Development 121, 1467-1474 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

Linkage of cardiac left-right asymmetry and dorsal-anterior development in *Xenopus*

Maria C. Danos and H. Joseph Yost*

Department of Cell Biology and Neuroanatomy, 4-135 Jackson Hall, 321 Church Street SE, University of Minnesota, Minneapolis, MN 55455, USA

*Author for correspondence (e-mail: yostx001@staff.tc.umn.edu)

SUMMARY

The left-right body axis is defined relative to the dorsal-ventral and anterior-posterior body axes. Since left-right asymmetries are not randomly oriented with respect to dorsal-ventral and anterior-posterior spatial patterns, it is possible that a common mechanism determines all three axes in a coordinate manner. Two approaches were undertaken to determine whether alteration in dorsal-anterior development perturbs the left-right orientation of heart looping. Treatments known to decrease dorsal-anterior development in *Xenopus laevis*, UV irradiation during the first cell cycle or *Xwnt-8* DNA injections into dorsal blastomeres, caused an increase in cardiac left-right reversals. The frequency of left-right reversal was correlated with the severity of dorsal-anterior perturbation and with the extent of anterior notochord regression. Injection of *Xwnt-8* DNA into dorsal midline cells resulted in decreased dorsal-anterior development and a correlated increase in cardiac left-right reversals. In contrast, injection of *Xwnt-8* DNA into cardiac progenitor blastomeres did not result in left-right reversals, and dorsal-anterior development and notochord formation were normal. Disrupting development of dorsal-anterior cells, including cells that give rise to the Organizer region and the notochord, results in the randomization of cardiac left-right asymmetry. These results suggest dorsal-anterior development and the regulation of left-right orientation are linked.

Key words: axis formation, left-right asymmetry, cardiac development, situs inversus, *Xenopus*

INTRODUCTION

The vertebrate body plan develops along three geometric axes: anterior-posterior, dorsal-ventral, and left-right. Left-right asymmetries are not random with respect to the dorsal-ventral and anterior-posterior axes, suggesting that the mechanisms determining all three body axes might be linked. In sea urchin embryos, lineage tracing and cell separation experiments indicate that left-right asymmetry is specified with respect to and coincident with dorsal-ventral axis specification (McCain and McClay, 1994). In vertebrates, the left-right axis is most evident in the heart and viscera, and the orientation of left-right cardiac asymmetry is highly conserved (Burggren, 1988). Examples in humans and mice indicate that left-right axis formation may be vital to the organism since congenital heart defects and early death often accompany problems in left-right organization (Brueckner et al., 1991; Casey et al., 1993; Horwich and Brueckner, 1993; Yokoyama et al., 1993). Since the left-right axis is geometrically defined with respect to the other two axes, it might be developmentally influenced by mechanisms determining anterior-posterior and dorsal-ventral axes.

Most embryonic structures are symmetric across the midline in early development, and only display left-right asymmetries well after dorsal-ventral and anterior-posterior differences are visible (reviewed by Brown and Wolpert, 1990). However, left-right axial information is set up early in development, well before its morphological expression is discernible (reviewed by Yost, 1994). In *Xenopus laevis* the heart forms from two primordia that move to the ventral midline during gastrula and early neurula stages and fuse to form a symmetric cardiac tube. The cardiac tube then loops, breaking symmetry and giving rise to an S-shaped organ in the tadpole stages (Nieuwkoop and Faber, 1967). The left-right orientation of the heart is dependent upon the extracellular matrix lining the blastocoel roof of early gastrulae and appears to be transmitted to the cardiac and visceral primordia as they move across this matrix to the ventral midline. Experimental perturbations of the matrix during early gastrula stages by microsurgical wounding, treatment with RGD peptides or heparinase, result in random orientations of the heart and gut (Yost, 1992). During early neurula stages, when the pre-cardiac mesoderm cells move across the matrix to the ventral midline, proteoglycan synthesis is necessary for looping of the cardiac tube, which occurs 1.5 days later in development (Yost, 1990). Thus, early events in the embryo appear to establish the left-right embryonic axis well before the cardiac tube is formed.

Results presented here indicate that orientation of cardiac left-right asymmetry is coupled with dorsal-anterior development. The dorsal-ventral and anterior-posterior embryonic

axes are established during gastrulation by signals from cells at the dorsal midline (for reviews, Spemann, 1938; Slack et al., 1992). During late gastrulation, the mesoderm cells on the dorsal midline form the notochord. During neurulation, signals from the notochord specify cell fates along the anterior-posterior axis of the neural tube (Hemmati Brivanlou et al., 1990) and the dorsal-ventral axis of both the neural tube (Hatta et al., 1991; Krauss et al., 1993; Yamada et al., 1993; Roelink et al., 1994) and the somites (Dietrich et al., 1993; Halpern et al., 1993; Pourquié et al., 1993).

The results presented here suggest a novel role for dorsal-anterior midline cells: determining the orientation of left-right cardiac asymmetries during development. Perturbation of dorsal-anterior development, leading to diminished anterior-dorsal structures and loss of anterior notochord, causes reversals in the orientation of cardiac left-right asymmetry.

MATERIALS AND METHODS

Embryos

Xenopus laevis pigmented females (Xenopus I, Ann Arbor, MI) were induced to ovulate by injection into the dorsal lymph sac of 50 units of pregnant mare's serum gonadotropin (Sigma), followed by 800 units of human chorionic gonadotropin (Sigma) 24 hours later. Eggs were stripped from the ovulating females and fertilized with a minced testis suspension in one third strength modified Ringers containing 50 μg/ml gentimycin sulfate, subsequently denoted as R/3. The fertilized eggs were dejellied in 2% cysteine, pH 8.0. All embryo incubations and operations were done in R/3 unless otherwise noted. Embryos were cultured at either 21°C or 15°C and were staged according to Nieuwkoop and Faber (1967). Heart orientation was scored in anesthetized embryos. Embryos and tadpoles were fixed in MEMFA (0.1 M Mops, pH 7.4; 2 mM EGTA; 1 mM $MgSO_4$; 3.7% formaldehyde) for 2 hours and stored in methanol at −20°C for in situ hybridization or immunohistochemistry. To photograph hearts, embryos were stained with MF20 (gift from David Bader) (González-Sánchez and Bader, 1984) according to the whole mount immunohistochemistry protocol of Hemmati-Brivanlou and Harland (1989), and epidermis was removed from the ventral trunk of the embryos.

UV irradiation

Fertilized eggs were dejellied and placed vegetal hemispheres down on quartz slides in R/3 within 25 minutes of fertilization and irradiated with a ultraviolet (UV) mineralite lamp at 254 nm for 15-30 seconds (Scharf and Gerhart, 1980). The embryos were not disturbed until the first cleavage had occurred and were cultured until they were ready for scoring of DAI and heart looping. Asymmetry of the gut was not scored in these or any of the subsequent experiments since it does not complete development in treated embryos. A description of the DAI scale follows: DAI 5, normal in all external respects; DAI 4, reduced forehead, eyes smaller and sometimes joined; DAI 3, eyes fused or cyclopic, at least some retinal pigment visible; DAI 2, no visible retinal pigment, at least one otic vesicle present (Scharf and Gerhart, 1983; Kao and Elinson, 1988).

Embryo injections

Four-cell stage embryos were transferred into a solution of 5% ficoll (Sigma) in R/3. Prospective dorsal blastomeres are smaller and pigmented more lightly than their ventral counterparts at this stage (Nieuwkoop and Faber, 1967), and can be readily distinguished. The two dorsal blastomeres were each injected with 100 pg of CSKA-$X8^{myc}$ (*Xwnt-8*) DNA plasmid, or as a control the CSKA-pTCAT DNA construct, or water (Christian and Moon, 1993). For injections into prospective heart blastomeres, 100 pg of DNA was injected into each of the two lateral blastomeres on the third tier adjacent to the dorsal midline (C2 blastomeres) in 32-cell stage embryos with regular cleavage patterns. For midline cell injections, each of the two dorsal midline blastomeres on the third tier (C1 blastomeres) in 32-cell stage embryos were injected. For lineage labelling, 100 pg of CSKA-$X8^{myc}$ DNA and 15 ng of rhodamine dextran (polyanionic, 70×10^3 M_r from Molecular Probes in Eugene, OR) were injected per blastomere. After injections, the embryos were transferred to R/3 and incubated until control embryos reached stages 42 to 45 for scoring heart orientation.

Whole-mount in situ hybridizations

RNA probes for collagen II, labelled with digoxigenin-rUTP (Boehrigner Mannheim Biochemical), were synthesized from the p7XK500 plasmid (Amaya et al., 1993). This plasmid was linearized with *Xho*I and transcribed with T7 polymerase for the antisense probe or linearized with *Kpn*I and transcribed with Sp6 polymerase for the sense control. RNA probe synthesis and whole-mount in situ hybridization were performed as described by Harland (1991), with the exception of omitting the proteinase K step. Embryos were made transparent in benzyl benzoate/benzyl alcohol (2:1) and photographed.

RESULTS

UV irradiated embryos lack dorsal-anterior structures and have disturbed left-right development

To obtain embryos defective in dorsal-anterior structures, fertilized eggs were briefly treated with UV radiation, and grown to stages 42-45. Treatment of the vegetal pole of *Xenopus* embryos with UV radiation during the first cell cycle blocks formation of microtubule arrays in the embryo. This prevents cortical rotation and embryos fail to develop dorsal-anterior structures such as head, central nervous system, notochord, and somites (reviewed by Gerhart et al., 1989). External deficiencies in dorsal-anterior structure can be classified into a dorsal-anterior index (DAI) (Kao and Elinson, 1988). A short exposure time (15-30 seconds) to UV light resulted in a heterogeneous population of embryos ranging from DAI 5 (normal embryos) to DAI 0 (ventralized, radial embryos). Embryos at DAI 2 or lower had either a severely diminished heart or no discernible heart (319 of 322 embryos), so left-right cardiac asymmetry was scored for embryos at DAI 3 or above. Fig. 1 depicts the hearts and the external morphologies of normal (DAI 5) and slightly dorsal-anterior reduced (DAI 4) embryos obtained from UV treatment. In normal heart orientation (Fig. 1A; DAI 5 embryo), the ventricle is on the embryo's left and the outflow tract on the embryo's right; the atrium is positioned dorsally and leads into the ventricle from the embryo's left side. In contrast, some embryos derived from UV irradiation displayed reduced dorsal-anterior development (compare Fig. 1D with 1C) and reversal of the cardiac left-right orientation (Fig. 1B); the ventricle is on the embryo's right, the outflow tract is on the embryo's left, and the atrium is located dorsally and leads into the ventricle from the embryo's right side.

Left-right reversals were correlated with UV-induced loss of dorsal-anterior structures. Embryos that had lower DAIs showed the highest frequencies of reversed hearts (Fig. 2). Embryos with a DAI of 4 showed a 22% reversal frequency, while a decrease in dorsal-anterior structures to DAI 3 resulted

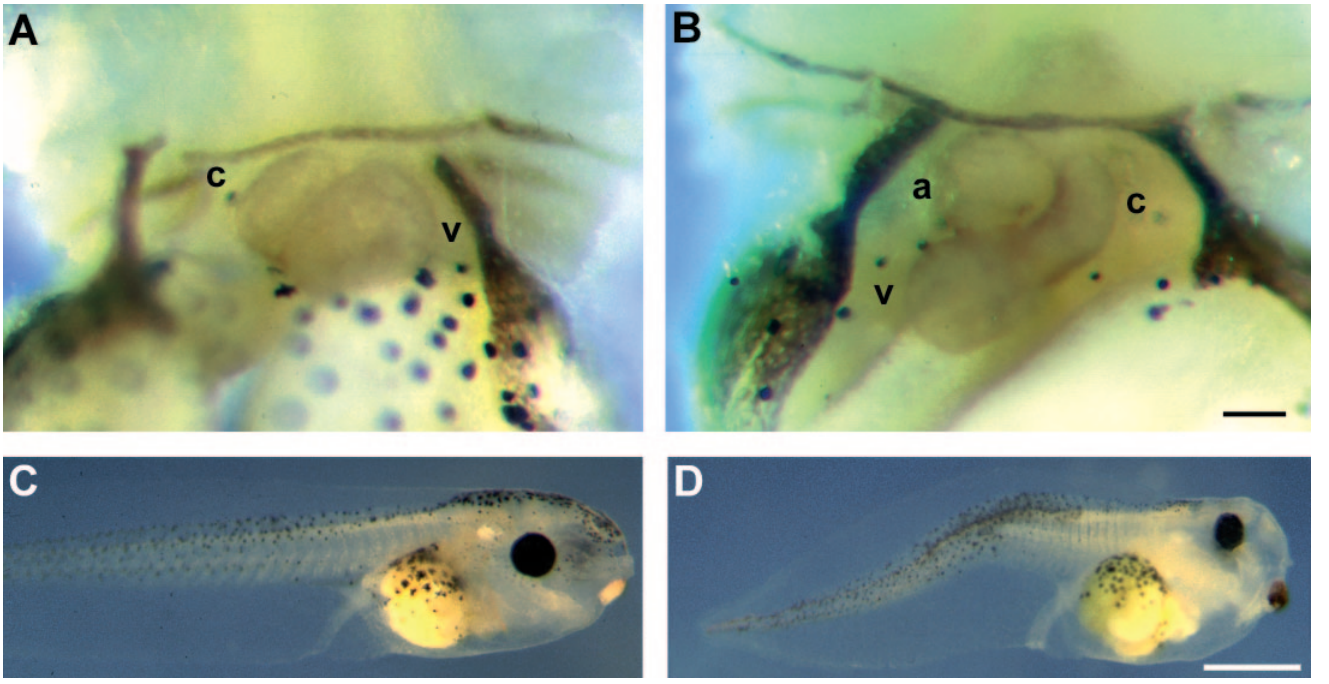


Fig. 1. Embryos derived from UV treatment, shown at stage 45. (A) Ventral view of a normal heart in a DAI 5 embryo. (B) Ventral view of a left-right reversed heart in a DAI 4 embryo. Ventricle (v), atrium (a) and conus or outflow tract (c) are indicated; hearts were stained with MF20 antibody and epidermis was removed. (C,D) Lateral view of a DAI 5 embryo and a DAI 4 embryo for comparison of dorsal-anterior development. Scale bars, 0.1 mm (A and B); 1.0 mm (C and D).

in a 45% reversal frequency. The DAI 5 class served as an internal control for this experiment; UV treated DAI 5 embryos and untreated embryos (also DAI 5) both had basal frequencies of cardiac reversals (3% and 1%, respectively). In an additional control group, embryos treated with UV irradiation late in the first cell cycle, after the cortical rotation had occurred, developed normal dorsal anterior structures (DAI 5) and normally oriented cardiac left-right asymmetries (29 of 29). Thus, there was a striking correlation between the frequency of cardiac left-right reversals and the extent of dorsal-anterior deficiencies due to perturbation of cortical rotation during the first cell cycle.

Misexpression of *Xwnt-8* alters cardiac left-right orientation

UV irradiation inhibits dorsal-anterior development by blocking the cortical rotation that occurs during the first cell cycle. We wanted to diminish dorsal-anterior development using another method; one that occurs later in development than UV treatment, that can be targeted to specific cell lineages, and that probably has an effect through a different mechanism. *Xwnt-8* ectopic expression was utilized in these experiments to assess the effects of diminished dorsal-anterior structures on heart orientation. *Xwnt-8* is a growth factor inducible agent that is thought to be involved in signaling the ventral-lateral mesoderm pattern in *Xenopus* (Christian et al., 1991; Christian and Moon, 1993). Ectopic expression of *Xwnt-8* in dorsal cells transcribed from an injected DNA plasmid expression construct after the mid-blastula transition (post-MBT), results in anterior defects and a deleted or aberrant notochord (Christian and Moon, 1993). This data suggests that misexpression of *Xwnt-8* in cells of the 'Organizer' field,

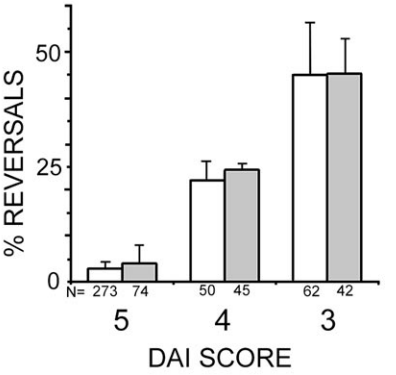


Fig. 2. The frequency of cardiac left-right reversal is correlated to the extent of dorsal-anterior deficiencies. The percentage of cardiac reversals was scored for each DAI from UV-treated embryos (white bars) and embryos in which *Xwnt-8* was injected into dorsal cells (grey bars). The number of embryos scored in each category are shown beneath its abscissa.

during the time when endogenous *Xwnt-8* is expressed in ventral-lateral cells, blocks the normal differentiation of head and notochord (Christian and Moon, 1993).

Embryos between the four-cell and 32-cell stages were injected in the two bilateral dorsal blastomeres near the dorsal midline at the pigmentation boundary with 100 pg of *Xwnt-8* plasmid DNA (Fig. 3). This region is fated to contribute to dorsal structures: notochord, central neural tissue and archenteron (Keller, 1975, 1976; Bauer et al., 1994). Control injections were performed using CSKA-pTCAT plasmid or water. Embryos were grown to tadpole stages and scored for DAI and

heart looping. As was seen with the UV irradiation, the extent of diminished dorsal-anterior structures correlated with higher frequencies of reversals. DAI 5 embryos had a basal frequency of heart reversals (Fig. 2). In embryos with diminished dorsal-anterior structures, scored as DAI 4 or 3, the heart reversal frequencies were 24% and 45% respectively (Fig. 2).

Two different treatments, one during the first cell-cycle and the other post-MBT, resulted in an inverse correlation between the frequency of cardiac reversals and the extent of dorsal-anterior development. In embryos for which the dorsal-ventral and anterior-posterior axes were severely disrupted (DAI 3 embryos), left-right orientation was stochastically determined. The 45% heart reversal frequency for DAI 3 embryos, from either UV treatments or *Xwnt-8* injections, was statistically identical to the predicted frequency of randomized left-right asymmetries (50%, $P>0.25$ by χ-square analysis). In these embryos, left-right asymmetry was generated but the mechanism that orients the left-right asymmetry with respect to remnants of the other axes was lost.

Xwnt-8 ectopic expression in dorsal midline cells, but not heart precursor cells, causes cardiac reversals

Xwnt-8, as a member of the Wnt family, is thought to be a secreted protein (Christian et al., 1991). One possible explanation of heart reversals was that the Xwnt-8 expressed from the injected plasmids was present within or secreted to the heart precursor cells and directly altered their development. To assess this possibility, the blastomeres in the thirty-two-cell stage embryo that are fated to contribute to heart (Keller, 1975, 1976; Bauer et al., 1994) were injected with *Xwnt-8* expression plasmid or with water or CSKA-pTCAT plasmid as controls (Fig. 3, blastomeres C2). To confirm that these injections targeted the heart precursor cells, rhodamine dextran lineage label was mixed with the DNA and injected into C2 blastomeres. In accordance with the fate maps, injection of C2 blastomeres resulted in lineage labelled hearts (10 of 14 embryos), somites, lateral mesoderm, and endoderm. These results indicated that C2 injections were into cells that contributed to heart or surrounding tissue. Xwnt-8 ectopic expression in or near heart precursor blastomeres (C2, Fig. 3) did not result in heart reversals or in dorsal-anterior defects (Table 1). In contrast, injection of *Xwnt-8* expression plasmid into C1 blastomeres (Fig. 3) at the dorsal midline in 32-cell stage embryos resulted in lower DAIs and a corresponding increase in heart reversals (Table 1). These results indicate that ectopic expression of *Xwnt-8* specifically in dorsal midline cells, which normally give rise to part of the Organizer region and notochord, results in altered dorsal-anterior development and a corresponding loss of left-right orientation in the heart.

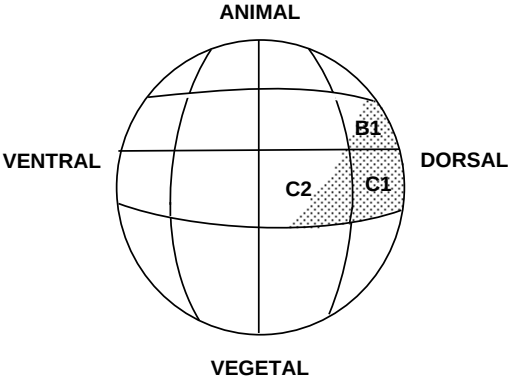


Fig. 3. Drawing of 32-cell stage embryo to show injection locations, labelled using the nomenclature of Nakamura and Kishiyama (1971). The C2 blastomeres are fate mapped to give rise to precursor heart cells. The stippling shows the region that will give rise to the notochord (Keller, 1975, 1976; Bauer et al., 1994). Injections done at this stage were either into C1 blastomeres to target the notochord and other axial structures (see text), or into C2 blastomeres to target the heart.

Table 1. Xwnt-8 injections into the dorsal midline but not the heart progenitor cells at the 32-cell stage cause higher incidence of heart reversals and lower DAI scores

Injection site	*n*	% Reversal	Average DAI
Dorsal midline (C1) - *Xwnt-8*	110	16	3.8
Dorsal midline (C1) - Control	130	2	4.9
Heart progenitor (C2) - *Xwnt-8*	122	5	4.8
Heart progenitor (C2) - Control	108	7	5
Uninjected	151	2.6	5

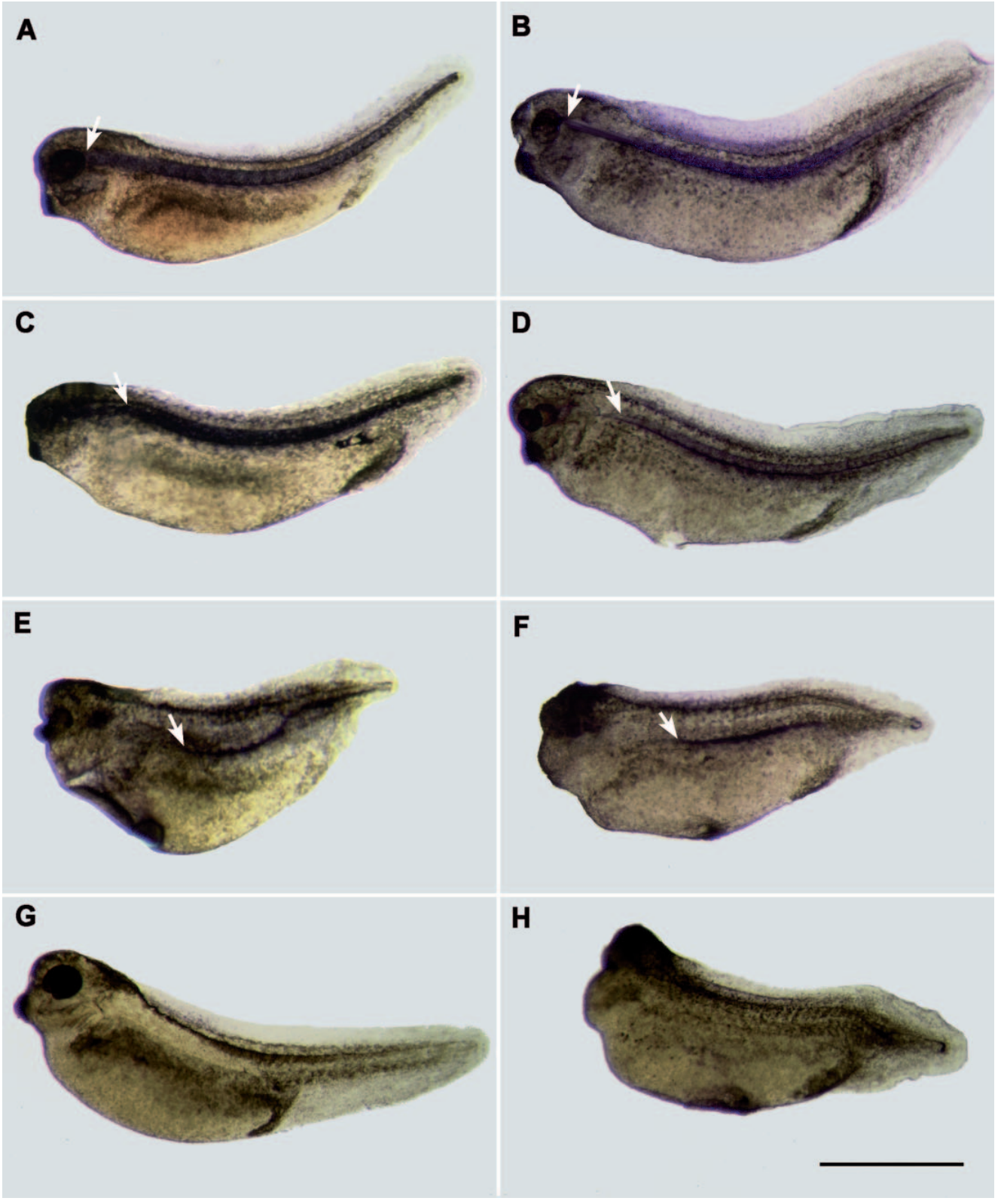
Embryos with lower DAIs lack anterior notochord

The above results suggest that dorsal midline cells, which are fated to contribute to part of the Organizer and to notochord, are involved in the developmental regulation of cardiac left-right orientation. To assess the extent of notochord development in axial-deficient embryos, in situ hybridization was performed using a collagen II mRNA probe that stains the notochord (Su et al., 1991; Amaya et al., 1993). Embryos (stages 34-38), from the UV treatment and *Xwnt-8* injections described above, were fixed and in situ hybridizations were performed (Harland, 1991). Notochords were present in DAI 4 embryos derived from both treatments (Fig. 4C,D). This is in contrast to a report that notochords were not detected by scanning electron microscopy of younger embryos (stages 22-24) that had received mild UV treatment, including embryos with very little loss of dorsal-anterior structure (DAI 4; Youn and Malacinski, 1981). In the present study, both UV treatment and *Xwnt-8* injections during early development gave rise to embryos that had specific deficiencies in notochord development. Notochords in DAI 4 embryos did not extend as far anteriorly as in DAI 5 embryos (Fig. 4C,D and A,B). Notochords in DAI 3 embryos from either treatment were more severely regressed, ventrally displaced and lacked the vacuoles seen in normal embryos (Fig. 4E,F and A,B).

The deficiency in notochord development was correlated with a decrease in DAI as assessed by a ratio of notochord length to body length. Embryos from both UV treatment and

Fig. 4. Notochord deficiencies in dorsal-anterior deficient embryos (stages 33-38) were detected by in situ hybridizations with the collagen II probe. Embryos treated with UV during the first cell cycle, scored as DAI 5 (A), DAI 4 (C), and DAI 3 (E), stained with the antisense probe. Embryos dorsally injected with *Xwnt-8*, scored as DAI 5 (B), DAI 4 (D), or DAI 3 (F), stained with the antisense probe. Arrows mark the anterior extent of the notochords. As hybridization controls, DAI 5 (G) and DAI 3 (H) embryos (obtained from *Xwnt-8* injections) were stained with the sense probe. Scale bar, 1.0 mm.

Xwnt-8 injections were stained with the collagen II probe, sorted by DAI, and photographed. Measurements of body length and notochord length were made, respectively, from the anterior extent of the head and the notochord to the blastopore, on embryos between stages 34 and 38. The ratio of notochord length to body length was calculated per embryo for each DAI and treatment. The two different treatments had similar effects on the extent of anterior notochord regression (Fig. 5); decrease in DAI was correlated with regression of anterior notochord. Both an increase in the frequencies of left-right reversals



(Fig. 2) and progressive loss of anterior notochord (Fig. 5) were correlated with a decrease in DAI. Therefore, loss of notochord was correlated with loss of normal left-right orientation.

DISCUSSION

Linkage of left-right and dorsal-anterior development

In order to co-ordinate the formation of the embryo as it develops along three geometric axes, at some point in development the mechanisms that establish asymmetries along one axis must interact with the mechanisms that establish asymmetries along the perpendicular axes. Most studies of embryo development focus on one axis. Experimental results described here demonstrate that there is linkage between left-right development and dorsal-anterior development in vertebrates.

There is a striking correlation between experimentally diminished dorsal-anterior development, as scored on the DAI scale, and increased frequencies of cardiac left-right reversal. Embryos with reduced dorsal-anterior development (lower DAIs) were obtained either by perturbation of the cytoplasmic rotation in the first cell cycle that establishes the dorsal-anterior axes or by ectopic expression of *Xwnt-8* in the dorsal regions of the embryo after the mid-blastula transition. Although these two treatments were applied at different stages and presumably act through different mechanisms, the effects on both dorsal-anterior and left-right development were the same. Dorsal-anterior development occurs along a continuum from normal (DAI 5) to the absence of all dorsal-anterior asymmetry (DAI 0). Development of left-right asymmetries is apparently discontinuous; it requires both a mechanism by which asymmetry is generated and a mechanism that regulates the orientation of asymmetry along the left-right geometric axis. Dorsal-anterior development is linked with the latter: in DAI 3 embryos, partial reduction of dorsal-anterior development results in loss of the mechanism that regulates the orientation of left-right asymmetries, resulting in stochastically oriented left-right structures, but the mechanism by which left-right asymmetries are generated is retained.

The roles of dorsal midline cells in left-right development

The misexpression of Xwnt-8 can be regionally specified by injection of selected cells. Ectopic expression of Xwnt-8 in dorsal-most blastomeres (C1 blastomeres, Fig. 3) resulted in anterior-dorsal defects (decreased DAI), regression of anterior notochord, and loss of cardiac left-right orientation. In contrast, injections into heart progenitor cells (C2 blastomeres, Fig. 3) did not alter anterior-dorsal development and did not cause cardiac reversal. These results indicate that loss of left-right orientation is not due to a direct influence of *Xwnt-8* in developing heart cells, but is correlated with perturbed anterior-dorsal development and defective notochord development. By fate-mapping, the dorsal-most (C1) blastomeres have been shown to give rise to subblastoporal endoderm, bottle cells and dorsal blastoporal lip (Organizer region) at the gastrula stage. In the neurula stages, the progeny of C1 blastomeres are interdigitated throughout the notochord, head mesoderm and archenteron (Keller, 1975, 1976; Bauer et al., 1994). The progeny of the dorsal midline cells could regulate cardiac left-right development as Organizer cells during gastrulation or as notochord during neurulation, although it should be noted that less-well-characterized dorsal midline cells might be involved in left-right cardiac development.

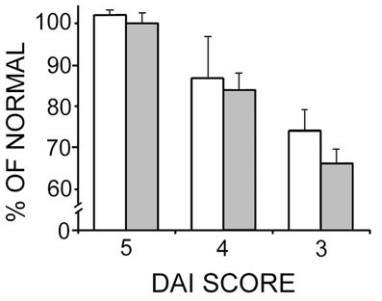


Fig. 5. Decrease in anterior notochord development is correlated with dorsal-anterior deficiencies. The amount of notochord for each DAI from UV-treated embryos (white bars) and embryos in which *Xwnt-8* was injected into dorsal cells (grey bars) was measured. The ratio of notochord length to body length was calculated for each embryo (10-14 embryos in each category) and normalized by dividing this ratio by the mean ratio in untreated embryos (83±1, from measurements of 10 untreated embryos).

An effect that is common to UV treatment during the first cell cycle and Xwnt-8 misexpression in post-MBT dorsal midline cells is deficient anterior notochord development (Figs 3 and 4). Although the present results indicate that there is a correlation between defective notochord development and loss of cardiac left-right orientation, it cannot be concluded that notochord directly regulates left-right development. The most likely explanation for progressive loss of anterior notochord, caused by either UV treatment or Xwnt-8 ectopic expression, is that the amount of Organizer activity is diminished. Embryo recombinant experiments show a correlation between the amount of Organizer and the extent of dorsal-anterior development, as assessed by DAI (Stewart and Gerhart, 1990). It is possible that the Organizer directly regulates left-right development during gastrulation. Then, diminished notochord and loss of left-right orientation would be independent consequences of diminished Organizer activity. The Organizer interacts with dorsolateral mesoderm to allow it to develop heart-forming potency (Sater and Jacobson, 1990); perhaps it also interacts with pre-cardiac mesoderm to specify left-right orientation. If decreased DAI reflects decreased Organizer activity (Stewart and Gerhart, 1990), the threshold of Organizer activities required to make a normal-sized heart (DAI 3 and above) must be lower than that required to consistently establish cardiac left-right orientation (DAI 5). Alternatively, Organizer activity could indirectly establish cardiac left-right asymmetry by working through intermediary tissues. For example, planar signals from the Organizer are transmitted through the ectoderm to establish the anterior-posterior axis of the neural plate (for reviews, Doniach, 1992; Ruiz i Altaba and Jessell, 1993). The extracellular matrix of the ectoderm is necessary for normal left-right development (Yost, 1992). Perhaps the Organizer transmits planar signals through the ectoderm to establish left-right asymmetries, which are then transmitted to the cardiac mesoderm by way of the ectodermal extracellular matrix. UV-treated embryos that have no

Organizer activity (DAI 0) (Stewart and Gerhart, 1990) appear to form normal ectodermal extracellular matrix, at least as assayed by fibronectin immunohistochemistry (Yost, 1992). However, other aspects of ectodermal matrix might be regulated by planar signals from the Organizer to regulate left-right development of the heart.

The notochord is derived from the Organizer, and has inductive properties for establishing the floor plate and organizing dorsal-ventral polarity of neural structures in chick (Placzek et al., 1990; Yamada et al., 1991, 1993). By analogy, the notochord might inductively signal left-right asymmetry to the cardiac cells. Alternatively, the elongation and stiffening of the notochord in neurula stages (Keller et al., 1989; Adams et al., 1990) mechanically stretches the embryo along the anterior-posterior axis. Embryo shape changes may be necessary for orientation of left-right asymmetries, perhaps by aligning the extracellular matrix that is necessary for normal cardiac left-right asymmetry (Yost, 1992).

In *Xenopus* mid-neurulae stage embryos, extirpations of dorsal-anterior tissue, including but not exclusive to the notochord, result in randomization of cardiac asymmetry (Danos and Yost, unpublished data), suggesting that the orientation of left-right cardiac asymmetry can be perturbed after the Organizer activity is diminished. In order to distinquish between the roles of the Organizer and subsequent roles of dorsal midline cells in regulating left-right development, it is important to identify the developmental periods during which left-right orientation is dependent on dorsal midline cells. The activities of notochord and other dorsal-anterior tissues in left-right orientation are currently being explored in explant and in vitro systems.

Vertebrates are not bilaterally symmetric; the left side differs from the right, and the orientation of left-right asymmetries is highly conserved in vertebrates. The present results indicate that the developmental regulation of left-right orientation in *Xenopus* is linked to the developmental regulation of the other axes. The mechanism of linkage between the embryonic axes is not yet elucidated, but the linkage is dependent upon normal development of dorsal midline cells.

We are grateful to M. L. Condic and A. L. Teel for comments on the manuscript, and to them and members of our laboratory for insights and encouragement throughout the course of this study. We thank E. Amaya and J. L. Christian for DNA clones, and D. Bader for monoclonal supernatants. This work was supported by a grant-in-aid from the American Heart Association.

REFERENCES

Adams, D.S., Keller, R. and Koehl, M.A.R. (1990). The mechanics of notochord elongation, straightening, and stiffening in the embryo of *Xenopus laevis*. *Development* **110**, 115-130.

Amaya, E., Stein, P.A., Musci, T.J. and Kirschner, M.W. (1993). FGF signalling in the early specification of mesoderm in *Xenopus*. *Development* **118**, 477-487.

Bauer, D.V., Huang, S. and Moody, S.A. (1994). The cleavage stage origin of Spemann's Organizer: analysis of the movements of blastomere clones before and during gastrulation in *Xenopus*. *Develpment* **120**, 1179-1189.

Brown, N.A. and Wolpert, L. (1990). The development of handedness in left/right asymmetry. *Development* **109**, 1-9.

Brueckner, M., McGrath, J., D'Eustachio, P. and Horwich, A.L. (1991). Establishment of left-right asymmetry in vertebrates: genetically distinct steps are involved. In *Biological Asymmetry and Handedness* vol. 162 (ed. G.R. Bock and J. Marsh), pp. 202-218. Chinchester: John Wiley.

Burggren, W.W. (1988). Cardiac design in lower vertebrates: what can phylogeny reveal about ontogeny? *Experientia* **44**, 919-930.

Casey, B., Devoto, M., Jones, K.L. and Ballabio, A. (1993). Mapping a gene for familial situs abnormalities to human chromosome Xq24-q27.1. *Nature Genetics* **5**, 403-407.

Christian, J.L., McMahon, J.A., McMahon, A.P. and Moon, R.T. (1991). *Xwnt-8*, a *Xenopus Wnt-1*/*int-1*-related gene responsive to mesoderm-inducing growth factors, may play a role in ventral mesodermal patterning during embryogenesis. *Development* **111**, 1045-1055.

Christian, J.L. and Moon, R.T. (1993). Interactions between Xwnt-8 and Spemann Organizer signalling pathways generate dorsoventral pattern in the embryonic mesoderm of *Xenopus*. *Genes Dev.* **7**, 13-28.

Dietrich, S., Schubert, F.R. and Gruss, P. (1993). Altered *Pax* gene expression in murine notochord mutants: the notochord is required to initiate and maintain ventral identity in the somite. *Mech. Dev.* **44**, 189-207.

Doniach, T. (1992). Induction of anteroposterior neural pattern in *Xenopus* by planar signals. *Development* **Supplement**, 183-193.

Gerhart, J., Danilchik, M., Doniach, T., Roberts, S., Rowning, B. and Stewart, R. (1989). Cortical rotation of the *Xenopus* egg: consequences for the anteroposterior pattern of embryonic dorsal development. *Development* **107 Suppl**, 37-51.

González-Sánchez, A. and Bader, D. (1984). Immunochemical analysis of myosin heavy chains in the developing chicken heart. *Dev. Biol.* **103**, 151-158.

Halpern, M.E., Ho, R.K., Walker, C. and Kimmel, C.B. (1993). Induction of muscle pioneers and floor plate is distinguished by the zebrafish no tail mutation. *Cell* **75**, 99-111.

Harland, R.M. (1991). In situ hybridization: an improved whole-mount method for *Xenopus* embryos. In *Methods in Cell Biology* vol. 36 (ed. B.K. Kay and H.B. Peng), pp. 685-695. New York: Academic Press, Inc.

Hatta, K., Kimmel, C.B., Ho, R.K. and Walker, C. (1991). The cyclops mutation blocks specification of the floor plate of the zebrafish central nervous system. *Nature* **350**, 339-341.

Hemmati Brivanlou, A., Stewart, R.M. and Harland, R.M. (1990). Region-specific neural induction of an engrailed protein by anterior notochord in *Xenopus*. *Science* **250**, 800-802.

Hemmati-Brivanlou, A. and Harland, R.M. (1989). Expression of an *engrailed*-related protein is induced in the anterior neural ectoderm of early *Xenopus* embryos. *Development* **106**, 611-617.

Horwich, A. and Brueckner, M. (1993). Left, right, and without a cue. *Nature Genetics* **5**, 321-322.

Kao, K.R. and Elinson, R.P. (1988). The entire mesodermal mantle behaves as Spemann's Organizer in dorsoanterior enhanced *Xenopus laevis* embryos. *Dev. Biol.* **127**, 64-77.

Keller, R., Cooper, M.S., Danilchik, M., Tibbetts, P. and Wilson, P.A. (1989). Cell intercalation during notochord development in *Xenopus laevis*. *J. Exp. Zool.* **251**, 134-154.

Keller, R.E. (1975). Vital dye mapping fo the gastrula and neurula of *Xenopus laevis*. I. Prospective areas and morphogenetic movements of the surperficial layer. *Dev. Biol.* **42**, 222-241.

Keller, R.E. (1976). Vital dye mapping of the gastrula and neurula of *Xenopus laevis*. II. Prospective areas and morphogenetic movements of the deep layer. *Dev. Biol.* **51**, 118-127.

Krauss, S., Concordet, J.P. and Ingham, P.W. (1993). A functionally conserved homolog of the Drosophila segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* **75**, 1431-44.

McCain, E.R. and McClay, D.R. (1994). The establishment of bilateral asymmetry in sea urchin embryos. *Development* **120**, 395-404.

Nakamura, O. and Kishiyama, K. (1971). Prospective fates of blastomeres at the 32-cell stage of *Xenopus laevis* embryos. *Proc. Japan Acad.* **47**, 407-412.

Nieuwkoop, P.D. and Faber, J. (1967). *Normal Table of Xenopus laevis (Daudin)*. Amsterdam: North-Holland.

Placzek, M., Tessier-Lavigne, M., Yamada, T., Jessell, T. and Dodd, J. (1990). Mesodermal control of neural cell identity: floor plate induction by the notochord. *Science* **250**, 985-988.

Pourquié, O., Coltey, M., Teillet, M., Ordahl, C. and Douarin, N.M.L. (1993). Control of dorsoventral patterning of somitic derivatives by notochord and floor plate. *Proc. Natl. Acad. Sci. USA* **90**, 5242-5246.

Roelink, H., Augsburger, A., Heemskerk, J., Korzh, V., Norlin, S., Ruiz i Altaba, A., Tanabe, Y., Placzek, M., Edlund, T. and Jessell, T.M. (1994). Floor plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the notochord. *Cell* **76**, 761-775.

Ruiz i Altaba, A. and Jessell, T.M. (1993). Midline cells and the organization of the vertebrate neuraxis. *Curr. Opin.Genet. Dev.* **3**, 633-640.

Sater, A.K. and Jacobson, A.G. (1990). The role of the dorsal lip in the induction of heart mesoderm in *Xenopus laevis*. *Development* **108**, 461-470.

Scharf, S.R. and Gerhart, J.C. (1980). Determination of the dorsal-ventral axis in eggs of Xenopus laevis: complete rescue of UV-impaired eggs by oblique orientation before first cleavage. *Dev. Biol.* **79**, 181-198.

Scharf, S.R. and Gerhart, J.C. (1983). Axis determination in eggs of *Xenopus laevis*: a critical period before first cleavage, identified by the common effects of cold, pressure, and ultraviolet irradiation. *Dev. Biol.* **99**, 75-87.

Slack, J.M.W., Isaacs, H.V., Johnson, G.E., Lettice, L.A., Tannahill, D. and Thompson, J. (1992). Specification of the body plan during *Xenopus* gastrulation: dorsoventral and anterioposterior patterning of the mesoderm. *Development* **Supplement,** 143-149.

Spemann, H. (1938). *Embryonic Development and Induction*. New Haven: Yale University Press.

Stewart, R.M. and Gerhart, J.C. (1990). The anterior extent of dorsal development of the *Xenopus* embryonic axis depends on the quantity of organizer in the late blastula. *Development* **109**, 363-372.

Su, M.-W., Suzuki, H.R., Biecker, J.J., Solursh, M. and Ramirez, F. (1991). Expression of two nonallelic type II procollagen genes during *Xenopus laevis* embryogenesis is characterized by stage-specific production of alternatively spliced transcripts. *J. Cell Biol.* **115**, 565-575.

Yamada, T., Pfaff, S.L., Edlund, T. and Jessel, T.M. (1993). Control of cell pattern in the neural tube: motor neuron induction by diffusible factors from notochord and floor plate. *Cell* **73**, 673-686.

Yamada, T., Placzek, M., Tanaka, H., Dodd, J. and Jessell, T.M. (1991). Control of cell pattern in the developing nervous system: polarizing activity of the floor plate and notochord. *Cell* **64**, 635-647.

Yokoyama, T., Copeland, N.G., Jenkins, N.A., Montgomery, C.A., Elder, F.F.B. and Overbeek, P.A. (1993). Reversal of left-right asymmetry: a situs inversus mutation. *Science* **260**, 679-682.

Yost, H.J. (1990). Inhibition of proteoglycan synthesis eliminates left-right asymmetry in *Xenopus laevis* cardiac looping. *Development* **110**, 865-874.

Yost, H.J. (1992). Regulation of vertebrate left-right asymmetries by extracellular matrix. *Nature* **357**, 158-161.

Yost, H.J. (1994). Breaking symmetry: left-right cardiac development in *Xenopus laevis*. In *Fourth International Symposium on Etiology and Morphogenesis of Congenital Heart Disease - Developmental Mechanisms* (ed. M.M. Markwald, E.B. Clark and A. Takao), pp. 505-511. New York: Futura, N.Y.

Youn, B.W. and Malacinski, G.M. (1981). Axial structure development in ultraviolet-irradiated (notochord defective) amphibian embryos. *Dev. Biol.* **83**, 339-352.

(Accepted 6 February 1995)