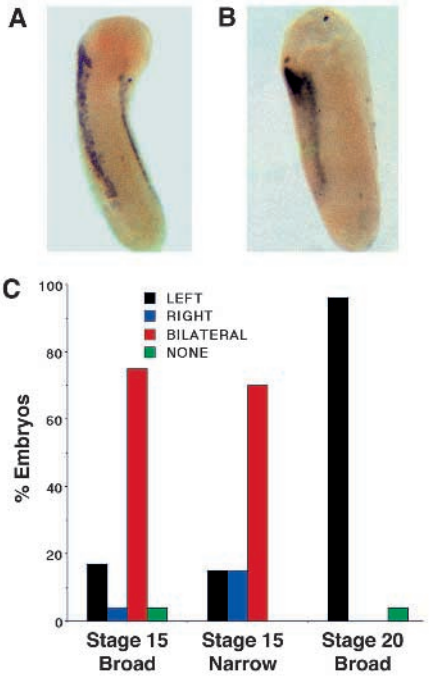

Fig. 4. *Xnr-1* expression pattern in *Xenopus* embryos after extirpation of midline dorsoanterior structures depends on the stage of extirpation. (A) Bilateral *Xnr-1* expression in a stage 24 embryo from which dorsal cells had been extirpated at stage 15. (B) Normal left-sided *Xnr-1* expression in a stage 24 embryo from which dorsal cells had been extirpated at stage 20. Dorsal view, anterior at top in both panels. (C) *Xnr-1* expression patterns are predominately bilateral (red bars) in embryos from which dorsal midline cells were extirpated at stage 15, either in broad extirpations (75% bilateral, s.d.=8%; *n*=24) or in narrow extirpations (72% bilateral, s.d.=8%; *n*=27) focused on notochord and prospective floorplate (see Methods), but normal (black bars) in embryos from which dorsal midline cells were extirpated at stage 20 (95% left only, s.d.=8%; *n*=26). Untreated control embryos from the same experiments demonstrated *Xnr-1* expression on the left only in 86% of cases (s.d.=11%; *n*=52). Results from broad extirpations were statistically identical to those from narrow extirpations at stage 15 ($z=0.375$, $P>0.16$) and were significantly different from those at stage 20 ($z=5.75$, $P<0.001$).

Table 1. *Xnr* expression in lateral plate mesoderm

	Left LPM		Right LPM	
Explant	% with *Xnr-1* expression	*n*	% with *Xnr-1* expression	*n*
Stage 15 + Endo	95*	19	89	19
Stage 15 –Endo	75	20	74	19
Stage 20 + Endo	95	21	11**	18
Stage 20 –Endo	94	16	8**	13

LPM, lateral plate mesoderm; Endo, endoderm.
*$P<0.10$ vs left LPM –Endo; **$P<0.001$ vs left LPM –Endo.

explanted with and without endoderm at stage 20 and assayed for *Xnr-1* expression at stage 24. Left explants expressed *Xnr-1* (Fig. 5C). In contrast, very few of the right lateral plate mesoderm explants expressed *Xnr-1* (Fig. 5D; Table 1). The difference between left and right lateral plate mesoderm was

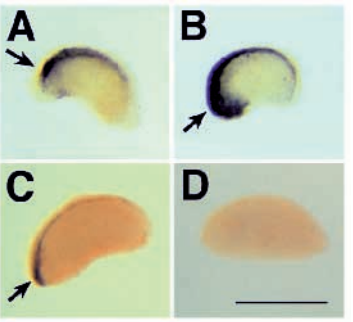

Fig. 5. *Xnr-1* asymmetric expression is specified in stage 20 lateral plate mesoderm. (A) Left lateral mesoderm explanted at stage 15 (B) Right lateral mesoderm explanted at stage 15. (C) Left lateral mesoderm explanted at stage 20. (D) Right lateral mesoderm explanted at stage 20. Note no *Xnr-1* expression in (D) and strong *Xnr-1* expression in (A-C). All explants were cultured until sibling embryos were at stage 24, and then fixed and assayed in parallel by in situ hybridization for *Xnr-1* expression. Scale bar, 1 mm.

statistically significant ($P<0.001$, using the z-test of independent proportions). Endoderm had no effect on *Xnr-1* expression in either group at stage 20 (Table 1). These data are consistent with the hypothesis that the inhibitory effect of dorsal midline structures on *Xnr-1* expression in the right lateral plate mesoderm is stage-dependent, and occurs after stage 15 (early neural fold) and before stage 20 (neural tube stage). Thus, the specification of left-right asymmetric *Xnr-1* expression in lateral plate mesoderm occurs by stage 20 and coincides with the developmental period during which specification of cardiac left-right orientation is dependent on midline structures (Danos and Yost, 1996).

DISCUSSION

Left-right asymmetric expression of *nodal*-related genes has been described in embryos of three vertebrate species, mouse, chick and frog, and appears to be highly conserved (Levin et al., 1995; Collignon et al., 1996; Lowe et al., 1996; Lustig et al., 1996; this study). Here, alterations of the normal left-right asymmetric expression patterns of a *nodal*-related gene in *Xenopus*, *Xnr-1* (Lowe et al., 1996; Lustig et al., 1996), are shown to be correlated with alterations in cardiac left-right development. Furthermore, results of extirpation and explant studies in *Xenopus* embryos indicate that the regulation of asymmetric *Xnr-1* expression is dependent on signals from midline cells and that the right lateral plate mesoderm becomes specified during neurulation to repress *Xnr-1* expression.

Asymmetric *Xnr-1* expression and specification of cardiac left-right orientation

Our description of *Xnr-1* left-right asymmetric expression patterns in *Xenopus* embryos, with expression in the left lateral plate mesoderm during tailbud stages, is consistent in general with others (Lowe et al., 1996; Lustig et al., 1996). However, examination of a large population of embryos revealed variability. Less than 1% of the population exhibited a reversal of the normal expression pattern. A larger percentage (11%) exhibited loss of normal asymmetric *Xnr-1* expression: either

bilateral *Xnr-1* expression in both the left and right lateral plate mesoderm or bilateral absence of *Xnr-1* expression in the lateral plate mesoderm. In chick, bilateral expression or absence of *cNR-1* expression has been correlated with randomization of cardiac orientation (Levin et al., 1995). Similarly, mouse *iv/iv* homozygotes have randomized cardiac orientation (Seo et al., 1992; McGrath et al., 1992) and a high incidence of either symmetric or absent expression patterns of *nodal* (Lowe et al., 1996).

If cardiac left-right orientation is dependent on the orientation of asymmetric *nodal* expression, with either bilateral expression or absence of expression resulting in randomization of cardiac situs, then the incidence of cardiac reversals in a population can be estimated as follows:

$$\text{predicted cardiac reversal rate} = \frac{r + (b + n)/2}{N}$$

where r = expression only in the right lateral mesoderm; b = bilateral expression in both left and right lateral plate mesoderm; n = no expression in the lateral plate mesoderm; and N = population size. The *Xnr-1* expression patterns in a large *Xenopus* population correlates well with the observed spontaneous cardiac reversal rates of 3-8% in untreated *Xenopus* embryos (Danos and Yost, 1995; unpublished observations), and is also predictive of observed cardiac reversal rates in experimentally manipulated embryos with altered dorsoanterior development (Fig. 6).

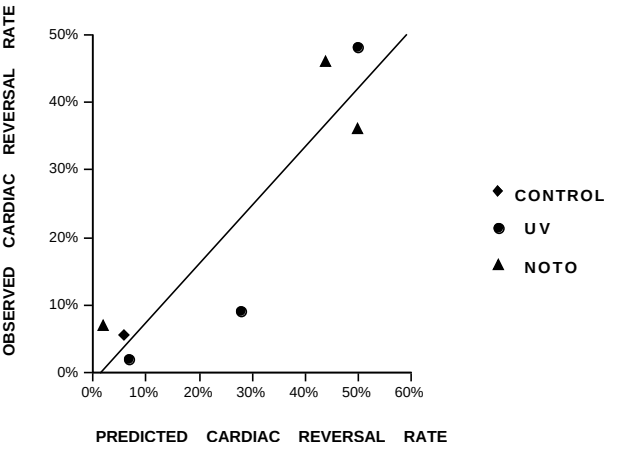

Fig. 6. Comparison of predicted cardiac reversal rates (based on the equation as defined in the text) versus observed cardiac reversals in control embryos (diamonds), UV-treated embryos (DAI 5-DAI 3) (circles), and midline extirpations (noto) (triangles). The predicted cardiac reversal rates in the data set correlate positively with the observed cardiac reversal rates as defined by the equation y=0.866x - 0.012, r^2=0.839.

If the determination of cardiac left-right orientation is dependent on normal *Xnr-1* asymmetric expression, then early experimental interventions that result in randomization of cardiac orientation in *Xenopus* should also alter the expression patterns of *Xnr-1*. This is the case in four separate interventions. First, *Xnr-1* expression is altered in embryos with diminished dorsoanterior development (Figs 2, 3). As the DAI score is decreased to 3, there is an increased incidence of bilateral *Xnr-1* expression patterns. More severely ventralized embryos lacked *Xnr-1* expression, which probably reflects the severe disruption of axis formation and the loss of lateral plate mesoderm. Second, *Xnr-1* expression is bilateral and cardiac orientation is randomized in embryos from which midline structures, including notochord, hypochord and floorplate, were extirpated at stage 15 (Fig. 4). Third, injection of RGD peptides into early gastrula randomizes left-right orientation (Yost, 1992) and results in bilateral *Xnr-1* expression (data not shown). Fourth, early perturbations in the maternal Vg1 signaling pathway result in randomization of left-right orientation and bilateral expression of *Xnr-1* (Hyatt et al., 1996). Thus, there is a strong correlation between altered *Xnr-1* expression and randomization of cardiac left-right orientation in *Xenopus.* This suggests two possibilities: one, that asymmetric *Xnr-1* expression in the lateral plate regulates specification of cardiac left-right orientation; or two, that asymmetric *Xnr-1* expression and cardiac left-right orientation are parts of separate, parallel pathways that are both regulated by a common factor. Recently, bilateral expression of the *nodal*-related gene *cNR-1* in the chick has been shown to alter cardiac orientation (M. Levin and C. Tabin, personal communication) implicating the *nodal*-related genes in the regulatory pathway of cardiac left-right determination. Overexpression of *Xnr-1* in 4-cell *Xenopus* embryos (Jones et al., 1995) or in single cells on the left or right side of 8- to 16-cell embryos (data not shown) results in hyperdorsalization. These results reflect an earlier developmental role in dorsoventral axis formation (Jones et al., 1995) but complicate the assessment of a direct role for *Xnr-1* in cardiac left-right orientation, since cardiac orientation is dependent on dorsoventral development (Danos and Yost, 1995) and is perturbed in embryos with secondary axes (Hyatt et al., 1996).

Specification of autonomous *Xnr-1* asymmetric expression

It is not known what signals in the early embryo are necessary for the establishment of asymmetric *nodal*-related gene expression in the lateral plate mesoderm, such that *Xnr-1* expression is on in the left and off in the right. Here, extirpation and explant culture experiments address two issues: what cell interactions are necessary for specification of *Xnr-1* asymmetric expression and when does the lateral plate become specified, as a result of these interactions, for asymmetric expression of *Xnr-1*. Extirpation of dorsal midline cells at stage 15 results in altered expression of *Xnr-1* (Fig. 4). Somewhat surprisingly, when midline cells are removed, *Xnr-1* is expressed ectopically in the right lateral plate mesoderm, so that *Xnr-1* expression becomes bilateral. The timing of the midline extirpations is crucial; extirpation at stage 15 results in bilateral *Xnr-1* expression and randomized cardiac asymmetry, whereas extirpation of the same midline cells at stage 20 does not (Fig. 4). Broad extirpations at stage 15 gave no increase in incidence of altered *Xnr-1* expression patterns relative to narrow extirpations at the same stage, implicating the most medial structures, the neural floor plate, notochord and hypochord, in the regulation of asymmetric *Xnr-1* expression.

Explantation of the lateral plate mesoderm and culturing in the absence of the rest of the embryo gave results that concur with the stage-dependent influence of midline structures on the expression of *Xnr-1* in the right lateral plate mesoderm. Both left and right lateral plate mesoderm autonomously expressed

Xnr-1 when explanted at stage 15 and grown in culture. In contrast, when explanted at stage 20, right lateral plate mesoderm did not express *Xnr-1*. Although the endoderm slightly improved the rate of expression of *Xnr-1* in the left lateral plate mesoderm at stage 15, endoderm appears to have no major role in the regulation of *Xnr-1* in this assay system (Table 1). The positive effect of endoderm in the early left lateral plate explants may be due to an increase in the overall health of the explant as opposed to a specific regulatory effect.

The extirpation and explantation results suggest that a negative regulatory signal is emitted from the midline between stages 15 and 20 to suppress expression of *Xnr-1* in the right, but not left, lateral plate mesoderm. Since lateral plate mesoderm on both sides of the embryo expresses *Xnr-1* in the absence of midline cells, it is likely that they receive an earlier inductive signal to initiate the capability of *Xnr-1* expression, which is then countered on the right side by an antagonistic signal from the midline. Explantation of lateral plate mesoderm at stage 15 or extirpation of midline structures at stage 15 separates the right lateral plate mesoderm from the proposed suppressing signal emitted from the midline and allows subsequent ectopic expression of *Xnr-1*.

Model of sequential signaling from dorsal midline cells to ventral cells

Previous embryological and genetic experiments in *Xenopus* and zebrafish indicated that dorsal midline cells are necessary for normal cardiac left-right orientation (Danos and Yost, 1995, 1996). Given the geometry of the embryo, it is not clear how signals from the dorsal midline are transmitted to cardiac precursor cells at the ventral midline. The results from the extirpation and explantation studies here indicate that the stage-dependent effect of midline structures on *Xnr-1* expression is concurrent with or slightly precedes the stage-dependent effects of these structures on the specification of cardiac orientation (Danos and Yost, 1996). Midline cells regulate both asymmetric *Xnr-1* expression and cardiac orientation between stages 15 and 20. The lateral mesoderm is positioned between the dorsal midline and the ventral midline, and might serve as an intermediary for passing signals between cells on opposite sides of the embryo. Thus, we propose that signals pass from the midline, possibly via somitic tissue, to the lateral plate to specify *Xnr-1* expression. Once *Xnr-1* asymmetry is specified, it can then either directly or indirectly signal the heart primordia to specify cardiac left-right orientation. In this model, cardiac primordia that are presented with either bilaterally symmetric expression of *Xnr-1* or with no *Xnr-1* expression would not be specified for left-right orientation and, as a population, would be randomized for left-right orientation.

Although left-right body axis formation is initiated early in development (Danos and Yost, 1995; Yost, 1991, 1995; Hyatt et al., 1996), the left-right signaling pathway is probably dependent on subsequent checkpoint regulation by the midline cells at later stages of development and thus can be altered by experimental manipulations until the point of specification of left-right orientation within an organ primordium. This checkpoint regulation would allow for consistent alignment of the left-right axis in each of many organ primordia relative to the dorsal-ventral and anterior-posterior axes. Little is known about the mechanisms by which developing organ systems, including the heart, respond to these left-right signals to generate morphologic asymmetry.

In summary, the variability in left-right expression of *Xnr-1* in a untreated populations is consistent with observed rates of spontaneous cardiac reversals. Four experimental interventions in early embryos, known to cause cardiac randomization in *X. laevis*, also alter *Xnr-1* expression, with a resulting increase in bilateral expression patterns. The molecular nature of the signals from the notochord and neighboring midline structures that are necessary for the specification, during neurula stages, of subsequent *Xnr-1* asymmetric expression is currently being investigated.

The authors thank M. L. Condic for suggestions on data analysis and comments on the manuscript, Brian Hyatt for general discussions regarding left-right asymmetry, Judy Liddell for technical assistance, Bruce Lingren for advice on statistical analysis, and K. Sampath and C. Wright for *Xnr-1* DNA. We would also like to thank M. Levin, K. Sampath, C. Tabin and C. Wright for sharing results prior to publication. J. L. L. was supported by fellowships from Variety Club Children's Association and the American Academy of Pediatrics. M. C. D. was supported by a NSF-RTG predoctoral traineeship and a U. of M. Dissertation Fellowship. This work was supported in part by a Grant-In-Aid from the American Heart Association and was done in part during the tenure of an Established Investigatorship from the American Heart Association to H. J. Y.

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(Accepted 1 February 1997)