Cell, Vol. 82, 803–814, September 8, 1995, Copyright © 1995 by Cell Press

A Molecular Pathway Determining Left-Right Asymmetry in Chick Embryogenesis

Michael Levin,* Randy L. Johnson,* Claudio D. Stern,† Michael Kuehn,‡ and Cliff Tabin*
*Department of Genetics
Harvard Medical School
Boston, Massachusetts 02115
†Department of Genetics and Development
College of Physicians and Surgeons
Columbia University
New York, New York 10032
‡Experimental Immunology Branch
National Institutes of Health
Bethesda, Maryland 20892

Summary

While significant progress has been made in understanding the molecular events underlying the early specification of the antero-posterior and dorso-ventral axes, little information is available regarding the cellular or molecular basis for left-right (LR) differences in animal morphogenesis. We describe the expression patterns of three genes involved in LR determination in chick embryos: *activin receptor IIa*, *Sonic hedgehog* (*Shh*), and *cNR-1* (related to the mouse gene *nodal*). These genes are expressed asymmetrically during and after gastrulation and regulate the expression of one another in a sequential pathway. Moreover, manipulation of the sidedness of either activin protein or *Shh* expression alters heart situs. Together, these observations identify a cascade of molecular asymmetry that determines morphological LR asymmetry in the chick embryo.

Introduction

One of the most conspicuous features of animal morphology is asymmetry along one or more dimensions. Antero–posterior (AP) and dorso–ventral (DV) asymmetry have been studied in detail (Hunt and Krumlauf, 1992; Niehrs et al., 1994). However, vertebrates and some invertebrates are also morphologically asymmetric along the left–right (LR) axis. The sidedness of the asymmetric organs is invariant among normal individuals, suggesting that there exists a LR positional information system. Several mechanisms for generating LR asymmetry have been proposed, including a maternal anisotropic distribution of protein or mRNA (Wilhelmi, 1921), a cytoskeletal component that is inherently asymmetric (Yost, 1991), an asymmetric imprinting and segregation of DNA (Klar, 1994), or a molecule with a directional biochemical activity that is oriented relative to the AP or DV axes (Brown et al., 1991; Yost, 1992).

The LR axis is probably specified after the AP and DV axes and is determined with respect to them (McCain and McClay, 1994; Danos and Yost, 1995). Several morphological markers of LR asymmetry are apparent in many vertebrates: heart, direction of embryo rotation, gut, liver, lungs, et cetera. In the chick, although Hensen's node is slightly asymmetric at stages 5–7 (Hara, 1978; Cooke, 1995), the first grossly asymmetric feature to appear is the heart tube, which forms from the fusion of cardiac primordia at the midline. Subsequently, the initially symmetric heart acquires a dextral loop. While the mechanism for establishing the direction of heart looping is not understood, it is known that precardiac cells from the left and the right sides differentially contribute rostral and caudal portions of the heart tube, as shown by radiolabeling cells at stages 5–7 (Stalsberg, 1969), which may influence direction of looping.

The molecular mechanisms underlying LR axial patterning in vertebrate embryos are currently not understood. However, there is likely to be a genetic basis for LR asymmetry, as several types of unlinked mutations affecting LR laterality exist in mice and human beings: *iv* (Hummel and Chapman, 1959), where 50% of the offspring are phenotypically situs inversus, or mirror-image with respect to the LR axis; *inv* (Yokoyama et al., 1993), where 100% of the offspring are inverted; heterotaxia (Layton et al., 1993), where every organ makes an independent decision as to its LR orientation; and *legless* (Schreiner et al., 1993), where the phenotypic expressivity of genetic limb malformations is related to visceral organ situs.

Any mechanism for generating consistently biased LR asymmetry is likely to involve differential gene expression. Since chick heart sidedness has been experimentally demonstrated to be determined during gastrulation (at stages 5–6; Hoyle et al., 1992), and rat heart situs is likewise determined just before neurulation (Fujinaga and Baden, 1991a), it would be reasonable to expect to find asymmetric gene expression at late gastrulation. Genes exhibiting asymmetric expression during these early embryological stages would be excellent candidates for regulating LR patterning, participating in it, or both. Here, we describe three such genes, *activin receptor IIa* (*cAct-RIIa*), *Sonic hedgehog* (*Shh*), and chicken *nodal-related 1* (*cNR-1*).

As genes encoding signaling molecules and receptors that exhibit LR asymmetries during early chick embryogenesis, at the time that LR asymmetry is established, these three molecules are plausible candidates for being part of the LR determination pathway. In this study, we show that an activin-like protein, *Shh*, and *cNR-1* interact to form part of a pathway responsible for determination of morphological LR asymmetry in the chick embryo.

Results

Asymmetric Gene Expression at Hensen's Node

Since late gastrulation is the period when LR asymmetry is most likely to be determined (Fujinaga and Baden, 1991a; Hoyle et al., 1992), we examined the early expression pat-

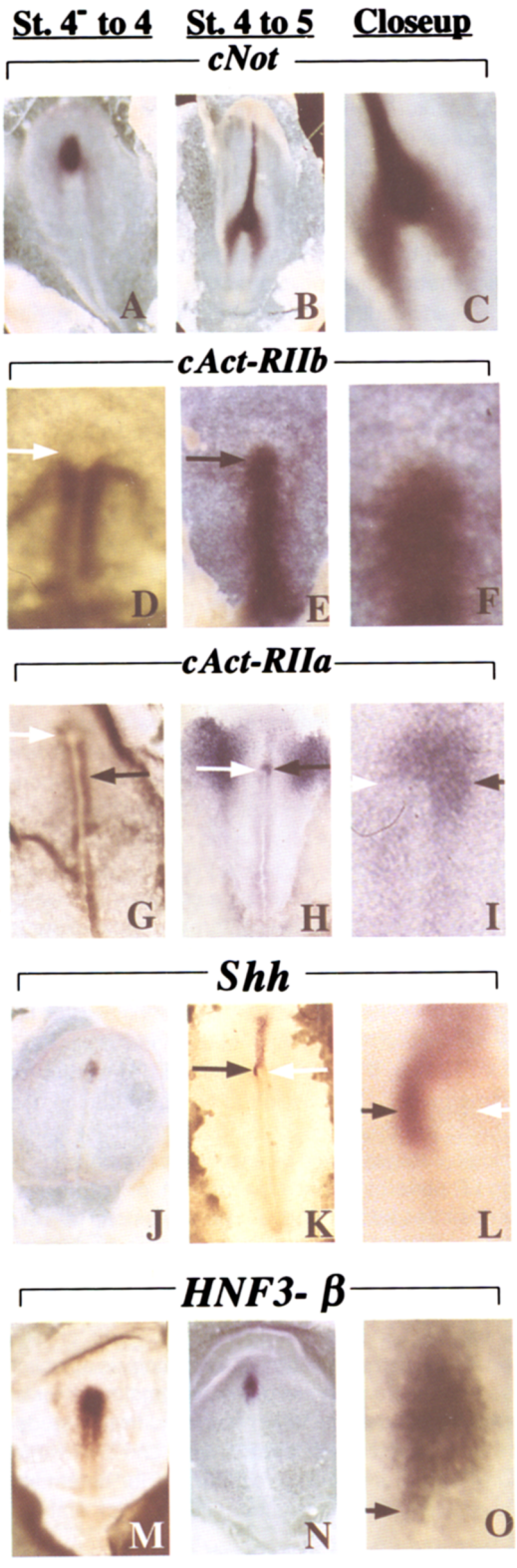

Figure 1. Identification of Asymmetric Gene Expression during Gastrula Stages 4^-, 4^+, and 5

(A–C) In situ hybridization with *cNot* probe. Note that expression is uniform on both sides of the node (A) and is present in the notochord (B). A closeup of Hensen's node at stage 5 shows the symmetric expression (C).

(D–F) In situ hybridization with *cAct-RIIb* probe. Note that expression is present in the primitive streak at stage 4^-, but is specifically excluded from the node (arrow, D). At stage 4 and onward, it is strongly expressed in the streak and node (arrow, E). A closeup of Hensen's node at stage 4^+ shows the symmetric expression (F).

(G–I) In situ hybridization with *cAct-RIIa* probe. Note that expression is stronger in the right half of the streak (black arrow) at stage 4, and that there is no expression in the node (white arrow, G). From stage 4 onward, expression is seen in the right half of the node (black arrow), as well as symmetrically in a localized region of anterior–lateral mesoderm (H). A closeup of Hensen's node at stage 4 shows the asymmetric expression (I).

(J–L) In situ hybridization with *Shh* probe. Note that expression is uniform throughout the node at stage 4^- (J) and becomes restricted to a sickle on the left side of the node at late stage 4^+ (black arrow, K), as well as in the head process cells at stage 5 (K). A closeup of Hensen's node shows the asymmetric expression (L).

(M–O) In situ hybridization with *HNF3β* probe. Expression is symmetric in the node at stage 4 (M); note the transient asymmetry on the left side of the primitive ridge just posterior to the node at stage 4 (black arrow in [N] and [O]). A closeup of Hensen's node at stage 4 shows the asymmetric expression (O). All embryos are shown with the ectoderm (dorsal) side upward.

terns of several genes involved in embryonic patterning by performing whole-mount in situ hybridizations on chick embryos harvested at stages 4–5. Most genes examined displayed expression patterns that are symmetric about the LR axis. For example, *cNot*, a homeobox gene hypothesized to specify notochordal identity (Stein and Kessel, 1995), is uniformly expressed in both sides of Hensen's node during these stages (Figures 1A–1C). Other LR symmetrically expressed genes include *FGF4* and *goosecoid* within the node, and *Msx1*, *Hoxb-8*, and *engrailed* outside the node (data not shown).

An additional symmetrically expressed gene was observed when we examined genes in the activin-related signaling pathway. Activin is a TGFβ family member and has been implicated as an important signaling molecule in early embryogenesis (Ziv et al., 1992; Slack, 1994). Although it is known to be expressed in gastrulation-stage chick embryos (Mitrani et al., 1990), its distribution has not been described in detail. One molecule capable of acting as a receptor for this signal is the *activin receptor IIb* (*cAct-RIIb*), which is first detected in the primitive streak at stage 4^- but is specifically excluded from the node (Figure 1D). Subsequently (at stage 4^+), *cAct-RIIb* is strongly expressed within the node (Figures 1E and 1F). In marked contrast with these symmetrically expressed genes, previous observations indicated an asymmetry in the expression of *cAct-RIIa*, another potential activin receptor (Stern et al., 1995). *cAct-RIIa* is expressed more strongly in the primitive ridge on the right side of the primitive streak at stage 4 (Figure 1G), and then exclusively in the right side of the node (Figures 1H and 1I). At stage 5, it is also expressed symmetrically in areas lateral to the node that roughly coincide with the areas that have heart-forming potency (Figures 1H and 1I).

Previous observations indicated an asymmetry in the expression of a second gene, *Shh* (Johnson et al., 1994). *Shh*, a vertebrate homolog of Drosophila melanogaster *hedgehog*, is another important signaling molecule during embryogenesis (Riddle et al., 1993; Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994; Johnson et al.,

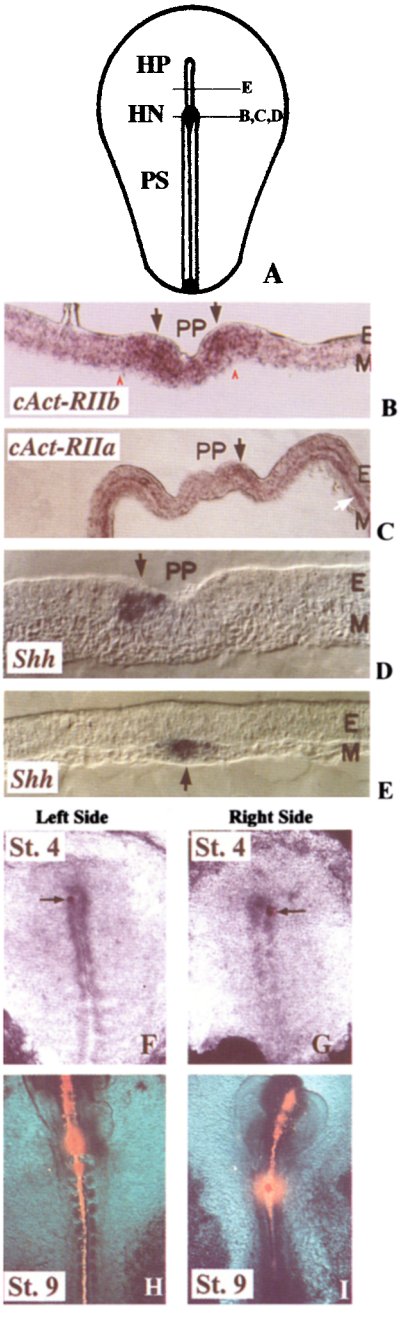

Figure 2. Expression of Asymmetric Genes in Sections at Hensen's Node

Black arrows indicate domain of expression. Red arrowheads indicate borders of expression domain.

(A) Schematic of a stage 4 embryo, showing the levels at which sections (B) through (E) were taken. HP, head process; HN, Hensen's node; PS, primitive streak; PP, primitive pit; E, ectoderm; M, mesoderm.

(B) Cryosection at the level of Hensen's node taken through an embryo that had been hybridized with a probe for *cAct-RIIb*, showing that the expression is present on both the left and right sides of the node.

(C) Cryosection at the level of Hensen's node taken through an embryo that had been hybridized with a probe for *cAct-RIIa*, showing that the expression is present only on the right side of the ectoderm of Hensen's node.

(D) Cryosection at the level of Hensen's node taken through an embryo that had been hybridized with a *Shh* probe. Note that expression is present only on the left side of the ectoderm of Hensen's node.

(E) Cryosection of the same embryo at the level of the head process, showing that the expression is in the mesodermal cells of the head process.

Cells on both sides of Hensen's node contribute to the notochord. Cells on the left side (F) or the right side (G) of Hensen's node were labeled with DiI at stage 4^- (arrows indicate site of label). At stage 9, the embryo that had cells in the left side of the node labeled at stage 4 shows DiI label in the notochord (H), as does the embryo that had cells in the right side labeled (I). Fluorescent photographs were taken with ectoderm (dorsal) side upward.

1994). It is strongly implicated in the control of AP polarity in the limb and in the control of DV polarity in the neural tube and somites. Examining its early expression pattern in more detail, we find that *Shh* is initially symmetrically expressed throughout the node (Figure 1J), but with the onset of the expression of *cAct-RIIa* in the right side of the node, *Shh* mRNA becomes restricted to the left side (Figures 1K and 1L). This striking asymmetric pattern persists until stage 7, when *Shh* is expressed in the notochord cells, but not in the regressing node (data not shown).

HNF3β, a winged-helix transcription factor that may regulate *Shh*, be regulated by it, or both in the notochord and neural tube (Echelard et al., 1993; Krauss et al., 1993), is symmetrically expressed in the node by stage 5 (data not shown), but exhibits a brief and transient period of asymmetric expression during stage 4^-. This asymmetry consists of a small part of the left side of the primitive ridge, just posterior to the node (Figures 1N and 1O). These expression patterns are consistent with the idea that left-sided *Shh* induces a domain of *HNF3β* expression in the primitive ridge that abuts the expression in the node. Indeed, *Shh* can induce ectopic *HNF3β* when stage 4 chick embryos are globally infected with a retrovirus producing SHH protein (data not shown).

Hensen's node is the chick organizer and is functionally homologous to the dorsal lip of the amphibian blastopore (reviewed by Streit et al., 1994). Epiblast cells migrate through Hensen's node and the primitive streak and contribute to the mesodermal layer between the epiblast and the hypoblast. To determine the tissues that asymmetrically express the activin receptor and *Shh*, embryos at stages 4^+ and 5 were hybridized with probes to *cAct-RIIb*, *cAct-RIIa*, or *Shh* in whole mount and then sectioned (Figure 2A). *cAct-RIIb* expression is present in all three layers of the node, on both the left and right sides at stage 4^+ (Figure 2B). In contrast, *cAct-RIIa* is expressed only in the ectoderm, and only on the right side of Hensen's node at this stage (Figure 2C). Asymmetric *Shh* expression at stage 4^+ is confined to the ectoderm on the left side of the node (Figure 2D). The midline expression of *Shh* anterior to the node at stage 5 (as well as *HNF3β* and *cAct-RIIa*; data not shown) is exclusively in mesodermal cells (see Figures 1H, 1K, and 1N; Figure 2E).

Positional Fate of Cells Lateral to Hensen's Node

The expression of *Shh* in the ectoderm at Hensen's node and in axial mesoderm anterior to the node is consistent with the possibility that *Shh*-expressing cells at the node are the exclusive precursors of the notochord. In such a case, the asymmetric distribution of *Shh*-expressing cells

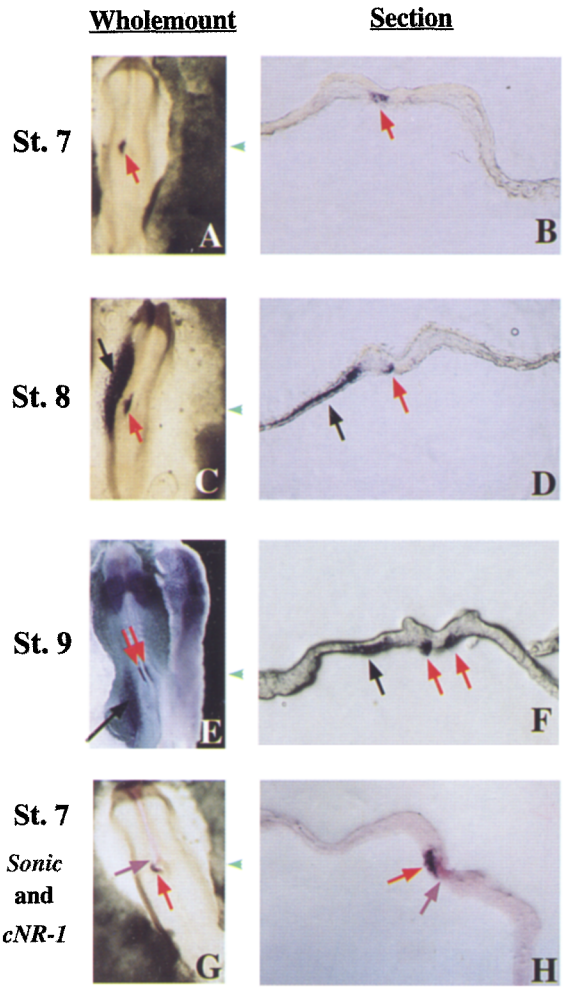

Figure 3. Endogenous Expression Pattern of *cNR-1*

Whole-mount in situ hybridization on control embryos was performed using the *cNR-1* probe (A–F), or the *cNR-1* probe and the *Shh* probe (G–H), after which the embryos were cryosectioned. Red arrows indicate the medial domain of expression of *cNR-1*; black arrows indicate the lateral mesoderm domain of expression of *cNR-1*; magenta arrow indicates *Shh* expression; green arrows indicate level of section.

(A–B) At stage 7, *cNR-1* is expressed in a small domain to the left of the notochord.

(C–D) At stage 8, *cNR-1* is expressed in a much wider domain on the left of the midline (C), along with the small medial domain seen in (B).

(E–F) At stage 9, *cNR-1* acquires a right-sided domain of expression lateral to the notochord, but the wide expression domain remains asymmetric. The stain within the vitelline veins (just posterior to the head) is an artifact caused by probe pooling.

(G–H) At stage 7, *Shh* (magenta stain) is still asymmetrically expressed on the left side of the ectoderm at Hensen's node, while *cNR-1* (purple) begins to be expressed in the endodermal tissue adjacent to it. All embryos are oriented as in Figure 2.

in the node might reflect a differential fate of cells on the left and right sides of the node, rather than playing a signaling role in LR asymmetry. To test this possibility, we labeled ectoderm cells on the right and left sides of Hensen's node (Figures 2F and 2G) of a stage 4 embryo with the carbocyanine dye DiI (for 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) and observed the label at stages 9–10. Both of these sites of injection resulted in strong labeling of the entire notochord (Figures 2H and 2I), showing that there is no difference in positional fate between cells on the left and cells on the right of Hensen's node (consistent with the findings of Selleck and Stern, 1991). Thus, the asymmetric expression of *Shh* around the node does not simply act to demarcate an asymmetric source of notochord precursors. Rather, it seems likely that *Shh* acts as a signal to affect target cells asymmetrically.

Expression of *cNR-1*, a Third Asymmetrically Expressed Gene

To examine signaling during chick gastrulation further, we investigated the expression of *cNR-1*, a chick homolog of the mouse gene *nodal*. *nodal*, like activin, is a member of the TGFβ superfamily. It is required for formation of mesoderm and is expressed at the node during gastrulation in the mouse (Zhou et al., 1993; Conlon et al., 1994; Toyama et al., 1995). A highly related gene, *cNR-1*, has recently been identified in the chick by medium-stringency hybridization (M. K., unpublished data). *cNR-1* is expressed symmetrically in and lateral to the middle two thirds of the primitive streak until stage 4^{-}, but not in Hensen's node (data not shown). This initial phase of *cNR-1* expression disappears by stage 4^{+}.

Subsequently, however, *cNR-1* expression reappears (Figures 3A and 3B) during stage 7, only on the left side and just lateral and anterior to the node. This is followed by a much larger patch of expression in the lateral plate mesoderm (Figures 3C and 3D). This large lateral patch remains asymmetric (only on the left side) until at least stage 11, while the smaller medial region of expression eventually appears on the right side as well, at stage 9 (Figures 3E and 3F). Sectioning (Figures 3B, 3D, 3F, and 3H) reveals that the expression in the large lateral domain is mesodermal.

The asymmetries in the expression profiles of *Shh* and *cNR-1* overlap temporally very briefly: at the time of *cNR-1* induction, at stages 6^{+} to 7. To determine the spatial relationship between these two domains of expression, we performed in situ hybridization on late stage 6 embryos with probes for *Shh* and *cNR-1* that could be detected by magenta and purple stains, respectively. Sectioning (Figure 3H) shows that at this time, the ectodermal expression domain of *Shh* is directly adjacent to the endodermal domain of *cNR-1*.

Activin Can Establish *Shh* Asymmetry

The intriguing asymmetric expression patterns observed in our study suggest the hypothesis that they might be part of a cascade of signals involved in establishing LR asymmetry. Prior to full elongation of the primitive streak, neither activin receptor nor *Shh* is expressed (data not shown). *Shh* expression is then initiated throughout the whole node before becoming restricted to the left side (Figure 1J; compare 1K). This restriction occurs precisely at the time at which *cAct-RIIa* becomes expressed on the right side of the node. Another activin receptor, *cAct-RIIb*,

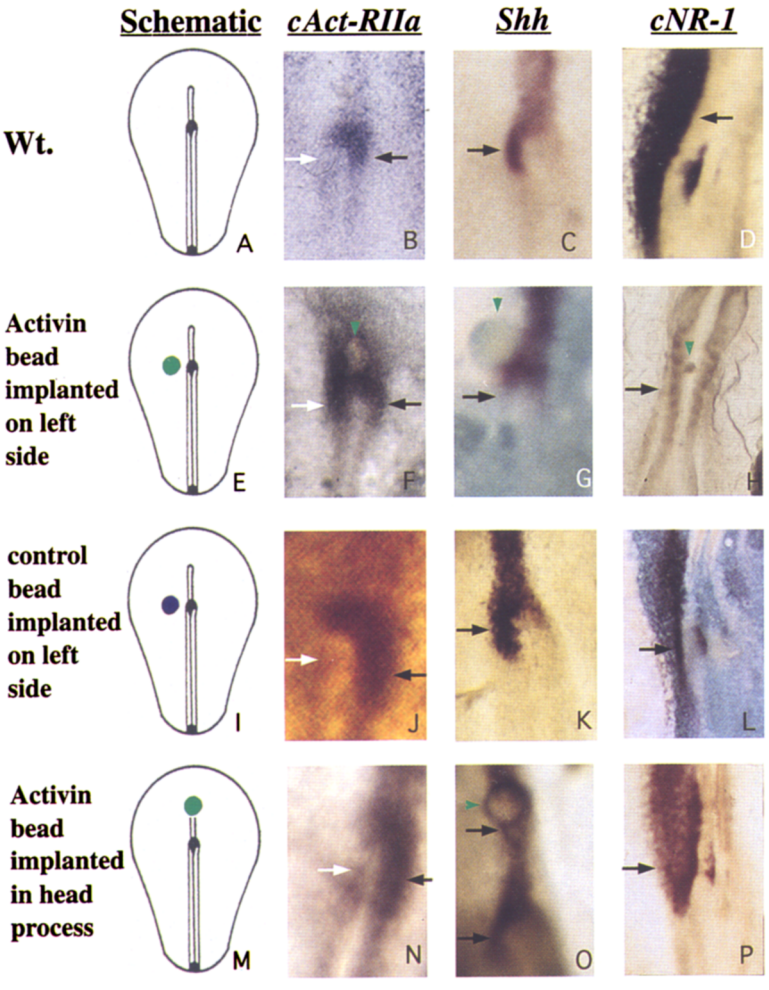

Figure 4. Ectopic Activin Down-Regulates *Shh* and *cNR-1*

White arrows indicate region where expression is not present in control embryos; black arrows indicate wild-type expression domain; green arrows indicate activin-coated bead. In (J), (K), and (N), the bead is not visible, because it became detached during processing for the in situ hybridization. (F) shows two beads, with the central bead just below Hensen's node.

(A–D) In situ hybridization of control embryos (A) with *cAct-RIIa* probe at stage 3$^+$ (B), *Shh* probe at stage 4$^+$ (C), and *cNR-1* probe at stage 8 (D). Note the asymmetries in expression.

(E–H) An activin-soaked bead was implanted on the left side of the node in stage 4$^-$ embryos (E). This resulted in ectopic expression of *cAct-RIIa* on the left side of the node (F), in repression of *Shh* expression in the left side of the node at stage 4$^+$ (G), and in repression of *cNR-1* expression in the lateral mesoderm at stage 8 (H).

(I–L) A control bead was implanted on the left side of the node in stage 4 embryos (I). The patterns of *cAct-RIIa* (J), *Shh* (K), and *cNR-1* (L) are the same as in control embryos (A–D).

(M–P) An activin-soaked bead was implanted in the head process in stage 4 embryos (M). The patterns of *cAct-RIIa* (N), *Shh* (O), and *cNR-1* (P) are the same as in control embryos (A–D).

All embryos are oriented as in Figure 2.

has been previously shown to be activin-inducible (Stern et al., 1995). Although this has not been previously reported for *cAct-RIIa*, it suggests the interesting possibility that an activin-like molecule may be responsible for inducing asymmetric *cAct-RIIa* expression and setting up the LR asymmetry in the expression of *Shh*.

To test this hypothesis, beads soaked in activin protein were implanted on the left side of Hensen's node at stage 4. Six hours later, treated embryos were fixed, processed for in situ hybridization, and compared with unoperated embryos (Figures 4A–4D). An activin bead implanted on the left side of the node (Figure 4E) caused ectopic expression of *cAct-RIIa* (Figure 4F) on the left side of the node, and, as predicted, concomitantly caused a disappearance of *Shh* message from its normal domain on the left side of Hensen's node (Figure 4G). The activin bead also caused a partial reduction in expression of *cAct-RIIa* in the large lateral domain on the right side of the embryo (data not shown). Control beads (soaked in phosphate-buffered saline [PBS] instead of activin, Figure 4I) had no effect on *cAct-RIIa* or *Shh* expression (Figures 4J and 4K). The ability of activin to down-regulate *Shh* seems to be restricted to the node, since activin beads placed in the notochord (Figure 4M) have no effect on the asymmetric expression of *cAct-RIIa* nor on *Shh* in the node (Figures 4N–4O).

Shh Acts Upstream of *cNR-1*

The identification of *cNR-1* as an asymmetric gene expressed after *Shh* in a domain that is initially in contact with the asymmetric *Shh* domain raised the intriguing possibility that *cNR-1* might be a downstream member of the activin–*Shh* cascade. Consistent with this hypothesis, we observed that the repression of endogenous *Shh* expression caused by activin bead implants (Figure 4E) also caused the disappearance of the normal *cNR-1* expression domain (Figure 4H; compare with 4D). Control beads (soaked in PBS instead of activin, Figure 4I), and placement of activin beads in locations that did not affect *Shh* expression (Figures 4M and 4O), had no effect on *cNR-1* expression (Figures 4L and 4P).

To test directly whether *Shh* expression at Hensen's node can induce expression of *cNR-1*, chick embryo fibroblast cells expressing *Shh* (Riddle et al., 1993) were implanted as cell pellets at stage 4 between the epiblast and endoderm. Embryos were then incubated until stage 8 and processed for in situ hybridization with the *cNR-1* probe.

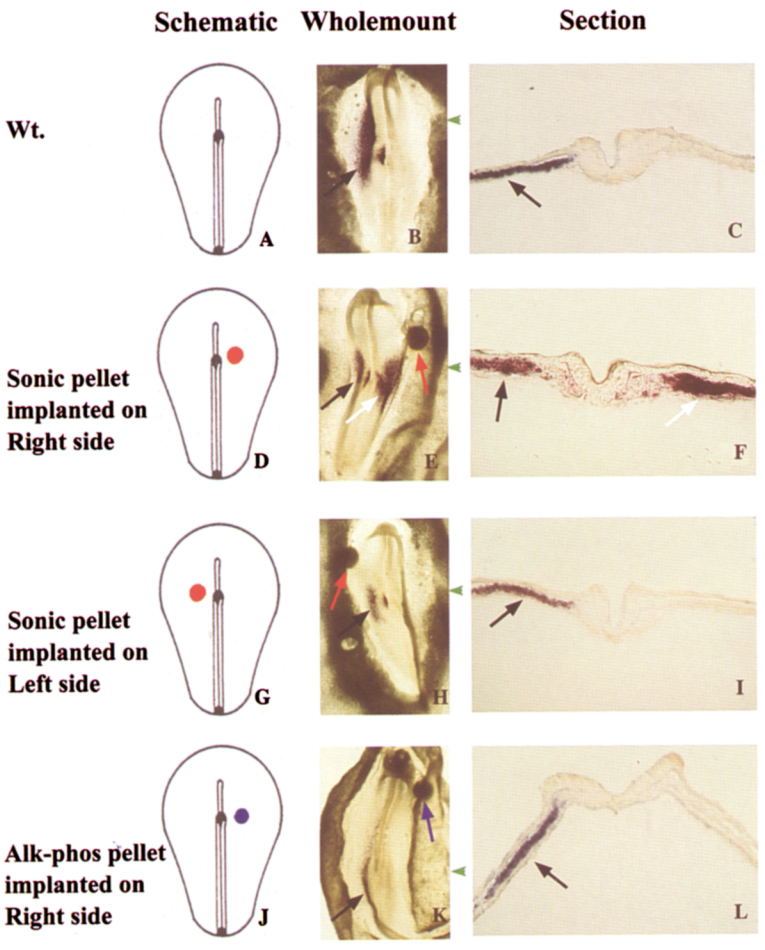

Figure 5. Ectopic *Shh* Expression Causes Ectopic *cNR-1* Expression

Black arrows indicate endogenous domain of expression; white arrow indicates ectopic domain; red arrows indicate implanted *Shh* cell pellet; blue arrow indicates implanted control (alkaline phosphatase) cell pellet; green arrows indicate level of section.

(A) A schematic showing that in the absence of cell implantation or ectopic expression of *Shh*, the normal expression of *cNR-1* is asymmetric, as seen by in situ hybridization in wholemount (B) and section (C).

(D) A schematic showing that a pellet of chicken embryo fibroblast cells infected with a *Shh*-expressing retrovirus was implanted on the right side of Hensen's node at stage 4. This results in an ectopic domain of *cNR-1* expression on the right side of the embryo at stage 9, as seen by in situ hybridization in wholemount (E) and section (F).

(G) A schematic showing that a pellet of *Shh*-expressing cells implanted on the left side of the node (where *Shh* is normally expressed) has no effect on the expression pattern of *cNR-1* (H, I).

(J) A schematic showing that a pellet of cells infected with a nonspecific (alkaline phosphatase) virus implanted on the right of Hensen's node at stage 4 has no effect on the expression pattern of *cNR-1* (K, L).

Implanted cells themselves do not express *cNR-1* (Figures 5A–5L). The implanted cells integrate into the surrounding tissue and participate (as a coherent pellet) in the anterior cell migration that takes place at this time.

When implants were placed on the right side of the node (Figure 5D), where *Shh* is not normally expressed, an ectopic patch of *cNR-1* expression on the right side of the stage 8 embryo was observed (Figures 5E and 5F, compared with control *cNR-1* expression in Figures 5B and 5C). When implants are placed on the left side (Figure 5G), the side where *Shh*, and later *cNR-1*, are normally expressed, the endogenous *cNR-1* expression domain is unaffected, and no ectopic *cNR-1* domain is formed on the right side (Figures 5H and 5I); control implants, infected with a retrovirus bearing alkaline phosphatase (Figure 5J), have no effect on the normal *cNR-1* pattern and do not cause ectopic expression on the contralateral side (Figures 5K and 5L). The same result is obtained when *Shh*-expressing implants are placed next to the posterior parts of the streak (data not shown).

Bilateral Exposure to Either Activin or *Shh* Protein Randomizes Heart Laterality

To determine whether this asymmetric molecular cascade plays a role in determining large-scale morphological asymmetry in the chick embryo, we first examined the later effects of misexpressing *Shh* on the right side of Hensen's node. *Shh*-expressing or control cell pellets were implanted on the right side of the node in stage 4 embryos in New culture, and embryos were scored for heart sidedness at stage 12. We find that the process of placing embryos in culture has a somewhat destabilizing effect on the sidedness of heart development. The embryo culture approach was necessitated in our experiments because chick embryos at early gastrulation stages do not survive in ovo following puncture of the vitelline membrane during the process of implanting beads or pellets. Control embryos in New culture have approximately a 15% incidence of left-sided heart looping, which is consistent with previous reports (Salazar, 1974; Cooke, 1995). When control cell pellets (infected with a retrovirus bearing alkaline phosphatase) are implanted on the right side of Hensen's node (Figure 6B), there is no change in the incidence of correctly sided heart formation (87%; Figure 6D). In contrast, *Shh* pellets (Figure 6A) caused a randomization of heart situs (50% inversion; Figures 6C and 6D). The effect was significant to $p < 0.05$ ($\chi^2 = 5.4$), thus demonstrating that *Shh* expression is a causal factor in determining heart situs.

Since we had found that the asymmetric expression of

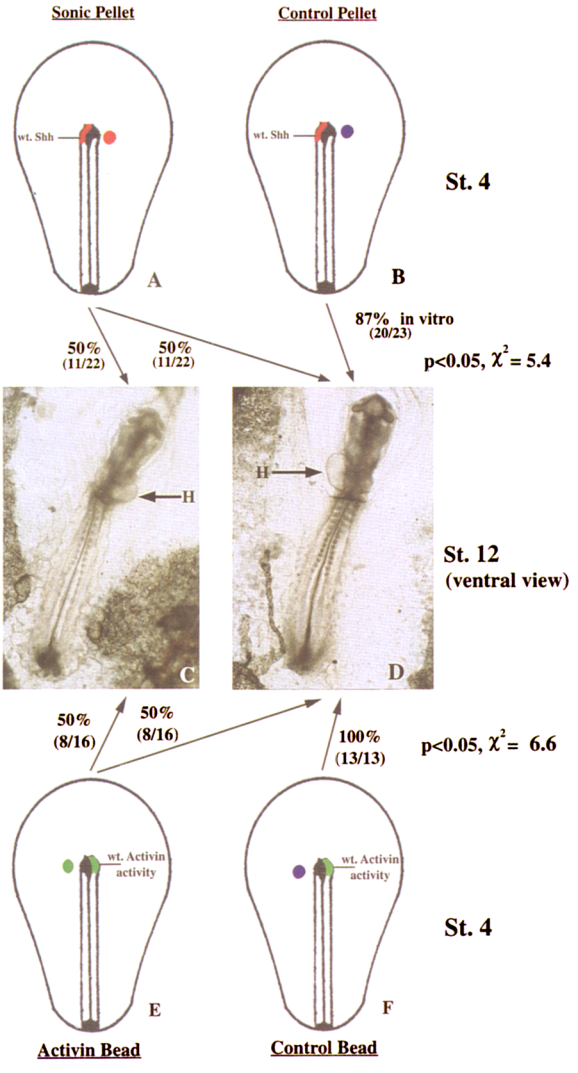

Figure 6. Ectopic *Shh* Expressed on the Right Side, or Activin Placed on the Right Side, Causes a Randomization of Heart Situs

When a pellet consisting of cells infected with the *Shh* virus is implanted on the right side of Hensen's node (A), normal (C) and left-sided (D) hearts are seen with a frequency of 50% each. When a control pellet (B) is implanted, left-sided hearts (C) are only observed with a frequency of 13%. When a control bead is implanted on the left side of Hensen's node (F), centered embryos exhibit correct right-sided heart looping with a frequency of 100% (D). An activin-soaked bead implanted on the left side of the node (E) results in a 50% frequency of left-sided heart tubes (C).

Abbreviations: H, heart; Wt. *Shh*, endogenous *Shh* expression; Wt. Activin activity, presumed endogenous localization of activin-like activity.

Shh at Hensen's node is regulated by an activin-like activity, we wanted to see whether mislocalization of activin could also influence heart situs. However, these experiments are technically more demanding than the *Shh* cell implants. It would therefore be difficult to obtain sufficient numbers to establish a significant change in laterality above the 15% in vitro background of left-sided heart formation. We therefore reexamined this problem. The 15% inversion rate in heart looping of chick embryos in New culture is probably due to alteration of tension forces on the vitelline membrane (Salazar, 1974). The sensitivity of heart sidedness to such extrinsic factors in culture is consistent with the apparent decoupling of heart situs from the node's morphological asymmetry (Cooke, 1995). When placed in New culture, embryonic membranes are stretched across a glass ring. We empirically found that by carefully centering the embryo with respect to the ring, and eliminating from consideration any embryos that had grown to touch the side of the ring, we could reproducibly obtain embryos with normal right-sided looping (100%).

Using this modified procedure, we placed embryos in New culture between stages 3^+ and 4. Heparin–acrylic beads were washed in saline and then either grafted directly or first loaded in 50 μl of activin solution. The grafts were placed just lateral to the node on the left-hand side at the point where the edges of the node bulge out. This exact placement is important both because it had been shown in our earlier experiments that activin in that location affects *Shh* expression, and also because by fate map there is no precardiac mesoderm directly adjacent to the node (Selleck and Stern, 1991); hence, a bead in that position will not physically interfere with the migration of the forming heart tissue. Control beads (Figure 6F) produced no change in heart situs (100% right-sided, Figure 6D). In contrast, activin-loaded beads (Figure 6E) caused a significant ($\chi^2 = 6.6$, $p < 0.05$) randomization of heart situs (50% inversion, Figures 6C and 6D), demonstrating that an asymmetric distribution of the activin-like activity identified above is necessary for proper determination of heart situs.

Discussion

In spite of the general bilateral symmetry of the vertebrate body plan, a number of internal structures are asymmetric with respect to the LR axis. We have characterized four genes that are LR-asymmetrically expressed in developing chick embryo prior to overt morphological asymmetry (which first manifests as dextral bending of the heart tube). The existence of many symmetrically expressed genes shows that this asymmetric expression is not due to some simple morphological asymmetry within the node. Moreover, we demonstrate that three of these genes, *cAct-RIIa*, *Shh*, and *cNR-1*, are part of a signaling cascade that influences heart situs. While genes of these three families have never been previously linked together in an inductive network, each of them is known to play important roles in embryonic patterning.

Activin

Activin has been implicated in many early patterning events in vertebrate embryos, including mesodermal induction in frogs (reviewed by Slack, 1994) and initiation of primitive streak formation in the chick (Ziv et al., 1992).

The activin receptor *cAct-RIIa* manifests the first known sign of LR asymmetry in the chick embryo, long before any morphological asymmetries are evident (Stern et al., 1995; Figures 1G–1I). Its expression is stronger on the

right side of the primitive streak and is restricted to the right side of Hensen's node. These observations suggest that members of the activin family of factors (or antagonists) may also be asymmetrically expressed at early stages. *cAct-RIIb* may be required for activin or an activin-like signal to induce *cAct-RIIa*, since *cAct-RIIb* expression immediately precedes *cAct-RIIa* expression at the node, and activin is unable to down-regulate *Shh* expression in the notochord where *cAct-RIIb* is not expressed (data not shown).

When activin is ectopically applied to the left side of the node, *cAct-RIIa* is induced in that region. *cAct-RIIa* is therefore an activin-inducible gene. Hence, the asymmetric expression of *cAct-RIIa* at the node strongly suggests that activin or an activin-like molecule is asymmetrically distributed and plays an early role in LR determination. Alternatively, follistatin or other signals might specifically antagonize symmetrically produced activin on the left side of the node, thereby allowing *Shh* to be expressed. Further studies will be required to examine the expression and function of both activin and follistatin in the early embryo.

Shh

Shh has been strongly implicated in patterning along the AP (Riddle et al., 1993) and DV (Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994; Johnson et al., 1994) axes in vertebrates. Hensen's node is known to serve as an important signaling center in embryonic patterning. Hence, the discovery that the node expresses *Shh* is suggestive that *Shh* also plays a key role during gastrulation. We can eliminate the possibility of the involvement of *Shh* in several of the inductive functions of the node. For example, *Shh* is not sufficient for the ability of transplanted Hensen's node to induce an ectopic embryonic axis (Waddington, 1932, 1933; Izpisúa-Belmonte et al., 1993), since implanted *Shh* pellets do not have this effect. Likewise, *Shh* is not likely to be involved in neural induction, because its expression starts when the node loses its ability to induce neural tissue (Storey et al., 1992).

On the other hand, the fact that *Shh* is expressed in a LR asymmetric pattern during gastrulation makes it a candidate for an agent that establishes LR differences. The ability of *Shh* to induce *cNR-1*, a later left-side marker, and to randomize heart sidedness when misexpressed on the right side strongly supports this hypothesis. *Shh* expression does not correlate with an asymmetric fate map (consistent with the results of Selleck and Stern, 1991), as DiI fate mapping indicates that both *Shh*-expressing and -nonexpressing cells contribute to notochord.

Shh is initially expressed throughout Hensen's node. The subsequent asymmetric repression of *Shh* on the right side of the node occurs concomitantly with the activation of expression of *cAct-RIIa*, an activin-inducible gene. Additionally, we find that ectopic activin is capable of repressing *Shh* in the left side of the node. These results strongly suggest that the normal asymmetric expression of *Shh* at Hensen's node is generated by an endogenous asymmetric activin-related activity. This repression of *Shh* by activin contrasts with the ability of activin to induce *Shh* when applied to Xenopus laevis animal caps (Yokota et al., 1995). However, the repression of *Shh* in our experiments is observed in the ectoderm of the node, while the activation in the Xenopus system occurs in the context of mesoderm formation and likely represents induction of notochord tissue.

The asymmetric distribution of *Shh* at Hensen's node is observed in embryonic chick, quail, and duck (data not shown). However, the asymmetry in *Shh* expression has not been detected in mice or zebrafish (A. McMahon, personal communication; P. Ingham, personal communication). It is possible that in these species a transient asymmetric distribution of *Shh* exists for a shorter time window and has been missed, since such a basic mechanism might be expected to be conserved among those species. Alternatively, this aspect of molecular regulation of asymmetry might differ among vertebrate species, implying that fish, birds, and mammals have different mechanisms underlying LR asymmetry, as they do in establishing the AP and DV axes (Gurdon, 1992). Consistent with this possibility, in preliminary experiments we observed no effect in chick embryos from exposure to pharmacologic doses of several drugs that are known to produce situs inversus in mammals and frogs (phenylephrine [Fujinaga and Baden, 1991b]; p-nitrophenyl-α-D-xylopyranoside [Yost, 1990]; methoxamine, etc.; data not shown), suggesting that some components of the LR pathway differ between birds and mammals. Moreover, it has been reported that some aspects of early heart formation differ between mice and chicks (Kaufman and Navaratnam, 1981).

cNR-1

Nodal is a *TGFβ*-related gene expressed in the node during mouse embryogenesis. *cNR-1* is a related gene expressed asymmetrically during chick gastrulation. The induction of a small patch of *cNR-1* lateral to the node is followed by a much larger domain in more lateral mesoderm, which represents an amplification step of the initially subtle molecular asymmetry in the node. The induction of a right-sided ectopic domain of *cNR-1* expression in response to ectopic *Shh*, together with its concomitant loss of expression along with *Shh* in response to activin, strongly suggests that the induction of *cNR-1* by *Shh* occurs endogenously in the embryo. Since *cNR-1* is itself likely to be a secreted protein, its induction may be part of the pathway by which *Shh* influences heart situs.

Spatiotemporal Specificity of Gene Interactions

Activin is only capable of repressing *Shh* expression within a discrete spatial domain. Cells in the notochord do not down-regulate *Shh* in response to ectopic activin. This may be due to the absence of *cAct-RIIb* from the notochord, in contrast with *cAct-RIIa*, the expression of which overlaps with *Shh* in the head process. This spatially restricted response to activin is consistent with the findings of Sokol and Melton (1991) and Kinoshita et al. (1993), who reported a prepattern of competence to activin in the frog.

A tight spatial regulation is likewise seen in the responsiveness of cells to *Shh*, as ectopic expression of *cNR-1*

is induced only when *Shh*-expressing cells are placed near Hensen's node (data not shown). This localized response allows us to address the timing of induction of *cNR-1* by *Shh*. Implanted *Shh*-expressing cell pellets quickly join the anterior migration of surrounding tissue, and thus, by the time that ectopic *cNR-1* is expressed, a considerable distance between the implant and the area of ectopic induction of expression is evident (Figure 5E). Ectopic *cNR-1* expression is induced at a time consistent with the endogenous expression of the lateral domain of *cNR-1* and suggests that there is a 6–8 hr delay between the time the lateral cells receive the *Shh* signal and the time that they begin to express *cNR-1*. As evidenced by the distance between the *Shh*-expressing pellet of cells and the induced *cNR-1* expression in lateral mesoderm, *Shh* is not constantly required to induce or maintain *cNR-1*; rather, a single exposure appears to be sufficient to initiate the *cNR-1* expression domain.

In general, the above experiments suggest inductions over short and moderate distances, each limited to one side of Hensen's node. The fact that these inductive influences do not spread across the midline to the other side suggests that there is some sort of barrier. Possible candidates include the primitive pit and notochord. Consistent with this, removal of the notochord destabilizes LR asymmetry (Danos and Yost, 1995). These structures may serve to maintain LR compartments that are defined by the expression of various asymmetric genes.

Molecular and Morphological Asymmetry

The dextral bending of the heart loop is the earliest gross anatomical feature displaying asymmetry in the chick embryo, closely followed by rotation of the embryo. This is preceded by the asymmetric expression of several genes that we have connected in an asymmetric regulatory cascade. Manipulation of several points in this cascade can alter heart situs. *Shh* is normally expressed exclusively on the left side of Hensen's node. Expression of *Shh* on both sides of the node randomizes the direction of bending of the heart tube. On the basis of the expression of the activin-inducible marker *cAct-RIIa*, there is an endogenous activin-like signal asymmetrically localized in the right side of the node. Placement of activin protein on the left side results in an absence of *Shh* expression at the node and concomitantly in a randomization of the direction of heart looping. The fact that both bilateral expression of *Shh* and lack of expression of *Shh* correlate with randomization of heart situs indicates that *Shh* is neither responsible for inducing heart formation nor for instructing its morphogenesis. Indeed, the reversed hearts obtained in both activin and *Shh* experiments are morphologically normal other than their reversal of chirality. Rather, activin and *Shh* seem to act to provide a pivotal influence determining the handedness of the heart.

Another asymmetric gene detected prior to morphological asymmetry is *cNR-1*, widely expressed in the lateral plate mesoderm, which contains cardiac precursors. This *cNR-1* expression may be significant in inducing asymmetry in heart development, since experimentally produced bilateral *Shh* expression, which causes double-sided expression of *cNR-1*, also leads to randomization of heart situs. One possible mechanism by which *cNR-1*, as a growth factor, could affect heart situs is by influencing the proliferation and migration of committed cardiac precursor cells. In the chick, cardiac progenitors from both sides migrate to the midline and form the nascent heart tube. However, normally more progenitors of the rostral portion of the heart (which forms first) originate on the right side (Stalsberg, 1969), and the heart tube subsequently bends in that direction. More progenitors from the left side contribute to the caudal heart tube, which is formed subsequently. In the presence of bilateral *cNR-1* expression, cardiac precursors from both sides might contribute in equal numbers to the rostral and caudal portions of the heart, leaving the heart tube unbiased with respect to direction of looping. Regardless of whether or not *cNR-1* affects heart development, the observation that left-sided application of activin and right-sided expression of *Shh* are specifically able to induce reversals of large-scale organ asymmetry strongly suggests that these factors are endogenous causal agents in controlling such asymmetry.

A Model of the LR Determination Pathway

While none of the interactions elucidated here need be direct, the expression and induction/repression data suggest a model for the early events in establishment of LR asymmetry (Figure 7): activin or some other related factor is asymmetrically expressed at stage 3. Perhaps acting through the symmetrically expressed *cAct-RIIb*, it induces *cAct-RIIa* on the right side of Hensen's node. An activin-like factor, perhaps acting through *cAct-RIIa*, then restricts *Shh* expression to the left side of the node. This regulation of *Shh* may involve the transient asymmetric expression of *HNF3β*. During the several hours of asymmetric *Shh* expression, the *Shh* protein serves as a signal to cells that are lateral to the left side of the node at that time. As the *Shh*-expressing cells migrate anteriorly, and the notochord and head-fold form, Hensen's node begins to regress. At this time, *cNR-1* begins to be expressed in the lateral plate mesoderm (which is known to contain cardiogenic material at this stage [Viragh et al., 1989]), adjacent to the level where *Shh* was asymmetrically expressed. *cNR-1* is a member of the TGFβ family and is likely to encode a secreted signaling molecule. Thus, it is likely to act in a cell-nonautonomous manner, affecting surrounding cells and ultimately influencing the situs of asymmetric organs including the heart and perhaps the liver, the spleen, et cetera. It is interesting that the morphology of the heart and embryonic development in general were not disturbed by the ectopic expression of such powerful inducing factors as *Shh* and activin, suggesting further that these molecules play a specific role in providing LR information to tissues and organs whose development in other respects is regulated by other means.

The existence of a molecular cascade of asymmetric signaling molecules during gastrulation demonstrates that the early chick embryo is LR asymmetric long before morphological asymmetries appear. The randomization of

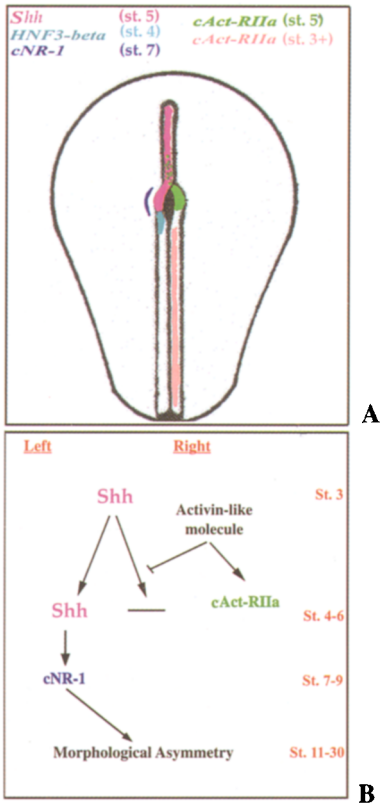

Figure 7. A Model of a Molecular Pathway of LR Determination

(A) A mosaic composite of stage 4⁻ through 6⁺ embryos, showing the expression of various asymmetric genes. The symmetric lateral patches of *cAct-RIIa* are omitted.

(B) A model of gene interactions leading to morphological asymmetry.

heart situs caused by misexpression of activin and *Shh* makes it very likely that this pathway functions in vivo to set up LR asymmetry. The elucidation of a pathway determining LR asymmetry, involving known patterning molecules such as *Shh* and several TGFβ family members, will make it possible to dissect the molecular systems regulating vertebrate LR asymmetry.

Experimental Procedures

Cloning of *HNF3β* and *cNR-1* cDNAs

Approximately 10^6 plaques from a stage 10–15 chick embryo cDNA library (Nieto et al., 1994) were screened under conditions of medium stringency with a murine *HNF3β* cDNA (Sasaki and Hogan, 1993). Positively hybridizing clones were plaque-purified and rescreened with a 700 bp fragment of mouse *HNF3β* that does not contain the *fork head* domain. Several strongly hybridizing phage were further characterized. One of these, HNF14, showed significant sequence identity to murine *HNF3β* and displayed an appropriate expression pattern in chick embryos when used in whole-mount in situ hybridization (data not shown). This clone was used in all subsequent studies.

A single *cNR-1* cDNA clone of 1 kb was isolated from approximately 10^6 plaques from a stage 10–15 chick embryo cDNA library (Nieto et al., 1994) by using a 400 bp genomic fragment of chick *cNR-1* (M. K., unpublished data). This cDNA exhibited an identical expression pattern to the *cNR-1* genomic fragment.

In Situ Hybridization

Procedures for whole-mount in situ hybridization and sectioning of stained embryos were as described by Riddle et al. (1993). For simultaneous detection of multiple probes, one probe was labeled with digoxigenin and the other with fluorescein, as described by Jowett and Lettice (1994). Following detection of the digoxigenin-labeled probe, embryos were heat-inactivated at 70°C for 30 min. Detection of the fluoresceinated probe was carried out in exactly the same manner as for digoxigenin-labeled probes, except that an anti-fluorescein–alkaline phosphatase conjugate (Boehringer) was used to bind fluoresceinated label, and alkaline phosphatase activity was revealed with the chromogen Magenta-Phos (Biosynth). After fixing in 4% paraformaldehyde, 0.1% glutaraldehyde, some embryos were embedded in gelatin and sectioned at 20 μm on a cryostat. All whole-mount embryo photomicrographs were performed with the dorsal side of the embryo uppermost, so that the right and left of the embryo correspond to those of the photograph. The following cDNA clones were used to probe for *cNot*, *HNF3β*, *Shh*, *cNR-1*, *goosecoid*, *cAct-RIIa*, and *cAct-RIIb*, respectively: p37Cnot1 (3.5 kb; Stein and Kessel, 1995), *MS14* (1.2 kb), pHH-2 (1.4 kb; Riddle et al., 1993), *MS22* (1 kb), *gscL* (700 bp; Izpisúa-Belmonte et al., 1993), *cActRIIa* (1.5 kb; Stern et al., 1995), and *cActRIIb* (1.6 kb; Stern et al., 1995).

Embryo Culture

Chick embryos (Spafas) were grown in vitro according to the procedure of New (1955). In brief, eggs were cracked into a container of Panett–Compton saline and cleared of heavy albumen. The vitelline membrane was cut around the equator of the floating yolk and placed upside down onto a watch glass. A glass ring was placed on top of it, and the vitelline membrane was wrapped around its edges. The preparation was then taken out of the saline, and under a dissecting microscope, the remaining liquid was removed from the ring, leaving the embryo, endoderm upward, dry inside the ring. The ring was then placed in a petri dish of light albumen for culture.

DiI Labeling

DiI (Molecular Probes) at a concentration of 0.5% (w/v) in 100% ethanol was diluted 1:9 in 0.3 M sucrose. By use of a fine glass micropipette, groups of approximately 50 cells were labeled with DiI (Stern and Holland, 1993). This was done while the embryos were in New culture (New, 1955).

Activin Bead Implants

Heparin acrylic beads (from Sigma) were washed in PBS three times for 10 min and then placed on a petri dish on ice, with several microliters of activin at a concentration of 8000 Xenopus U/ml (a gift from J. Smith and M. Whitman). After 1 hr, beads were picked up by a micropipette and implanted between the epiblast and endoderm of a stage 4 embryo in New culture (New, 1955).

Retrovirus Production and Cell Implants

Line 0 chick embryo fibroblasts were infected at a ratio of approximately 10 viruses per cell with a retrovirus expressing *Shh* (Riddle et al., 1993). Cell pellets were made by scraping one confluent 60 mm tissue culture dish and centrifuging at 1000 rpm for 60 s. By using fine glass and tungsten needles (Fine Science Instruments), a small pocket was made between the ectoderm and endoderm of a stage 4 chick embryo (Spafas) in New culture, and a piece of cell pellet was inserted into the pocket. The embryos were then cultured until harvesting. All stages reported are according to the standard stage series of Hamburger and Hamilton (1951).

Acknowledgments

We thank Brigid Hogan for the mouse *HNF3β* clone, Michael Kessel for the *cNot-1* clone, David Wilkinson for the cDNA library, Connie Cepko and Sean Fields-Berry for the chicken embryo fibroblasts, Jim Smith and Malcolm Whitman for the activin protein, and members of the Cepko and Tabin labs for their technical help and advice. We are also grateful to Drs. Ron Evans, Kaz Umesono, and Ruth Yu for sharing their unpublished results with us, and to Luba Levin for helpful suggestions to M. L. This work was supported by a National Science Foundation predoctoral fellowship to M. L., by a National Institutes of Health postdoctoral fellowship to R. L. J., and by Public Health Service grants to C. T. and C. S.

Received May 9, 1995; revised July 13, 1995.

References

Brown, N. A., McCarthy, A., and Wolpert, L. (1991). Development of handed body asymmetry in mammals. Ciba Foundation Symp. *162*, 182–196.

Conlon, F. L., Lyons, K. M., Takaesu, N., Barth, K. S., Kispert, A., Hermann, B., and Robertson, E. J. (1994). A primary requirement for *nodal* in the formation and maintenance of the primitive streak in the mouse. Development *120*, 1919–1928.

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

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

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A., and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. Cell *75*, 1417–1430.

Fujinaga, M., and Baden, J. M. (1991a). Critical period of rat development when sidedness of asymmetric body structures is determined. Teratology *44*, 453–462.

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

Gurdon, J. B. (1992). The generation of diversity and pattern in animal development. Cell *68*, 185–199.

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

Hara, K. (1978). Spemann's organizer in birds. In Organizer: A Milestone of a Half Century since Spemann, O. Nakamura and S. Toivonen, eds. (Amsterdam: Elsevier/North Holland), pp. 221–265.

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

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.

Izpisúa-Belmonte, J. C., De Robertis, E. M., Storey, K. G., and Stern, C. D. (1993). The homeobox gene *goosecoid* and the origin of organizer cells in the early chick blastoderm. Cell *74*, 645–659.

Johnson, R. L., Laufer, E., Riddle, R. D., and Tabin, C. (1994). Ectopic expression of *Sonic hedgehog* alters dorso–ventral patterning of somites. Cell *79*, 1165–1173.

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.

Kaufman, M. H., and Navaratnam, V. (1981). Early differentiation of the heart in mouse embryos. J. Anat. *133*, 235–246.

Kinoshita, K., Bessho, T., and Asashima, M. (1993). Competence prepattern in the animal hemisphere of the 8-cell-stage *Xenopus* embryo. Dev. Biol. *160*, 276–284.

Klar, A. J. S. (1994). A model for specification of the left–right axis in vertebrates. Trends Genet. *10*, 292–295.

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*, 1431–1444.

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.

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

Mitrani, E., Ziv, T., Thomsen, G., Shimoni, Y., Melton, D. A., and Bril, A. (1990). Activin can induce the formation of axial structures and is expressed in the hypoblast of the chick. Cell *63*, 495–501.

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

Niehrs, C., Steinbeisser, D., and De Robertis, E. M. (1994). Mesodermal patterning by a gradient of the vertebrate homeobox gene *goosecoid*. Science *263*, 817–820.

Nieto, M. A., Sargent, M. G., Wilkinson, D. G., and Cooke, J., (1994). Control of cell behavior during vertebrate development by *Slug*, a zinc finger gene. Science *264*, 835–839.

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.

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

Salazar, D. R. J. (1974). Influence of extrinsic factors on development. J. Embryol. Exp. Morphol. *31*, 199–206.

Sasaki, H., and Hogan, B. L. (1993). Differential expression of multiple *fork-head* related genes during gastrulation and axial pattern formation in the mouse embryo. Development *118*, 47–59.

Schreiner, C. M., Scott, W. J., Jr., Supp, D. M., and Potter, S. S. (1993). Correlation of forelimb malformation asymmetries with visceral organ situs in the transgenic mouse insertional mutation, *legless*. Dev. Biol. *158*, 560–562.

Selleck, M. A. J., and Stern, C. D. (1991). Fate mapping and cell lineage analysis of Hensen's node in the chick embryo. Development *112*, 615–626.

Slack, J. M. (1994). Inducing factors in *Xenopus* early embryos. Curr. Biol. *4*, 116–126.

Sokol, S., and Melton, D. A. (1991). Pre-existent pattern in *Xenopus* animal pole cells revealed by induction with activin. Nature *351*, 409–411.

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

Stein, S., and Kessel, M. (1995). A homeobox gene involved in node, notochord and neural plate formation of chick embryos. Mech. Dev. *49*, 37–48.

Stern, C. D., and Holland, P. W. H., eds. (1993). Essential Developmental Biology: A Practical Approach (Oxford: IRL Press).

Stern, C. D., Yu, R. T., Kakizuka, A., Kintner, C. R., Mathews, L. S., Vale, W. W., Evans, R. M., and Umesono, K. (1995). Activin and its receptors during gastrulation and the later phases of mesoderm development in the chick embryo. Dev. Biol., in press.

Storey, K. G., Crossley, J. M., De Robertis, E. M., Norris, W. E., and Stern, C. D. (1992). Neural induction and regionalisation in the chick embryo. Development *114*, 729–741.

Streit, A., Thery, C., and Stern, C. D. (1994). Of mice and frogs. Trends Genet. *10*, 181–183.

Toyama, R., O'Connell, M. L., Wright, C. V. E., Kuehn, M. R., and Dawid, I. B. (1995). *Nodal* induces ectopic *goosecoid* and *lim1* expression and axis duplication in zebrafish. Development *121*, 383–391.

Viragh, S., Szabo, E., and Challice, C. E. (1989). Formation of the primitive myo- and endocardial tubes in the chicken embryo. J. Mol. Cell Cardiol. *21*, 123–137.

Waddington, C. H. (1932). Experiments in the development of chick and duck embryos cultivated in vitro. Phil. Trans. Roy. Soc. (Lond.) B *13*, 221.

Waddington, C. H. (1933). Induction by the primitive streak and its derivatives in the chick. J. Exp. Biol. *10*, 38–46.

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

Yokota, C., Mukasa, T., Higashi, M., Odaka, A., Muroya, K., Uchiyama, H., Eto, Y., Asashima, M., and Momoi, T. (1995). Activin induces the expression of the *Xenopus* homologue of *Sonic hedgehog* during mesoderm formation in *Xenopus* explants. Biochem. Biophys. Res. Commun. *207*, 1–7.

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.

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

Yost, H. J. (1991). Development of the left–right axis in amphibians. Ciba Foundation Symp. *162*, 165–176.

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

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

Ziv, T., Shimoni, Y., and Mitrani, E. (1992). Activin can generate ectopic axial structures in chick blastoderm explants. Development *115*, 689–694.