DEVELOPMENTAL BIOLOGY **189,** 57–67 (1997)
ARTICLE NO. DB978662

Left/Right Patterning Signals and the Independent Regulation of Different Aspects of *Situs* in the Chick Embryo

Michael Levin,*,[1] Sylvia Pagan,* Drucilla J. Roberts,*,† Jonathan Cooke,‡ Michael R. Kuehn,§ and Clifford J. Tabin*,[2]

**Department of Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, Massachusetts 02115; †Department of Pathology, Massachusetts General Hospital, Boston, Massachusetts 02114, and Division of Women's and Perinatal Pathology, Brigham and Women's Hospital, Boston, Massachusetts 02115; ‡Laboratory of Developmental Neurobiology, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom; and §Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892*

Recently, a pathway of genes which are part of a cascade regulating the side on which the heart forms during chick development was characterized (M. Levin *et al.*, 1995, *Cell* 82, 1–20). Here we extend these previous studies, showing that manipulation of at least one member of the cascade, *Sonic hedgehog* (*Shh*), can affect the *situs* of embryonic rotation and of the gut, in addition to the heart. Bilateral expression of *Shh*, which is normally found exclusively on the left, does not result in left isomerism (a bilaterally symmetrical embryo having two left sides) nor in a complete *situs inversus* phenotype. Instead, misexpression of *Shh* on the right side of the node, which in turn leads to bilateral *nodal* expression, produces a heterotaxia-like condition, where different aspects of laterality are determined independently. Heart *situs* has previously been shown to be altered by ectopic Shh and activin. However, the most downstream gene identified in the LR pathway, *nodal*, had not been functionally linked to heart laterality. We show that ectopic (right-sided) *nodal* expression is able to affect heart *situs*, suggesting that the randomization of heart laterality observed in *Shh* and *activin* misexpression experiments is a result of changes in *nodal* expression and that *nodal* is likely to regulate heart *situs* endogenously. The first defined asymmetric signal in the left–right patterning pathway is *Shh*, which is initially expressed throughout Hensen's node but becomes restricted to the left side at stage 4^{+}. It has been hypothesized that the restriction of *Shh* expression may be due to repression by an upstream activin-like factor. The involvement of such an activin-like factor on the right side of Hensen's node was suggested because ectopic activin protein is able to repress *Shh* on the left side of the node, as well as to induce ectopic expression of a normally right-sided marker, the activin receptor *cAct-RIIa*. Here we provide further evidence in favor of this model. We find that a member of this family, *Activin βB*, is indeed expressed asymmetrically, only on the right side of Hensen's node, at the correct time for it to be the endogenous asymmetric activin signal. Furthermore, we show that application of follistatin-loaded beads eliminates the asymmetry in *Shh* expression, consistent with an inhibition of an endogenous member of the activin–BMP superfamily. This combined with the previous data on exogenous activin supports the model that *Activin βB* functions in the chick embryo to initiate *Shh* asymmetry. While these data extend our understanding of the early signals which establish left–right asymmetry, they leave unanswered the interesting question of how the bilateral symmetry of the embryo is initially broken to define a consistent left–right axis. Analysis of spontaneous chick twins suggests that, whatever the molecular mechanism, left–right patterning is unlikely to be due to a blastodermal prepattern but rather is initiated in a streak-autonomous manner.

[1] Current affiliation: Department of Cell Biology, Harvard Medical School, Boston, MA 02115.

[2] To whom correspondence should be addressed. Fax: (617) 432-7595. E-mail: tabin@rascal.med.harvard.edu.

0012-1606/97 $25.00

INTRODUCTION

During development, embryos acquire complex patterns along each of the three axes. The resulting morphologies can display a variety of symmetry types, including spherical (as in volvox), radial (as in starfish), chiral (as in snails), bilateral (as in *Drosophila*), and pseudobilateral (as in man). Most vertebrates have a generally bilaterally symmetrical body plan, but this symmetry is broken into pseudosymmetry by the consistently asymmetric placement of various internal organs such as the heart, liver, spleen, and gut, and an asymmetric development of certain paired organs such as brain hemispheres or lungs. While the specification of anterior–posterior (AP) and dorsal–ventral (DV) axial asymmetries has been studied in some detail (reviewed in Hunt and Krumlauf, 1992), far less is known about the molecular mechanisms underlying left–right (LR) asymmetry.

The LR axis is probably specified after the anterior–posterior and dorsal–ventral axes and is determined with respect to them (McCain and McClay, 1994; Danos and Yost, 1995). Until recently, almost all available information on LR asymmetry centered around four lines of inquiry: a phenomenological literature describing various asymmetries (Neville, 1976, presents an extensive and fascinating survey), the genetics of chirality in snails (an unidentified cytoplasmic factor determines dextrality; Freeman and Lundelius, 1982), several drugs which cause alterations in LR patterning (for example, an adrenergic pathway is implicated by Fujinaga and Baden, 1991a), and mammalian mutants which have phenotypes associated with LR asymmetry, such as randomization or total reversal of internal *situs* (Brueckner *et al.*, 1989; Yokoyama *et al.*, 1993). Selection for LR asymmetries in *Drosophila*, in hopes of generating a genetically tractable mutant, have been unsuccessful (e.g., Tuinstra *et al.*, 1990).

The discovery of signaling molecules asymmetrically expressed prior to overt morphological LR asymmetry in vertebrates (Levin *et al.*, 1995) has opened the way to understanding how the LR axis is regulated. In the chick embryo *Sonic hedgehog* (*Shh*) is initially expressed throughout Hensen's node; however, it is subsequently expressed exclusively on the left, perhaps due to the influence of an *activin*-like activity on the right side of the node. Subsequently, the resulting asymmetric expression of *Shh* induces the chick homologue of the mouse gene *nodal* (a member of the TGF-β family, previously called *cNR-1*), which spreads throughout the lateral plate mesoderm on the left side. Experimental manipulation of these asymmetrically localized signals verified that they form a linear pathway (Levin *et al.*, 1995). Implanting a source of activin on the left side of the node can repress *Shh* on the left; together with the normal absence of *Shh* in the right, this results in a lack of *nodal* expression. Moreover, implanting a source of Shh on the right side, where it is normally repressed, results in bilateral expression of *nodal*. Either manipulation leads to randomization of heart *situs*, strongly suggesting that this molecular cascade is involved in the regulation of morphological asymmetry.

Several important aspects of this pathway remained unclear, however. For example, while heart *situs* is clearly one endpoint of this pathway, it is unknown whether it is a heart-specific cascade or whether other aspects of laterality (such as *situs* of the gut) utilize the LR information inherent in the cascade. If this is the case, it becomes interesting to ask whether perturbation of this pathway results in: (a) all of the organs making coordinated, albeit randomized, *situs* choice (*situs inversus;* Hummel and Chapman, 1959), (b) the organs making independent laterality decisions in response to the signals (heterotaxia), or (c) an embryo formed symmetrically with respect to the LR axis (isomerism). *Situs inversus*, heterotaxia, and isomerism all occur at a significant incidence in many vertebrates, including man (Winer-Muram, 1995). Each of these conditions, in theory, could result from symmetrical signaling. For example, left-isomerism (exemplified by polysplenia syndrome; Ivemark, 1955) might be predicted to result from double-sided *Shh* expression, since Shh is a left-specifying factor. In this study, we examine this question and address other upstream and downstream steps in the LR-determining pathway.

MATERIALS AND METHODS

In Situ Hybridization

After being fixed in 4% paraformaldehyde overnight, chick embryos were processed for whole-mount *in situ* hybridization as described in Levin *et al.* (1995). The clones used in the *Shh* and *nodal in situ* hybridizations are as described in Levin *et al.* (1995). The *nodal* gene was previously called chick nodal-related-1 (*cNR-1*). The nomenclature was changed to match that of the mouse gene in recognition of the fact that the asymmetric expression of this gene is shared among frogs, chicks, and mice (Levin *et al.*, 1995; Lowe *et al.*, 1996) and based on parallels in function described in this report. The DIG antisense probe for *Activin βB* (a gift from K. Patel) covers a 341-bp fragment of the *Activin βB* clone. Embryo staging was according to Hamburger and Hamilton (1951).

Nodal and Shh Misexpression

All experimental manipulations were performed on standard pathogen-free white Leghorn chick embryos obtained from SPAFAS (Norwich, CT). At the time these experiments were conducted our clone of chick *nodal* did not include the entire N-terminal pro portion of the protein which is removed in processing. To obtain a construct that would encode a protein which would be processed correctly and secrete a normal, mature nodal protein, we fused the *BMP-4* pro region (including the cleavage cite) to the *cNR-1* mature region. For misexpression, this construct was inserted into the RCAS-BP(A) vector, which encodes a replication-competent retrovirus (Hughes, 1987). Chick embryonic fibroblast (CEF) cells were infected with this construct (as in Riddle *et al.*, 1993) and pelleted. Pellets were implanted between the epiblast and the hypoblast on the right side of stage 5–6 embryos in New culture (New, 1955). *In situ* hybridization of tissue (including the grafted pellet) to a *cNR-1* riboprobe was used to ensure that the cells produce *cNR-1* mRNA (data not shown). *Shh* was misexpressed by implanting protein-soaked beads (270 μg/ml of N-terminus of human Shh protein produced in bacteria) on the right side of stage 4–5 embryos *in ovo*.

Extirpation of Presumptive Heart Region

The region of the 30-hr chick embryo fated to give rise to the heart has been well mapped (Stalsberg and DeHaan, 1969). By this time, these cells express several cardiac-specific markers, including Nkx-2.5, but they have not yet started to form a morphological heart tube. Embryos with 6–7 segmented somites (ca. 30 hr of incubation) were explanted into New culture (New, 1955), but with the stretched vitelline membrane left flattened, underlaid by only a small amount of albumen medium and overlaid by a 2-mm layer of 1:1 Hanks BSS:Liebovitz air-buffered TCM mixture. Under this condition, groups of embryos could be assembled and age matched for experimental vs sham versions of the operation, which were carried out with tungsten needles. Splanchic mesoderm and overlying endoderm/hypoblast of the precardiac regions were bilaterally excised up to their midline junction at the developing gut pocked. Sham-operated controls received transverse cuts across these regions anteriorly and posteriorly, but without removal of tissue. The space above the embryo within the ring was then drained and that beneath the vitelline membrane further filled with albumen to give convexity of the cultured blastoderm. The embryos were then allowed to grow in culture to the 18- to 20-somite stage, 20–24 hr later.

Follistatin Application

Heparin acrylic beads were soaked in follistatin protein (obtained from the National Hormone and Pituitary Program) at approximately 0.05 mg/ml in water or PBS. Control beads were soaked in water or PBS only. Beads were implanted between the epiblast and the hypoblast of stage 3^+ embryos in New culture (New, 1955) and processed for *in situ* hybridization at appropriate later stages.

Analysis of Twins

Twins occur spontaneously in chick eggs at a rate of approximately 1–2%. Of such twins, approximately 5–10% are in the 180° head-to-head orientation required for this experiment.

RESULTS

Shh Affects Situs of Multiple Organ Systems

It has been previously proposed (Waddington, 1937) that heart looping mechanically sets the *situs* of other morphological aspects of laterality. To test this possibility we surgically removed the prospective heart region from six- to seven-somite chick embryos and scored embryonic rotation. The stage when the heart tissue was removed was prior to the formation of the heart tube, let alone the looping of the tube which could exert physical forces on other organ primordia. Inspection at stage 13–14, when embryonic rotation was assayed, verified that the heart was completely removed by the surgical procedure. The results are summarized in Table 1. It is seen that the incidence of correct embryonic rotation in embryos receiving a sham operation, 88% (Fig. 1A), is not significantly altered ($\chi^2 = 0.126$, $P > 0.5$) in embryos having no heart whatsoever (76% correct embryonic rotation, Fig. 1B). While this experiment does not eliminate the possibility that cardiac cells signal other organs to instruct laterality, it does argue strongly against the possibility that the mechanical stress imparted by the bending heart tube has this effect, as following our surgical manipulations, no heart tube ever forms.

If the *situs* of organs other than the heart is not determined mechanically, then it could be set by a response to signaling molecules. The asymmetric signals described in early chick development have previously been shown to affect heart *situs* (Levin *et al.*, 1995). In principle, this signaling cascade could lie after the regulatory branch point specifically controlling heart morphogenesis or it could lie upstream, playing a more fundamental role in establishing LR asymmetry of the body plan. The original studies of this signaling pathway were carried out *in vitro* using the technique of New culture. This procedure allowed a large number of embryos to be surgically manipulated, since *in ovo* surgery is much more difficult. However, using New culture, embryos do not survive long enough to assay the morphology of organs other than the heart.

To determine whether the *activin–Shh–nodal* pathway is heart-specific or a general LR determination system, we implanted beads soaked in Shh protein on the right side of Hensen's node (opposite its normal expression) *in ovo*. We find that the *in vivo* procedure results in a much higher mortality rate than *in vitro*. Moreover, a lower percentage of surviving embryos show alterations in heart laterality than *in vitro* (12.1% instead of 50%; Table 2). Both the mortality and the decreased incidence of effect on heart morphogenesis among survivors are likely to be due to the deleterious long-term effects of successfully implanted *Shh* beads over the extended time of incubation required to allow other organs to develop.

We assayed surviving embryos for heart *situs* and for *situs* of the stomach as a representative second asymmetric organ. In addition, we examined the direction of embryonic rotation within the egg, a property which has previously been suggested to be mechanically linked to the bending of the heart tube (Waddington, 1937). For the purposes of this study, inverted heart *situs* is defined as the heart tube's bending to the left, inverted stomach *situs* as the stomach on the right, and inverted body rotation as the embryo turning to the left. Embryos were scored as inverted only when unambiguous, not including, for example, embryos which failed to rotate in either direction. This conservative approach results in underestimating rather than overestimating the effects of Shh on laterality decisions. In addition, the only embryos scored were those which survived long enough to assay other organs in addition to the heart.

Seventy-four experimental animals which survived the procedure were examined 4 to 6 days after implantation. We find that all three scored aspects of laterality (heart *situs*, gut *situs*, and direction of embryonic turning) are affected by the presence of ectopic Shh (Table 2). Approximately 10% of the treated embryos showed each individual type of *situs* alteration. In a similar number of surviving embryos implanted with control beads we observed only a 1.5% incidence of reversal in the direction of body rotation (1 embryo) and no examples of reversal in heart or stomach *situs*. Those controls were consistent with the low rates of spontaneous

TABLE 1

Group	wt embryonic rotation	Reversed embryonic rotation	Ambiguous or absent embryonic rotation	*N*	Statistics
Sham operation	88%	6%	6%	15	
Heart excision	76%	5%	19%	21	$\chi^2 = 0.126$, $P > 0.5$

Note. Heart territories from stage 8^+ to 9^- chick embryos in New culture were surgically removed (heart excision group) or transected without removal of tissue (control group). Embryos were allowed to develop and the *situs* of the embryonic rotation was scored.

reversal in body rotation (about 1%) and reversal of internal organ *situs* (less than 0.1%) we have observed in our laboratory. These results demonstrate that, in addition to heart *situs* (Levin *et al.*, 1995), bilateral exposure to Shh signaling has a significant effect on rotation of the embryo ($\chi^2 = 73$, $P < 0.005$) and stomach *situs* ($\chi^2 = 3.8$, $P < 0.05$). Thus the asymmetric cascade lies upstream of the branch point leading to laterality decisions for each individual organ.

Interestingly, the *situs* of the heart and stomach and direction of rotation appear to be influenced independently by Shh signaling, resulting in a heterotaxic phenotype. We found examples of treated embryos where each was the only aspect of laterality which was reversed. Moreover, only 2 of the 16 embryos showing laterality defects were concordant for all three aspects being reversed (true *situs inversus*). This is particularly surprising in the case of the direction of heart looping and embryonic rotation as these have been previously reported to be linked. However, in response to Shh application we observed 5 cases where both the heart and the embryonic rotation were reversed, 4 cases where the heart but not embryonic rotation was reversed, and 4 cases where rotation but not heart looping was altered. Thus, in our experiments, these two processes were affected independently.

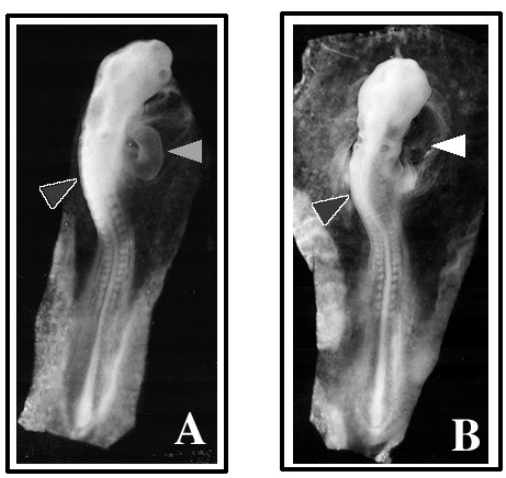

FIG. 1. The *Shh* pathway independently controls *situs* of organs other than heart. The prospective heart region was removed from embryos at stage 6–7. Embryos were allowed to develop until embryonic rotation took place. Embryos receiving a sham operation (A) rotate to the correct side in 88% of the cases; embryos whose heart region has been extirpated (B) likewise rotate correctly in 76% of the cases. Dark gray arrowheads show torsion, light gray arrowhead shows heart, and white arrowhead shows lack of heart.

Nodal Influences Heart Morphogenesis

The results indicate that Shh signaling can influence the *situs* of several asymmetric organs. This creates a paradox however, since Shh is only asymmetrically expressed in a very limited spatial and temporal domain in Hensen's node and is no longer asymmetrically expressed at the time when the organs are formed. This suggests that the asymmetric signaling by Shh must be mediated by a secondary signal. *Nodal* is an excellent candidate for such a secondary signal. Its asymmetric expression is induced by Shh (Levin *et al.*, 1995), correlating with the inductive effects of Shh on organ laterality. Alteration in *nodal* expression has similarly been correlated with changes in organ *situs* in the murine *iv* (Lowe *et al.*, 1996) and *inv* mutants (Collignon *et al.*, 1996). Moreover, the expression domain of *nodal* is quite broad, initiating (in the chick) in the anterior lateral plate mesoderm and subsequently spreading posteriorly while retracting rostrally (Levin *et al.*, 1995). At stage 8, it is expressed in a domain directly adjacent to cells expressing *Nkx-2.5*, a marker of cardiac progenitor cells (Schultheiss *et al.*, 1995), consistent with nodal's providing an asymmetric signal to lateralize the heart primordia.

To test directly whether *nodal* is indeed capable of influencing heart *situs*, pellets of CEFs infected with a retroviral vector expressing nodal were implanted into the right side of embryos at stage 6–7, the stage at which endogenous nodal is induced by Shh in the left side. To obtain higher frequency of survival, the experiments were carried out *in vitro*, in New culture (New, 1955). The results are summarized in Table 3. Under these conditions, in embryos receiving no implant or an implant of cells infected with a nonspecific control virus (alkaline phosphatase), 81% of the developing heart tubes bend to the right, as normal (Fig. 2A), while 19% were either inverted (Fig. 2B) or bilaterally symmetric (Fig. 2C), as previously seen under New culture conditions. There was no significant change in the percentage of hearts bending to the right (82%) when *nodal*-expressing cells were implanted in the left side, where *nodal* is normally expressed; however, when *nodal* was misexpressed

TABLE 2

	Control beads	Shh beads	Statistics
Heart *situs*	97% wt	87.8% wt	$\chi^2 = 150$
	0% reversed	12.1% reversed	$P = 0.005$
	2% abnormal	0% abnormal	
Stomach *situs*	100% wt	90.5% wt	$\chi^2 = 3.8$
	0% reversed	9.5% reversed	$P = 0.05$
Embryonic rotation	97% wt	81.2% wt	$\chi^2 = 73$
	1.5% reversed	13% reversed	$P = 0.005$
	1.5% unturned	5.8% unturned	
No. of embryos examined	65	74	Total $\chi^2 = 6.6$, $P < 0.01$

Note. Beads loaded with Shh protein by equilibrating for 24–48 hr were implanted into the right side of Hensen's node of stage 4 embryos *in ovo*. Embryos were allowed to develop and the *situs* of the heart, gut, and embryonic rotation was scored.

on the opposite side, approximately half that number bent to the right (38%), with a corresponding increase in both inverted and bilaterally symmetric hearts. This effect is significant to $P = 0.005$ ($\chi^2 = 23.9$). It should be noted that approximately twice as many affected hearts were bilaterally symmetric, looping in both directions (right-isomerism, 43%) as were inverted (19%). Since ectopic nodal expression can alter heart *situs*, these data implicate *nodal* as part of the functional cascade determining cardiac laterality. The ability of *nodal* to affect LR specification has been independently demonstrated in Xenopus (Sampath *et al.*, 1997).

Signaling Upstream of Shh in the LR Asymmetric Cascade

Identifying a series of signals directing the asymmetric morphogenesis of the visceral organs is important, in part, because it provides the opportunity to work backward toward addressing the origin of LR asymmetry during embryogenesis. The first described signal in the asymmetric cascade is *Shh*, which is uniformly expressed at Hensen's node at stage 4^-, but then is asymmetrically repressed on the right side at stage 4^+. An upstream activin-like signal mediating this repression was suggested by the finding that an activin-inducible marker (the activin receptor *cAct-RIIa*) is expressed on the right side of Hensen's node concomitant with *Shh* repression (Levin *et al.*, 1995). Consistent with an activin-like activity playing such a role, ectopic activin protein applied to the left side of the node can repress *Shh* and induce *cAct-RIIa*.

The model that an activin-like protein is involved in LR determination thus depended heavily on the effects of applying ectopic activin. If an endogenous activin-like signal is indeed critical for establishing the later asymmetric expression of *Shh* and *nodal*, then interfering with such signals at stage 3–4 should alter *Shh* and *nodal* expression. To test this, we implanted beads loaded with follistatin, an antagonist of signaling by activin and related molecules, including some BMPs (Hemmati-Brivanlou *et al.*, 1994; Yamashita *et al.*, 1995), on the right side of the forming node at stage 3. In 5 of 20 cases, *Shh*, which is normally repressed on the right side of Hensen's node (Fig. 3A), was symmetrically expressed on both sides of the node following follistatin application (Fig. 3B). Preliminary experiments indicate that symmetrical *nodal* expression can also result from follistatin treatment (data not shown). Neither *Shh* nor *nodal* was ever expressed bilaterally following control bead implants ($n = 23$).

To identify a candidate for the endogenous activin-like signal, we examined the expression of various *activin* and related *BMP* genes at stage 4 (including *BMP-2, 4, 6,* and *7* and *Activin βA* and *βB*), all of which except one either were

TABLE 3

	Control cells on *right* side	Nodal cells on *left* side	Nodal cells on *right* side
Right-sided (wt) hearts	81%	82%	38%
Left-sided (reversed) hearts	10%	0%	19%
Bilaterally symmetric hearts	9%	18%	43%
No. of embryos examined	31	22	21
Statistics		$\chi^2 = 5.3$, $P = 0.07$	$\chi^2 = 23.9$, $P = 0.005$

Note. Pellets of CEFs infected with the *nodal* virus were implanted into the right side of Hensen's node of stage 6 embryos in New culture. Embryos were allowed to develop and the *situs* of the heart was scored.

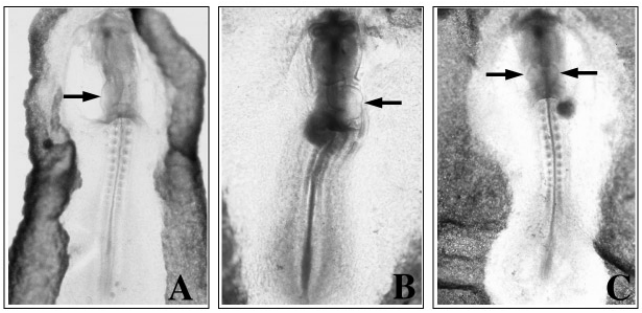

FIG. 2. *Nodal* determines heart asymmetry. Ventral views: when *nodal* is misexpressed on the right side of the node ($n = 21$), 38% of the resulting hearts are wt (A), while 19% show left-sided looping (B) and 43% are symmetrical (C). However, when cells infected with a control virus (alkaline phosphatase) are implanted on the right side of the node ($n = 31$), 81% of the resulting hearts are wt, 10% exhibit left-sided looping, and 9% are symmetrical. This effect is significant to $P = 0.005$, $\chi^2 = 23.9$. When a *nodal*-expressing pellet is implanted on the left side of the node ($n = 22$), 82% of the hearts are wt, 0% are left-sided, and 18% are symmetrical. These phenotypes are not statistically significantly different from control implants ($P = 0.07$, $\chi^2 = 5.3$). Arrows indicate looping of the heart tube.

not detectable at stage 4 or were expressed symmetrically (*BMP-6* was not detectable, while *BMP*s *2*, *4*, and *7* were expressed in the posterior third of the primitive streak, neural folds, and lateral mesoderm, respectively, data not shown). Chick *Activin βB* was seen in whole-mount *in situ* hybridization to be specifically expressed on the right side of Hensen's node from stage 3 to stage 5^+. Unfortunately the *Activin βB* probe gives a high, uniform, nonspecific background at all stages examined. Nonetheless, the right-sided expression can be clearly seen above background, most clearly at stage 4^+ (Fig. 3C). To verify that this signal was real, and was present prior to the asymmetry in *Shh* expression, we sectioned several entire stage 3 embryos in a plane perpendicular to the primitive streak and hybridized all sections with an *Activin βB* probe. Hybridization was exclusively detected on the right side in sections through the anteriormost tip of the primitive streak (Fig. 3D). Thus, the *Activin βB* gene is specifically expressed on the right side of the node at the correct stage to influence *Shh* expression and is therefore a candidate for the endogenous activin-like signal in the LR cascade.

Asymmetry Does Not Appear to Arise from a PrePatterned Blastoderm

Activin βB is currently the earliest molecular marker of LR asymmetry in the chick. This leaves unexplored the factors responsible for initiating LR asymmetries in gene expression in the chick embryo. At least two models for this have been proposed. One theory (Brown and Wolpert, 1990) suggests that LR asymmetry is determined within each cell (perhaps by a chiral molecule which is oriented with respect to the AP and DV axes, which are well established by stage 2 in the chick). An alternative hypothesis is that LR information arises from the maternal localization of a determinant in a LR asymmetric manner within the blastoderm (Wilhelmi, 1921). The analysis of spontaneous twin chick embryos gives a possible indication of which of these two hypotheses about the initial origin of LR asymmetry is more likely to be correct. The cell-autonomous theory predicts that in twin embryos that are oriented in a head-to-head fashion (180°), each twin will be correctly patterned along the LR axis because each node will contain cells which know left from right with respect to their own AP orientation (Fig. 4A). The prepattern theory, however, predicts that the twins will be mirror images of each other since the LR factors localized in the blastoderm will determine left and right sides regardless of the AP orientation of the embedded streaks, resulting in one correct and one reversed embryo (Fig. 4B). Examination of four such embryos probed with *Shh* (Fig. 4C) and *nodal* (data not shown) as markers of laterality, as well as six such sets of twins assayed by the morphology of the node at stage 5 (Fig. 4D; Cooke, 1995) and two sets of twins examined for embryo turning at stage 22 (Fig. 4E), shows that, in every such case, each embryo is correctly patterned with respect to its own orientation. This suggests that LR information is initiated with respect to the AP and DV axes of each embryo and is due neither to a maternally derived prepattern within the blastodisc nor to a zygotic decision prior to the establishment of the AP and DV axes.

DISCUSSION

The Shh Pathway and Heterotaxia

Shh has been previously shown to randomize heart *situs* (Levin *et al.*, 1995). We now find that when ectopic (right-sided) Shh protein is applied to the node *in ovo*, reversals of the *situs* of the heart, gut, and embryonic rotation are observed. Thus, *Shh* does not lie upstream of a heart-specific pathway, but rather provides a left–right reference by which multiple organs assess their laterality during morphogenesis.

In normal development, the morphogenesis of different organs is a tightly coordinated process. For the asymmetric organs to have an invariant orientation relative to each other, their primordia must respond to a common LR asymmetric set of cues. This is verified by the existence of *situs inversus* mutants (e.g., the *inv* mouse; Yokoyama *et al.*, 1993) where all of the internal organs are in an absolute reverse orientation but maintain the same relative configuration. On the other hand, ultimately each organ forms independently, and other, presumably downstream, mutations result in a randomization of asymmetric placement of each organ. This is seen in human heterotaxia syndromes (e.g., Afzelius, 1976).

In principle, asymmetric signals could affect all of the organ systems by initially acting on one, such as the heart

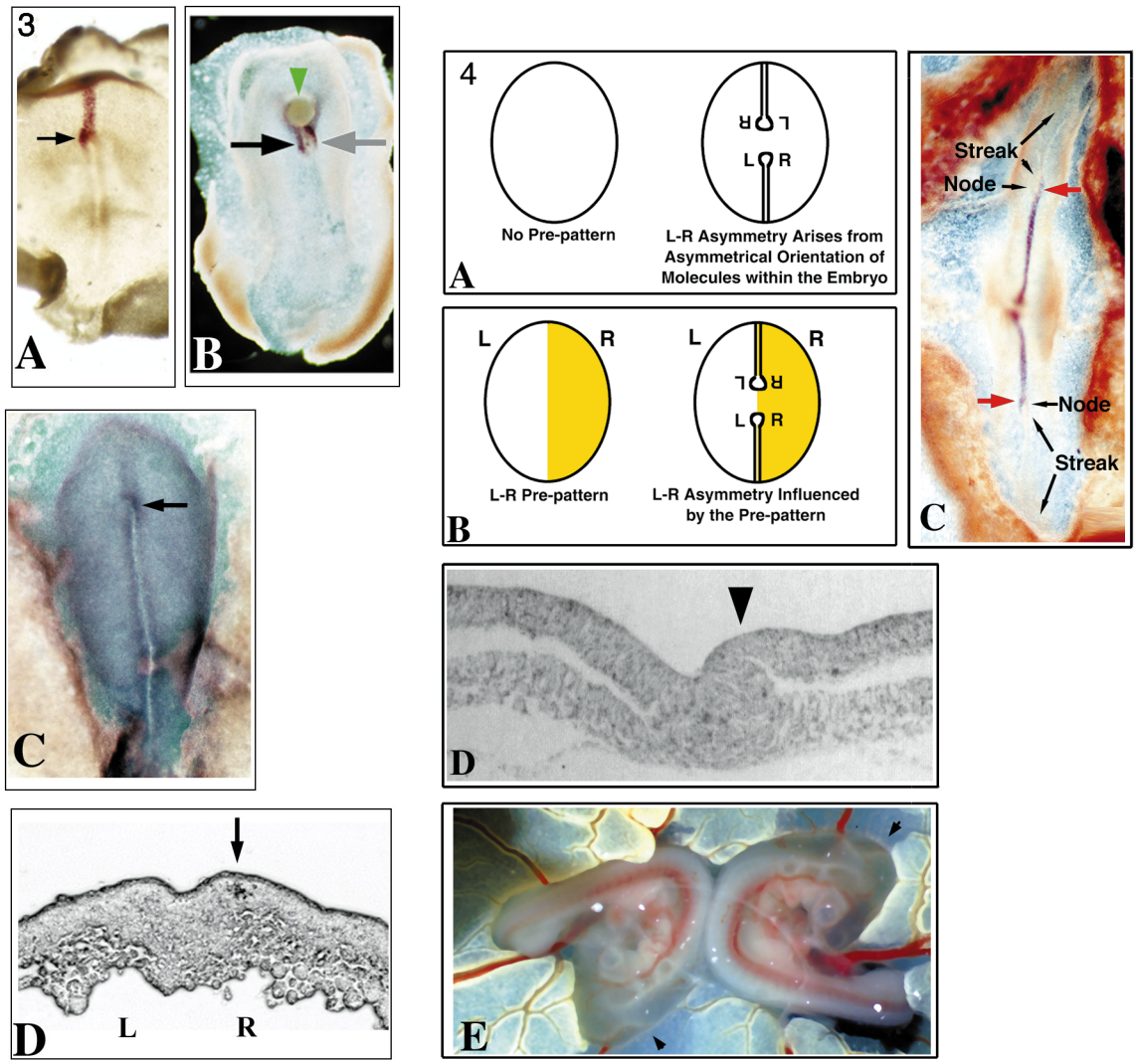

FIG. 3. Endogenous asymmetric activin-like activity. Control beads (soaked in PBS) or beads carrying follistatin protein were implanted into the right side of Hensen's node at stage 3, and the embryos were harvested at stage 5 and processed for *in situ* hybridization with the *Shh* probe. Control beads never caused symmetrical expression patterns of *Shh* ($n = 23$, A). In contrast, when a bead loaded with follistatin protein was implanted in the same manner, symmetrical expression of *Shh* is observed (5/20 cases, B). This result is significant to $P = 0.02$. The beads move anteriorly from their original implant location because of the cell migration which occurs during incubation (see B, green arrowhead). The bead in A is not visible because it became dislodged during *in situ* hybridization processing. Stage 4^+ embryos in whole mount (C) and cryosections at the level of the forming node of stage 3 embryos (D), were processed for *in situ* hybridization with the *Activin βB* probe. Signal was detected in the right side of Hensen's node (black arrow), consistent with its proposed role in repressing *Shh* there. Black arrows indicate endogenous expression domain. Gray arrows indicate ectopic domain. L and R, left and right sides of the primitive streak, respectively.

FIG. 4. LR asymmetry is apparently streak autonomous. The maternal prepattern theory suggests that such twins should result in mirror image embryos because the blastodisc is divided into left and right domains (shown here as yellow and white) containing distinct positional information, such as an asymetrically positioned factor which influences subsequent LR decisions (B). The chiral molecule theory predicts that embryos which are arranged head to head should have correct LR orientation with respect to their own axes (A). When such twins are hybridized to a *Shh* probe, it is seen that each embryo is correctly patterned with respect to itself (C). Red arrows point to wt left-sided *Shh* expression. The morphological asymmetry in the node, shown in section through a wt embryo (D, black arrowhead points to node asymmetry), as well as the direction of embryonic turning (shown in E, black arrowheads point to anterior) is likewise correct in each twin, with respect to its own AP and DV axes.

primordia, which could then mechanically influence the others (Waddington, 1937). In such a case, heterotaxia could be explained by a decoupling of different organs from this influence. Alternatively, each organ primordia could directly respond to the asymmetric signals, and heterotaxia would be a consequence of the failure to interpret these

cues (Brown and Wolpert, 1990). Our experiments support the latter scenario (consistent with the findings of Fujinaga and Baden, 1991b) since ectopic application of Shh results in independent alteration of *situs* of each of the properties we assayed.

In these experiments we placed Shh protein on the right side of Hensen's node resulting in bilateral Shh signaling and hence bilateral *nodal* expression (Levin *et al.*, 1995). Bilateral *nodal* expression has also been correlated with independent segregation of heart and gut orientation in the frog, following ectopic Vg1 expression (Hyatt *et al.*, 1996). It remains to be determined which organs respond to this nodal signal directly and which if any organs get their LR information from genes upstream of (*Shh*), downstream of, or parallel to *nodal* (for example, *lefty;* Meno *et al.*, 1996). The fact that heterotaxia was observed in these experiments instead of left isomerism (which might have been expected to result from the double-sided expression of *Shh,* a normally left-sided gene), suggests that the *Shh* pathway is involved in the biasing of random asymmetry, not in its generation (Brown and Wolpert, 1990).

Nodal Is a Causal Determinant of Heart Situs

Randomization of heart *situs* caused by ectopic Shh or activin has been correlated with double-sided and absent *nodal* expression respectively (Levin *et al.*, 1995). Similarly, recent studies showed that expression of *nodal* is altered in two mouse mutations (*iv,* Lowe *et al.*, 1996; and *inv,* Collignon *et al.*, 1996) which also display laterality defects. This placed the mutations upstream of *nodal,* but it did not distinguish between *nodal*'s being a causal factor in heart *situs* determination and its expression being a parallel effect of earlier parts of the asymmetric gene cascade. Here, we show that ectopic *nodal* expression itself results in inverted and double-sided hearts. This strongly suggests that *nodal* endogenously controls the laterality of cardiac looping.

In these experiments, a significant percentage of embryos display right-isomerized symmetric hearts following bilateral nodal expression achieved by implanting *nodal*-expressing cells. This is a striking contrast to the phenotypes observed following bilateral *nodal* expression generated by implanting *Shh*-expressing cells, in which heart laterality was randomized but no symmetric hearts were observed (Levin *et al.*, 1995). One possible explanation for this difference could be that *nodal* is but one component of the signals downstream of *Shh* necessary to fully specify heart *situs.* For example, *lefty,* another TGF-β family member, has been identified in mice (Meno *et al.*, 1996), has an expression pattern similar to that of *nodal,* and may work in concert with it. An alternative interpretation is that ectopic and endogenous nodal domains, when induced by Shh, are only transiently expressed in the cardiac-forming region; in contrast, *nodal*-expressing cell implants come to lie next to the heart tube and provide a constant source of signal adjacent to the forming heart, which can in some instances disrupt its morphogenesis. Consistent with this explanation, we also observe an increase in bilaterally symmetric hearts when *nodal* cell implants are placed on the left side, where *nodal* is normally expressed.

Nodal, a member of the TGF-β superfamily, encodes a secreted factor (Zhou *et al.*, 1993, and data not shown). Thus, it is plausible that it directly affects heart looping by providing an asymmetric signal to only one of the heart primordia. This could then affect heart morphogenesis by affecting the migration (Manasek, 1981), proliferation (Stalsberg, 1969), or cytoskeletal organization (Itasaki *et al.*, 1989, 1991) of cells descended from the left-side cardiac precursors.

An apparent paradox arises: Hoyle *et al.* (1992) report a difference (in the ability to bias the heart tube) between the left and right precardiac mesoderm as early as stage 5–6, while *nodal* is not expressed until somewhat later. The resolution of this issue is likely to be that cells become committed to express *nodal* earlier, at the time when *Shh* is expressed asymmetrically (stage 4^{+}). Consistent with this, when cells expressing *Shh* are ectopically implanted on the right side adjacent to Hensen's node, the cell pellet migrates anteriorly prior to *nodal* expression. *Nodal* is then induced next to the location where the *Shh* cells were originally implanted, demonstrating a commitment to express *nodal* prior to its actual expression. This commitment is probably what Hoyle *et al.* observed, assayed by them as the ability to bias the heart tube when transplanted.

An Endogenous Activin-like Signal Is Upstream of Asymmetric Shh Expression

Shh is asymmetrically expressed in the early chick embryo and is capable of inducing asymmetric *nodal* expression. The identification of this pathway of genes which are asymmetrically expressed leads to the question of further upstream factors: what is responsible for the asymmetry in the expression of *Shh?* Several lines of evidence suggest that an *activin*-related signal may play a critical role. First, previous studies demonstrated that exogenously applied activin is sufficient to repress *Shh* on the left side of the node and thereby also prevent *nodal* induction (Levin *et al.*, 1995). In other reported experiments, exogenous activin had somewhat different consequences for *Shh* expression, resulting in some cases of reversed *Shh* expression as well as bilaterally expressed *Shh* (Isaac *et al.*, 1997). The reason for this difference is unclear, but may reflect differences in timing or placement of the activin beads. In any case, both sets of experiments indicate that exogenous activin can act upstream of *Shh.* Here we provide evidence that this pharmacological effect likely reflects the action of a related endogenous signal normally present in the right side of the node, since ectopic follistatin, an inhibitor of activin and related factors, leads to symmetrical *Shh* expression. An excellent candidate for the endogenous *Shh*-repressing activity is *Activin βB. Activin βB* is expressed in the right side of the node just before and during the expression of *cAct-RIIa* there and, most importantly, before the disappearance of *Shh* expression from the right side. However, it should be noted that follistatin may also interfere with signaling by

related molecules such as BMP-7 (Yamashita *et al.*, 1995, Wilson and Hemmati-Brivanlou, 1995) and hence the endogenous activin-like activity upstream of *Shh* may in fact be a related molecule. We examined early chick embryos for the expression of a number of related signaling molecules (including BMP-2, 4, 6, and 7) and none were expressed in the node, or in a manner that would indicate a role in LR patterning. However, it remains possible that another member of this family exists which is asymmetrically expressed on the right side of Hensen's node at the same time as *Activin βB*. In any case, asymmetric expression of *Activin βB* is the earliest currently known marker of left–right asymmetry in the chick, expressed asymmetrically long before major organ LR asymmetry. The events further upstream that initiate LR asymmetry and lead to right-sided *Activin βB* expression remain enigmatic.

Vg1 has been reported to be a possible candidate for a signal upstream of *activin* (Hyatt *et al.*, 1996). *BVg1* misexpressed on the right side of *Xenopus* embryos, or expression of a dominant-negative (truncated) activin receptor on the left side, produces *situs* defects in the resulting embryos. It should be noted however, that the truncated activin receptor interacts with other ligands (Kessler and Melton, 1995; Hemmati-Brivanlou *et al.*, 1995); likewise, injected *BVg1* may cross-react with receptors for other TGF-*β* family members. Thus it is possible that these results reflect manipulation of an activin signal, not endogenous Vg1 signaling. It is unlikely that the experiments presented here reflect the activity of an endogenous chick *Vg1* homologue, as Vg1 signaling is not inhibited by follistatin (Kessler and Melton, 1995).

Activin and its receptors have not been reported to be asymmetric in any species other than chick. Moreover, several mice have been generated which carry null mutations for *Activin-βB* and *Activin receptor IIa* (Matzuk *et al.*, 1995), and these mice appear to have no phenotype associated with LR patterning. However, null mutations in *Activin receptor IIb* do result in laterality defects (En Li, personal communication), suggesting that this part of the pathway may indeed also be conserved in mammals. The target of asymmetrical activin signaling in chicks is *Shh*, but *Shh* does not appear to be asymmetric in the mouse node (Collignon *et al.*, 1996), and mice carrying a homozygous deletion of *Shh* have no laterality defects (Chiang *et al.*, 1996). These differences between chicks and mice may be due to different homologues playing the respective roles in LR signaling, or, perhaps the early steps involving *activin* and *Shh* are specific to avian species.

There Is Not an Irreversible LR Determination Prior to the Induction of the Primary Axis

The series of experiments described here is concerned with the investigation of the middle part of LR patterning: the cascade of differential gene expression which lies between initial LR asymmetry determination (the cause of the restriction of the very first gene to be asymmetrically expressed in any given embryo) and the final asymmetric morphogenesis of organs. The two most commonly discussed mechanisms of initial LR determination, a blastodermal prepattern of maternal origin and a chiral molecule within each cell, make opposite predictions for the laterality of 180° head-to-head twins. We have examined 12 cases of such spontaneously occurring twins. Determination of *situs* by means of molecular markers of laterality, embryonic turning, and morphological asymmetry at Hensen's node revealed that each twin was correctly patterned with respect to itself, not to a hypothesized blastodermal prepattern.

Although it is not clear with spontaneously arising twins exactly when the secondary streak was formed, in all cases examined the 180° twins appeared to be of identical stage and size, indicating that they arose at nearly the same time. In principle such spontaneous twins could have arisen within a common blastodisc or in two separate blastodiscs which subsequently fused. However, experimental protocols which induce 180° twins from within the same blastodisc give similar results to those we obtained, at least as assayed morphologically. For example, mechanical transection of a blastoderm can lead to formation of such head-to-head twins, each of which has correct *situs* (Lepori, 1967). Likewise, induction of ectopic streaks with activin and Wnt proteins (Cooke *et al.*, 1994) can produce such twins, which we found in preliminary experiments to give similar results to those described here. We chose not to pursue the experimentally induced twins because in that paradigm, the streak-inducing factors could themselves influence *situs*, making interpretation difficult.

Our examination of 180° twins suggests that their respective *situs* either is determined without regard to information in the blastodisc or is dominant to any underlying bias present as a blastodisc prepattern. This streak-autonomous model is consistent with the theory of Brown and Wolpert (1990) who propose that cells contain a tethered chiral molecule whose directed differential activity serves to produce LR asymmetries. The rarity of experimental material made it impossible to obtain sufficient numbers to confidently test a variety of other angular orientations in addition to 180°. This would have been useful to rule out more exotic prepattern geometries than the one set out in Fig. 4; however, the results of Lepori (1967), who produced duck twins in various angular orientations and observed very few cases of *situs inversus*, are consistent with our own. The one other relatively common class of spontaneous twins represents those arising in parallel or nearly parallel orientations. Under such circumstances the nodes and streaks of the two embryos are in close juxtaposition throughout their development and in those cases the asymmetric signals appear to be able to cross between embryos (Levin *et al.*, 1996). In the 180° twins the two primitive streaks form at opposite ends of the blastodisc. The nodes never become closely juxtaposed because even at full extension the primitive streak does not reach the anterior limit of what will be the embryo. We do not see evidence for cross-signaling in the 180° twins as downstream asymmetric markers and morphology are normal in each twin.

ACKNOWLEDGMENTS

We thank Susan Smith and Devon Smith for advice on *in ovo* surgery, Tiffany Heanue, Christine Hartmann, and other members of the Tabin and Cepko labs for providing spontaneous twin chick embryos, and Ketan Patel for providing the *Activin βB* clone. Follistatin protein was obtained from Dr. Philip Smith at the National Hormone and Pituitary Program. Sonic hedgehog protein was provided by Ontogeny, Inc. This work was funded by a grant from the NIH.

REFERENCES

Afzelius, B. A. (1976). A human syndrome caused by immotile cilia. *Science* **193,** 317–319.

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

Chiang, C., Ying, L. T. T., Lee, E., Young, K. E., Corden, J. L., Westphal, H., and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking sonic hedgehog gene function. *Nature* **383,** 407–413.

Collignon, J., Varlet, I., and Robertson, E. J. (1996). Relationship between asymmetric *nodal* expression and the direction of embryonic turning. *Nature* **381,** 155–158.

Cooke, J. (1995). Vertebrate embryo handedness. *Nature* **374,** 681.

Cooke, J., Takada, S., and McMahon, A. (1994). Experimental control of axial pattern in the chick blastoderm by local expression of Wnt and activin: The role of HNK-1 positive cells. *Dev. Biol.* **164,** 513–527.

Danos, M. C., and Yost, H. J. (1995). Linkage of cardiac left-right asymmetry and dorsal–anterior development in Xenopus. *Development* **121,** 1467–1474.

Freeman, G., and Lundelius, J. W. (1982). The developmental genetics of dextrality and sinistrality in the gastropod *Lymnaea peregra*. *Wilhelm Roux Arch.* **191,** 69–83.

Fujinaga, M., and Baden, J. M. (1991a). Evidence for an adrenergic mechanism in the control of body asymmetry. *Dev. Biol.* **143,** 203–205.

Fujinaga, M., and Baden, J. M. (1991b). Critical period of rat development when sidedness of body asymmetry is determined. *Teratology* **44,** 453–463.

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.

Hamburger, V., and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88,** 49–92.

Hemmati-Brivanlou, A., Kelly, O. G., and Melton, D. A. (1994). Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. *Cell* **77,** 283–295.

Hemmati-Brivanlou, A., and Thomsen, G. H. (1995). Ventral mesodermal patterning in Xenopus embryos: Expression patterns and activities of BMP-2 and BMP-4. *Dev. Genet.* **17,** 78–89.

Hoyle, C., Brown, N. A., and Wolpert, L. (1992). Development of left/right handedness in the chick heart. *Development* **115,** 1071–1078.

Hughes, S. H., Greenhouse, J. J., Petropoulos, C. J., and Sutrave, P. (1987). Adapter plasmids simplify the insertion of foreign DNA into helper-independent retroviral vectors. *J. Virol.* **61,** 3004–3012.

Hummel, K. P., and Chapman, D. B. (1959). Visceral inversion and associated anomalies in the mouse. *J. Hered.* **50,** 9–23.

Hunt, P., and Krumlauf, R. (1992). Hox codes and positional specification in vertebrate embryonic axes. *Annu. Rev. Cell Biol.* **8,** 227–256.

Hyatt, B. A., Lohr, J. L., and Yost, H. J. (1996). Initiation of vertebrate left–right axis formation by maternal Vg1. *Nature* **384,** 62–65.

Isaac, A., Sargent, M. G., and Cooke, J. (1997). Control of vertebrate left-right asymmetry by a snail-related zinc finger gene. *Science* **275,** 1301–1304.

Itasaki, N., Nakamura, H., and Yasuda, M. (1989). Changes in the arrangement of actin bundles during heart looping in the chick embryo. *Anat. Embryol.* **180,** 413–420.

Itasaki, N., Nakamura, H., Sumida, H., and Yasuda, M. (1991). Actin bundles on the right side in the caudal part of the heart tube play a role in dextro-looping in the embryonic chick heart. *Anat. Embryol.* **183,** 29–39.

Ivemark, B. (1955). Implications of agenesis of the spleen on the pathogenesis of conotruncal anomalies in childhood. *Acta Paediatr. Scand.* **44,** 590.

Jowett, T., and Lettice, L. (1994). Whole-mount *in situ* hybridization of zebrafish embryos using a mixture of digoxigenin- and fluorescein-labeled probes. *Trends Genet.* **10,** 73–74.

Kessler, D. S., and Melton, D. A. (1995). Induction of dorsal mesoderm by soluble, mature Vg1 protein. *Development* **121,** 2155–2164.

Klessinger, S., and Christ, B. (1996). Axial structures control laterality in the distribution pattern of endothelial cells. *Anat. Embryol.* **193,** 319–330.

Layton, W. M., Binder, M., Kurnit, D. M., Hanzlik, A. J., Van Keuren, M., and Biddle, F. G. (1993). Expression of the *IV* (reversed and/or heterotaxic) phenotype in SWV mice. *Teratology* **47,** 595–602.

Lepori, N. G. (1969). Sur la genèse des structures asymmétriques chez l'embryon des oiseaux. *Monit. Zool. Ital.* **3,** 33–53.

Levin, M., Johnson, R., Stern, C., Kuehn, M., and Tabin, C. (1995). A molecular pathway determining left–right asymmetry in chick embryogenesis. *Cell* **82,** 1–20.

Levin, M., Roberts, D. J., Holmes, L. B., and Tabin, C. (1996). Laterality defects in conjoined twins. *Nature* **384,** 321.

Lowe, L., Supp, D.-M., Sampath, K., Yokoyama, T., Wright, C. V. E., Potter, S. S., Overbeek, P., and Kuehn, M. R. (1996). Conserved left–right asymmetry of nodal expression and alterations in murine *situs inversus*. *Nature* **381,** 158–161.

Manasek, F. J. (1981). Determinants of heart shape in early embryos. *Fed. Proc.* **40,** 2011–2016.

Matzuk, M. M., Kumar, T. R., Vassalli, A., Bickenbach, J. R., Roop, D. R., Jaenisch, R., and Bradley, A. (1995). Functional analysis of activins during mammalian development. *Nature* **374,** 354–356.

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

Meno, C., Saijoh, Y., Fujii, H., Ikeda, M., Yokoyama, T., Yokoyama, M., Toyoda, Y., and Hamada, H. (1996). Left–right asymmetric expression of the TGFb-family member *lefty* in mouse embryos. *Nature* **381,** 151–154.

Neville, A. C. (1976). ''Animal Asymmetry.'' Arnold, London.

New, D. A. T. (1955). A new technique for the cultivation of the chick embryo *in vitro*. *J. Embryol. Exp. Morphol.* **3,** 326–331.

Riddle, R. D., Johnson, R. L., Laufer, E., and Tabin, C. (1993). *Sonic hedgehog* mediates the polarizing activity of the ZPA. *Cell* **75,** 1401–1416.

Sampath, K., Cheng, A. M. S., Frisch, A., and Wright, C. V. E. (1997). Functional differences among Xenopus nodal-related genes in left-right axis determination. *Development*, in press.

Schultheiss, T., Xydas, S., and Lassar, A. B. (1995). Induction of avian cardiac myogenesis by anterior endoderm. *Development* **121,** 4203–4214.

Stalsberg, H. (1969). The origin of heart asymmetry: Right and left contributions to the early chick embryo heart. *Dev. Biol.* **19,** 109–127.

Stalsberg, H., and DeHaan, R. L. (1969). The precardiac areas and formation of the tubular heart in the chick embryo. *Dev. Biol.* **19,** 128–159.

Talbot, W. S., Trevarrow, B., Halpern, M. E., Melby, A. E., Farr, G., Postlethwait, J. H., Jowett, T., Kimmel, C. B., and Kimelman, D. (1995). A homeobox gene essential for zebrafish notochord development. *Nature* **378,** 150–157.

Tuinstra, E. J., De Jong, G., and Scharloo, W. (1990). Lack of response to family selection for directional asymmetry in *Drosophila melanogaster:* Left and right are not distinguished in development. *Proc. R. Soc. London Series B* **241,** 146–152.

Waddington, C. H. (1937). The dependence of head curvature on the development of the heart in the hick embryo. *J. Exp. Biol.* **14,** 229–231.

Wilhelmi, H. (1921). Experimentelle untersuchungen uber *situs inversus viscerum*. *Arch. Entwicklungsmech. Org.* **48,** 517–532.

Wilson, P. A., and Hemmati-Brivanlou, A. (1995). Induction of epidermis and inhibition of neural fate by Bmp-4. *Nature* **376,** 331–333.

Winer-Muram, H. T. (1995). Adult presentation of heterotaxic syndromes and related complexes. *J. Thoracic Imaging* **10,** 43–57.

Yamashita, H., Tendijke, P., Huylebroeck, Sampath, T., Andries, M., Smith, J. C., Heldin, C-H., and Miyazono, K. (1995). Osteogenis protein-1 binds to activin type II receptors and induces certain activin-like effects. *J. Cell Biol.* **130,** 217–226.

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

Zhou, X., Sasaki, H., Lowe, L., Hogan, B. L., and Kuehn, M. R. (1993). Nodal is a novel TGF-beta-like gene expressed in the mouse node during gastrulation. *Nature* **361,** 543–547.

Received for publication April 10, 1997
Accepted June 10, 1997