The lefty-related factor Xatv acts as a feedback inhibitor of Nodal signaling in mesoderm induction and L-R axis development in *Xenopus*

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SUMMARY

In mouse, *lefty* genes play critical roles in the left-right (L-R) axis determination pathway. Here, we characterize the *Xenopus* lefty-related factor antivin (*Xatv*). *Xatv* expression is first observed in the marginal zone early during gastrulation, later becoming restricted to axial tissues. During tailbud stages, axial expression resolves to the neural tube floorplate, hypochord, and (transiently) the notochord anlage, and is joined by dynamic expression in the left lateral plate mesoderm (LPM) and left dorsal endoderm.

An emerging paradigm in embryonic patterning is that secreted antagonists regulate the activity of intercellular signaling factors, thereby modulating cell fate specification. *Xatv* expression is rapidly induced by dorsoanterior-type mesoderm inducers such as activin or Xnr2. *Xatv* is not an inducer itself, but antagonizes both Xnr2 and activin. Together with its expression pattern, this suggests that *Xatv* functions during gastrulation in a negative feedback loop with Xnr2 to affect the amount and/or character of mesoderm induced. Our data also provide insights into the way that *lefty/nodal* signals interact in the initiation of differential L-R morphogenesis. Right-sided misexpression of *Xnr1* (endogenously expressed in the left LPM) induces bilateral *Xatv* expression. Left-sided *Xatv* overexpression suppresses *Xnr1/XPitx2* expression in the left LPM, and leads to severely disturbed visceral asymmetry, suggesting that active ‘left’ signals are critical for L-R axis determination in frog embryos. We propose that the induction of *lefty/Xatv* in the left LPM by *nodal/Xnr1* provides an efficient self-regulating mechanism to downregulate *nodal/Xnr1* expression and ensure a transient ‘left’ signal within the embryo.

Key words: Nodal, lefty, Feedback inhibitor, Left-right axis, *Xenopus laevis*, antivin

INTRODUCTION

The vasculature and internal organs of vertebrates are arranged asymmetrically along the left-right (L-R) axis. In humans, deviations from the normal arrangement range from reversal of a few organs (situs ambiguus or heterotaxia), to reversal of all organs (situs inversus totalis). While the latter can be completely non-deleterious and remain undetected throughout life, other disturbances of asymmetry are often associated with complex cardiac defects, polysplenia, and venous malformations (Kosaki and Casey, 1998). The recent increase in our knowledge of the L-R axis determination process has come from two main directions.

First, a reverse genetic approach has led to the formulation of a basic network that regulates embryonic asymmetry, including intercellular signals such as Sonic hedgehog (*Shh*), *nodal*, *lefty1* and *lefty2*, and transcription factors such as *Pitx2* (for review, see Harvey, 1998). In chick, *Shh* expression is initially bilateral at Hensen’s node, but becomes stronger on the left at later stages, and at specific stages in chick and mouse, *nodal* also shows left-biased expression at the node (Collignon et al., 1996; Levin et al., 1995; Lowe et al., 1996). A relay factor ‘X’ has been proposed to propagate node-proximate asymmetries through the paraxial mesoderm outward to the left lateral plate mesoderm (LPM), which develops extensive left side expression of *nodal* and, with a slight delay, *Pitx2*. A candidate for factor X is Caronte (Car), a Cerberus-related secreted BMP antagonist so far studied only in chick embryos (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999; Zhu et al., 1999). Several features of *Car* (expression pattern, response to *Shh*, ability to induce *nodal* and randomize situs when misexpressed on the right side of the chick embryo), suggest that it relays node L-R asymmetries to the LPM. While the expression of *nodal* and *Pitx2* in the left LPM is conserved between vertebrates, the failure to find asymmetric *Shh* expression in mouse, *Xenopus*, and zebrafish highlights the need for further research into the regulation of L-R determination. In fact, very little is known about the very first events coordinating L-R axis specification in any vertebrate, although the asymmetric action of a L-R coordinator involving...
the TGFβ-related Vg1 factor has been suggested in *Xenopus* (Hyatt and Yost, 1998).

Second, the genes disrupted in two classical mouse mutants exhibiting situs defects, *iv* (inversus viscerum) and *inv* (inversion of turning), were recently identified. The gene mutated in *iv*, in which approximately 50% of the homozygous mutants show inverted laterality (plus some heterotaxia), encodes a protein similar to axonemal dynein heavy chains (Supp et al., 1997). In contrast, homozygous *inv* mutants usually have complete situs reversal, and the corresponding gene encodes a large putative extracellular protein with multiple ankyrin repeats (Mochizuki et al., 1998). Although their function remains unknown, *iv* and *inv* act quite early in the L-R pathway, since the laterality of *nodal*, *Pitx2* and lefty expression is drastically affected in the homozygous mutants (Campione et al., 1999; Lowe et al., 1996; Meno et al., 1996; Piedra et al., 1998; Ryan et al., 1998).

*Xenopus nodal* and *Pitx2* homologs have been characterized (Campione et al., 1999; Jones et al., 1995; Joseph and Melton, 1997; Ryan et al., 1998; Smith et al., 1995). Of the four *Xenopus nodal-related* genes (*Xnrs*), only *Xnr1* is expressed asymmetrically during early tailbud stages (Lowe et al., 1996), like mouse *nodal*, and seems to be involved in L-R determination (Sampath et al., 1997). Left LPM expression of *Xpitx2* begins later than *Xnr1* and continues on the left side of the heart and viscera during overt asymmetric morphogenesis. In addition, right-sided *Xnr1* misexpression induces *Xpitx2* expression and leads to the randomization of organ situs. Together, these observations place *Xnr1* upstream of *Xpitx2* and suggest that parts of the L-R determination cascade are conserved in *Xenopus*, chicken and mouse (Campione et al., 1999; Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998).

Two highly diverged TGFβ-like genes, *lefty1/2* and *lefty2*, play vital roles in the L-R axis determination pathway in mouse. During early somitogenesis, *lefty1* and *lefty2* are expressed predominantly in the left neural tube floor plate and left LPM, respectively (Meno et al., 1997). Mouse embryos deficient for *lefty1* have a variety of laterality defects, most commonly thoracic left isomerism (Meno et al., 1998). Moreover, most mutant embryos show bilateral expression of *lefty2*, *nodal* and *Pitx2*, suggesting that midline *lefty1* expression maintains left and right side identities by somehow restricting the passage of ‘left’ signals to the right side.

Several genes have been identified in chick and mouse that show unilateral expression on the right side of the embryo, including *activin*, *cSnR*, *FGF8*, and *nkx3.2*, and interfering with these patterns can reverse situs (Boettger et al., 1999; Meyers and Martin, 1999; Patel et al., 1999; Schneider et al., 1999). In chick, it has been proposed that right-sided activin signaling leads to the asymmetric perinodal *Shh* signal by repressing *Shh* expression on the right side of the node (Levin et al., 1995). The expression of *FGF8* and *nkx3.2* on opposite sides in chick and mouse is perplexing and underscores our rudimentary understanding of L-R determination. Overall, while significant data suggest important interplay between the L and R side gene cascades, a central conserved feature in all vertebrates studied to date is the left-sided expression of *nodal*.

Based upon the recent characterization of the lefty-related factor antivin (atv) from zebrafish (Thisse and Thisse, 1999), we searched for similar genes in *Xenopus*. Zebrafish antivin, like mouse lefty, can act to suppress mesoderm formation in embryos, and shows asymmetric left-sided expression during early somitogenesis. But, because of difficulties in misexpressing *atv* on the left or right in zebrafish embryos, and the absence of *atv* mutants, the role of lefty-related factors in non-mammals has not been fully defined. Here, we address the function of *Xenopus antivin* (*Xatv*), a probable left ortholog. We propose that *Xatv* acts during two phases of embryogenesis: the specification of mesendodermal fates during gastrulation and the establishment of the L-R axis at early tailbud stages. First, *Xatv* expression is induced by, and can antagonize, strong mesoderm inducing ligands such as Xnr2 and activin. Its expression pattern during gastrulation identifies *Xatv* as a likely endogenous negative regulator of mesoderm induction. At later stages, *Xatv* is expressed in the left LPM. Overexpression of *Xatv* on the left side of embryos suppresses left LPM expression of both *Xnr1* and *Xpitx2*, and results in severe defects in internal organ morphogenesis. We suggest that an active ‘left’ signal, involving *Xnr1/nodal*, is required for L-R axis determination during amphibian embryogenesis, and that negative feedback through the induction of *Xatv*/lefty ensures that the signal initiating asymmetric morphogenesis is transient.

**MATERIALS AND METHODS**

**Isolation of *Xenopus antivin***

Probing a *Xenopus* amplified dorsal lip library at low stringency with a 780 bp *Eco*47III-*XhoI* fragment of a zebrafish *antivin* cDNA (the ligand region and part of the 3′ UTR) yielded 53 overlapping *Xatv* cDNAs; the longest (848) was sequenced on both strands using the Sequenase II kit (USB). A 1.8 kb open reading frame represents the entire protein coding sequence, with 32 bp and 662 bp of 5′ and 3′ untranslated sequence, respectively. The nucleotide sequence of *Xatv* was deposited in GenBank (accession number AF209744).

**Embryo manipulations**

In vitro fertilization, manipulation, and staging were as described by Kay and Peng (1991) and Nieuwkoop and Faber (1967). To misexpress *Xnr1* or *Xatv* but minimize their influence on mesendoderm induction/gastrulation, we injected plasmid DNAs (Sampath et al., 1997), from which expression is first activated at midblastula transition (pXEX) or early gastrulation (pCSKA). Four-cell embryos with dorsal/ventral pigmentation differences (Nieuwkoop and Faber, 1967) were injected on the right or left with CsCl-purified pXEX or pCSKA vectors alone, or containing either *Xatv* or *Xnr1*. Injections were approx. 60-70° (site 1) or approx. 120° (site 2) from the dorsal midline and 20° above the equator. We described previously that pCSKA/β-galactosidase injections into site 1 or 2 gave similar lineage tracing patterns: labeling of anterior/posterior LPM, in addition to other tissues, and generally not crossing the midline as in Sampath et al. (1997). Data from injections into either site were pooled (Tables 1-4). pCSKA/Xatv or pXEX/Xatv were constructed by inserting the *Xatv* cDNA into the *Eco*RV site of pCSKA (Condie et al., 1990) or Smal site of pXEX (Johnson and Krieg, 1994). pCSKA/Xnr1 and pXEX/Xnr1 plasmids were described by Sampath et al. (1997). For scoring heart and gut looping at stage 43-45, embryos were fixed in MEMFA (Harland, 1991) and changed into methanol.

**Animal cap assays**

One-cell embryos were injected with RNA, animal caps explanted...
(stage 8/9), cultured in 0.75× normal amphibian medium (NAM), collected at stage 10.5 or 24, and flash frozen in dry ice/ethanol for RT-PCR analysis.

RT-PCR

RT-PCR analysis on various embryonic stages or animal caps was as described by Chang et al. (1997). Primers were:

RT-PCR analysis. 

Histological analysis

Embryos were dehydrated in an ethanol series, equilibrated to toluene:paraplast (Oxford Labware; 1:1 ratio) and paraplast embedded. Sections (10 μm) were counterstained with eosin (Sigma/Surgipath) or mounted directly.

**Fig. 1.** Structure and expression of Xatv.

(A) Xatv is 66%, 37% and 35% identical to zebrafish atv, mouse lefty-1 and lefty-2, respectively (overline indicates hydrophobic signal sequence; gray boxes, putative proteolytic cleavage sites; dashes, identical residues; dots, alignment gaps). Asterisks indicate cysteines of the TGFβ ligand ‘cysteine knot’; additional cysteines residues conserved between lefty/atv factors are indicated. (B) TGFβ/BMP, lefty, and Xnr1 expression during Xenopus embryogenesis (stages indicated; 0.1 embryo-equivalents/lane). FGFR, loading control; MBT, mid-blastula transition.
**Fig. 2.** Marginal zone and midline expression of Xatv. (A) Stage 10 embryo, dorsal view. Xatv transcripts are enriched dorsally in the marginal zone. (B-D) Stage 10.5, 11 and 12. Dorsovegetal views, dorsal up. Xatv expression within the forming dorsal midline. Arrowhead, dorsal lip; asterisk, yolk plug. (E) Stage 12, longitudinal section, approximate location shown in D. Xatv expression is seen in posterior neuroectoderm (open arrow), more anterior mesendoderm (solid arrow), and at lower levels in newly involuting mesendoderm (open arrowhead). Inset: higher magnification of posterior midline; Xatv is mostly expressed in deep neuroectoderm (bracket; dorsal lip indicated). (F) Stage 14. Midline expression, dorsal view, anterior left. Bracket shows the level of the section in G (arrowheads, circumblastoporal expression). (G) Stage 14, transverse section: Xatv expression in the prospective floorplate and underlying mesendoderm. (H) A more posterior section than in G shows Xatv expression only within the prospective floorplate. (I) Stage 17. Dorsal view, anterior left. (J) Stage 19. Dorsal view, anterior left. Inset: magnification of midline expression with transient notochord expression bracketed. (K,L) Stage 23 lateral view and stage 25 dorsal view, respectively (anterior left), showing Xatv expression in left dorsal endoderm (open arrowheads; see F for section), bilateral expression in the posterior dorsal endoderm (open arrows), and left LPM (solid arrowhead). (M) Stage 23. An anterior transverse section; Xatv expression in floorplate and hypochord. (N) Stage 25. (O) Stage 29/30. (P) Stage 25. Arrow, left dorsal endoderm expression. a, archenteron; b, blastocoel; fp, floorplate; n, notochord; h, hypochord.

**Fig. 3.** Asymmetric Xatv expression. (A-D) Uncleared embryos, (F-I) cleared embryos (stages indicated); lateral views, anterior left. Solid arrowheads, Xatv expression in left LPM; open arrowheads, Xatv expression in left dorsal endoderm. (E) Transverse section of stage 25 embryo; approximate location indicated in C. Note expression in floorplate and hypochord. Solid arrowhead, Xatv expression in the left LPM. (J,K) Stage 32. Sagittal and transverse section, respectively, of heart regions of an embryo double labeled for cardiac troponin I (pink) and Xatv (purple), showing Xatv expression in the looping heart tube (bracket). *sectioning artifact. Epidermal coloration is normal embryo pigmentation. c, cement gland; fp, floorplate; h, hypochord.
RESULTS

*Xenopus antivin*

The 367 amino acid Xatv is 66% identical (81% similar) to zebrafish atv, and approx. 35% identical (approx. 54% similar) to mouse lefty1/lefty2 (Fig. 1A), and has the following features characteristic of lefty proteins. (1) It contains 6 of the 7 cysteines of the TGFβ superfamily ‘cysteine knot’, but lacks the fourth cysteine normally involved in covalent ligand dimerization (Fig. 1B, Kingsley, 1994). (2) It lacks the α helix between the third and fourth cysteines thought to promote dimerization through non-covalent interactions (Kingsley, 1994), suggesting that both Xatv and lefty function as monomers. (3) The carboxyl terminus of most TGFβ-related ligands terminates CX₁CX₁, while Xatv ends CX₁CX₈, more similar to lefty (CX₁CX₁₃; Meno et al., 1996). (4) Mouse lefty1 and lefty2 contain two putative pro-mature region proteolytic cleavage sites (RXXR), which may be used cell-specifically (Meno et al., 1997); similarly located sites exist in Xatv (Fig. 1B).

*Xatv expression*

RT-PCR analysis (Fig. 1C) detected Xatv expression shortly after the onset of zygotic transcription and at all later stages, with slightly higher levels in gastrula and early tailbud embryos. Transcripts of approx. 2.7 kb were detected by northern blot analysis of poly(A)* RNA from stages 11 and 18 (data not shown).

The spatial expression pattern of Xatv was determined by in situ hybridization analysis (Figs 2, 3). Expression is first observed just prior to the onset of gastrulation (stage 10) in the marginal zone, with a dorsal emphasis (Fig. 2A). At gastrulation, Xatv expression is concentrated at the dorsal lip (Fig. 2B) and, compared to the organizer marker goosecoid, is more superficial and proximate to the lip, and extends more laterally (data not shown). As gastrulation continues, the Xatv signal forms a crescent over an approx. 30° region of the dorsal marginal zone (Fig. 2C) and, at stage 12, marks the forming dorsal midline (Fig. 2D). Lower expression encircles the yolk plug from stages 10.5 to 12 (Fig. 2B-D). In the midline of the neurula (Fig. 2E), Xatv is expressed in the anterior mesendoderm, and posteriorly in the neuroectoderm; expression in both germ layers occurs in a transitional domain at mid-trunk level. Expression in the neuroectoderm is mostly restricted to the deep layer, although labeled cells are found in the superficial layer (Fig. 2E, inset). Lower expression is apparent in the newly involving mesendoderm adjacent to the dorsal lip. In late neurula stage embryos (stage 14), Xatv is expressed predominantly in the dorsal axial midline (Fig. 2F). Anteriorly located sections show Xatv expression within the neuroectoderm and mesendoderm (Fig. 2G), while more posteriorly, Xatv is expressed in neuroectoderm only (Fig. 2H).

**Fig. 4.** Inhibition of Xnr2 and activin activity by Xatv. Animal caps injected with Xatv plus or minus Xnr2 or activin RNA (pg/embryo indicated) were assayed at stage 10.5 or stage 24. At 1:1 ratios (10 pg each RNA), marker induction was similar to that caused by Xnr2 alone. In contrast, a 10:1 mix of Xatv:Xnr2 RNA suppressed organizer-specific markers (cerberus, chordin, goosecoid and noggin). At 5:1 or 50:1 ratios of Xatv:activin RNA, Xatv suppressed all markers tested.

**Fig. 5.** Induction of Xatv in the LPM by Xnr1. pXEX/Xnr1 or pCSKA/Xnr1 was injected into the right side of 4-cell embryos to drive Xnr1 expression from late blastula or gastrula stages, respectively, and Xatv expression analyzed at stage 24-25. Dorsal views, anterior up. (A) Normal asymmetric expression of Xatv; (B) bilateral expression; (C) right side expression and (D) no expression. Bilateral expression predominated (Table 1). (E) Animal caps injected with Xnr1, Xnr2, or activin RNA were assayed at stage 10.5 for Xatv and Xbra. Xbra, a pan-mesodermal marker, measured induction efficiency. FGFR, loading control.
Consistent with the earlier expression around the yolk plug, expression is maintained around the posterior blastopore (Fig. 2F). During tailbud stages, Xatv is transiently expressed in the notochord anlage (Fig. 2J), but over a more prolonged period there is robust expression in the floorplate and the hypochord, a structure thought to originate from dorsal endoderm (Fig. 2I,J,M-P). Indeed, at earlier stages, 2 or 3 dorsal-most endodermal cells underlying the notochord are labeled per section (data not shown). The floorplate/hypochord expression begins posteriorly (Fig. 2I), spreads anteriorly, and at later stages essentially extends throughout the A-P axis (Fig. 2K,L). In both structures, however, expression is somewhat discontinuous, particularly at the mid-trunk level (Fig. 3F-I). Thus, individual sections show labeling in hypochord or floorplate alone, or in both tissues (Fig. 2M-P). From approx. stages 22-25, Xatv is expressed in the left dorsal endoderm over the anterior half of the embryo (Fig. 2K,L,P) with an anterior limit in the foregut region (data not shown). Posteriorly, dorsal endodermal expression is bilateral (Fig. 2L).

Xatv displays transient expression in the left LPM (Fig. 3A-D,F-I), and is not found in the paraxial or intermediate mesoderm (Fig. 2M,N) – a pattern similar to that of Xnr1 (Lowe et al., 1996). The LPM expression changes rapidly, initially appearing as a broad A-P band at the trunk level (stage 23; Fig. 3A,F), but becoming progressively restricted, anteriorly and ventrally, until it marks left ventral tissues within the heart precursor region (Fig. 3D,E,I). Sections of stage 28 embryos, when the myocardium is a simple epithelial sheet lying ventral to the just-formed endocardial tube, show that Xatv expression in the myocardium is not strictly unilateral, but is more extensive on the left than the right (data not shown). Later, Xatv is expressed on the right side of the definitive heart tube (Fig. 3K). In principle, the apparent left to right movement of expression within the cardiac tissue could arise from tissue movements and/or alterations in the expression domain. The precise relationship of Xatv expression with the L-R and A-P axes of the heart during the looping process is under investigation.

Xatv antagonizes Xnr2 and activin

Many TGFβ-like ligands can induce alterations in embryonic cell fate. Previously, chimeric BMP\textsuperscript{PRO}:lefty\textsuperscript{MAT} proteins, which may function non-physiologically, were shown to neutralize animal cap ectoderm, suggesting that lefty ligands have anti-BMP activity (Meno et al., 1998). We tested for potential inductive properties of Xatv by injecting RNA into embryos and assaying gene expression in explanted animal caps. Markers for several tissue types were analyzed: dorsal endoderm (cerberus), pan-endoderm (edd), organizer-specific (gsc, noggin, chordin, cerberus), pan-mesoderm (Xbra, muscle-specific actin), ventrolateral mesoderm (Xwnt8), and ventral mesoderm (globin). Xatv induced none of these markers (Fig. 4). Even high Xatv doses (1 ng/embryo) did not neutralize animal caps, as defined by the failure to induce NCAM/Koxt\textsubscript{2} and lack of effect on epidermal keratin expression (not shown).

To determine whether Xatv functions similarly to zebrafish atv, which antagonized mesoderm induction by activin in whole embryos (Thisse and Thisse, 1999), we coexpressed Xatv and a strong mesoderm inducer, Xnr2 (Fig. 4). At a 1:1 ratio (10 pg each RNA), marker induction was similar to Xnr2 alone. In contrast, a 10:1 ratio (100 pg Xatv: 10 pg Xnr2), resulted in the suppression of organizer-specific markers (gsc, noggin, chordin, cerberus) while mesodermal markers (Xbra, Xwnt8, or muscle-specific actin) were relatively unaffected (Fig. 4). Notably, globin, a ventral mesoderm marker, was reproducibly induced by 10:1 Xatv/Xnr2 RNA mixtures (Fig. 4). At higher ratios (500 pg Xatv:10 pg Xnr2), Xatv completely suppressed the induction of gsc, noggin, chordin and cerberus, while Xbra and Xwnt8 decreased slightly (not shown). Xatv also antagonized the dose-dependent mesoderm inducer activin (Fig. 4). At a 1:1 ratio (2 pg Xatv:2 pg activin RNA), marker induction was similar to activin alone, although globin was induced by this combination once (data not shown). There was some variability in the response to mixtures of 5:1 or 50:1 of Xatv/activin RNAs (2 pg activin RNA used in each case), perhaps reflecting different responsiveness of animal caps from separate embryo batches. In two cases (5:1 or 50:1 mixtures), there was complete suppression of all general and dorsal mesendodermal markers tested (Fig. 4); while in one (a 5:1 Xatv/activin mixture) only the dorsal markers chordin, cerberus and goosecoid were completely suppressed (not shown). Although saturation loading of the translational machinery usually occurs at doses of several nanograms of RNA, we tested whether the suppressive effects of Xatv could be attributed to non-specific competition. Marker induction by a mixture of a translatable, irrelevant RNA (β-globin) with Xnr2, at doses up to 500 pg β-globin:10 pg Xnr2, was almost identical to that caused by 10 pg Xnr2 alone (data not shown), indicating that Xatv specifically blocks nodal signaling pathways.

The potential for different translational efficiencies of these RNAs prevents conclusions on the amounts of active Xnr2, Xatv, or activin produced. Clarification of the stoichiometries of these antagonistic interactions may require purified functional proteins. Nonetheless, our data suggest that Xatv does not act as an inducer itself, but antagonizes potent mesoderm inducers such as Xnr2 and activin, thereby modulating cell fate specification during mesendodermal induction.

Xnr1 induces asymmetric Xatv expression

The unilateral expression during tailbud stages suggests that Xatv is involved in L-R axis determination. LPM expression of Xnr1 begins (stage 18/19) earlier than Xatv (stage 23), potentially placing Xatv downstream of Xnr1 in the L-R pathway. To test this hypothesis, we delivered plasmids encoding Xnr1 to either the left or right side of four-cell embryos and analyzed Xatv expression at stage 23-25 (see Fig. 3).

Embryos receiving left injections of Xnr1 plasmids retained Xatv expression in the left LPM (Fig. 5A; Table 1). In contrast, the majority of embryos receiving right-sided Xnr1 showed perturbed Xatv patterns, including expression bilaterally, or on the right only. The incidence of embryos bilaterally lacking Xatv expression was not altered significantly from controls (Fig. 5B-D; Table 1). A dose-dependent effect was seen (Table 1): at low doses (20 pg), most embryos retained left-sided Xatv expression, while 12% showed bilateral expression (Table 1). At higher doses (100 pg), most embryos had bilateral Xatv expression (55%; Table 1), and another 30% displayed right-side only expression. The induced ectopic expression pattern had mirror-image spatiotemporal characteristics of the
endogenous left-side expression of Xatv, suggesting that right-sided Xnr1 misexpression triggered a physiological left-side gene expression response. Consistent with previous reports (Sampath et al., 1997), section analysis showed that Xnr1 did not induce ectopic tissues or secondary axes that might induce additional Xatv expression.

The inductive relationship between Xnr1 and Xatv in tailbud stage LPM led us to test for a similar relationship during gastrulation. Xatv was robustly and rapidly induced in animal caps by Xnr1, Xnr2, or activin RNA (Fig. 5E), or activin protein (data not shown). Together with the overlapping spatiotemporal expression of Xnr1/Xatv during both stages of normal development, these data strongly suggest that Xnr1 functions upstream of Xatv in both mesendodermal specification and L-R determination.

### Table 1. Right-sided Xnr1 delivery perturbs asymmetric Xatv expression

<table>
<thead>
<tr>
<th>Injections</th>
<th>Side inj.</th>
<th>n</th>
<th>% left</th>
<th>% right</th>
<th>% bilateral</th>
<th>% neither</th>
</tr>
</thead>
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<tr>
<td>Uninjected</td>
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<td>65</td>
<td>88</td>
<td>-</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>Vector controls 100 pg</td>
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<td>42</td>
<td>95</td>
<td>-</td>
<td>-</td>
<td>5</td>
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<tr>
<td>pXnr1 20 pg</td>
<td>R</td>
<td>66</td>
<td>79</td>
<td>6</td>
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<td>3</td>
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<td>pXnr1 100 pg</td>
<td>L</td>
<td>72</td>
<td>89</td>
<td>-</td>
<td>3</td>
<td>8</td>
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</table>

Embryos injected (doses indicated) with pXEX/Xnr1 or pCSKA/Xnr1 on the left or right were analyzed for Xatv expression in the left LPM at stage 23-25.

*All surviving embryos were examined; this table includes only those with overall normal A-P and D-V axes (n scored; see Results text). Data were pooled from four experiments. -, not observed; neither, complete absence of expression. inj, injected; NA, not applicable.

### Table 2. Left-sided Xatv overexpression suppresses asymmetric XPitx2 expression

<table>
<thead>
<tr>
<th>Injections</th>
<th>Side inj.</th>
<th>n inj.</th>
<th>n scored*</th>
<th>% left</th>
<th>% right</th>
<th>% bilateral</th>
<th>% neither</th>
</tr>
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<td>NA</td>
<td>46</td>
<td>93</td>
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<td>119</td>
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<td>-</td>
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<td>92</td>
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<td>-</td>
<td>8</td>
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<tr>
<td>pXatv 100 pg</td>
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<td>124</td>
<td>90</td>
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<td>-</td>
<td>10</td>
</tr>
</tbody>
</table>

Embryos injected (doses indicated) with pXEX/Xatv or pCSKA/Xatv on the left or right were analyzed for XPitx2 expression in the left LPM at stage 24/25.

‡The alterations caused by Xatv injections are statistically significant compared to vector-injected controls (P<0.001, Fisher’s exact test). See Table 1 for other notations and abbreviations.

### Table 3. Left-sided Xatv overexpression suppresses asymmetric Xnr1 expression

<table>
<thead>
<tr>
<th>Injections</th>
<th>Side inj.</th>
<th>n inj.</th>
<th>n scored*</th>
<th>% left</th>
<th>% right</th>
<th>% bilateral</th>
<th>% neither</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninjected</td>
<td>NA</td>
<td>NA</td>
<td>79</td>
<td>95</td>
<td>-</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Vector controls 100 pg</td>
<td>R</td>
<td>79</td>
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<td>97</td>
<td>-</td>
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<td>1.5</td>
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<tr>
<td>pXatv 20 pg</td>
<td>L</td>
<td>75</td>
<td>73</td>
<td>99</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>pXatv 100 pg</td>
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<td>86</td>
<td>83</td>
<td>78</td>
<td>-</td>
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<td>22</td>
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Embryos injected (doses indicated) with pXEX/Xatv or pCSKA/Xatv on the left or right were analyzed for Xnr1 expression in left LPM at stage 21/22 (data pooled from four experiments).

‡Alterations caused by Xatv injections are statistically significant compared to vector-injected controls (P<0.001, Fisher’s exact test). Other notations and abbreviations as in Table 1.
may account for the loss of X Pitx2 (see Fig. 8); other approaches will be needed to test whether Xatv blocks X Pitx2 expression directly.

**Effect of Xatv misexpression on asymmetric organogenesis**

As reported previously, right-sided misexpression of Xnr1 (Sampath et al., 1997) and X Pitx2 (Campione et al., 1999; Ryan et al., 1998) can randomize the direction of heart and gut looping (e.g. Fig. 7C,D) when scored within a population of embryos. We assessed the consequences of Xatv misexpression on organ morphology and situs to correlate them with the molecular alterations in Xnr1/X Pitx2 expression described above. Embryos injected with pCSKA/Xatv or pXEX/Xatv on the left or right sides were cultured until stage 43-45 and scored for overall external/internal morphology, and situs of the heart and viscera (Fig. 7; Table 4). In these experiments, a small percentage of injected embryos failed to gastrulate, perhaps due to DNA toxicity; lower doses (20 pg) allowed greater survival than higher doses (100 pg; Table 4). In addition, higher survival occurred in embryos injected with pCSKA/Xatv than with pXEX/Xatv (Table 4), possibly reflecting a deleterious effect of earlier expression from pXEX (see Methods). Because defects in axial development affect L-R specification (e.g. Yost, 1998), embryos with abnormal A-P patterning (approx. 5% of injected embryos) were not analyzed for situs alterations. Of the remainder, 89% exhibited normal axial development (Fig. 7J), while 11% displayed ventral abnormalities (Fig. 7M), including edema and swelling. The latter may be associated with the defects in the visceral and cardiac systems described below.

Most embryos (63%; Table 4) injected on the left side with the highest Xatv dose (100 pg) developed indeterminate situs and/or dysmorphogenesis of both the heart and gut (54%), or gut alone (9%; Fig. 7I-O; Table 4). This incidence of visceral dysmorphogenesis (63%) is highly significant (P < 0.001) compared to vector controls (16%; vector, 100 pg L) using Fisher’s exact test, and agrees quite well with the 74% and 69% incidence of bilateral absence of Xnr1 and X Pitx2, respectively (Tables 2, 3). If the bilateral absence of Xnr1/X Pitx2 expression caused by left-sided Xatv misexpression were to cause global situs randomization, an approx. 35% incidence of combined heart/gut reversal would be predicted. In contrast, only a small fraction of left side-injected embryos developed situs reversals of both heart and gut (2/127; approx. 2%), heart alone (19/127; 15%), or gut alone (2/127; approx. 2%). Of the 19 with reversed hearts alone, gut situs was normal in seven, but indeterminate in the remaining twelve. Interestingly, a significant number of embryos (32%, compared to 20% in vector controls; P < 0.05) receiving high doses of Xatv on the right also displayed visceral organ malformations (not shown) similar to those arising from left-sided injections. This result is consistent with the observation that some right-side injected embryos developed bilateral absence of Xnr1 (22%) and X Pitx2 (19%) at tailbud stages (Tables 2, 3). One possibility is that the

<table>
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<th>Injections</th>
<th>Side inj.</th>
<th>n inj.</th>
<th>n scored*</th>
<th>Heart/gut reversed</th>
<th>Gut reversed</th>
<th>Heart reversed</th>
<th>Gut reversed</th>
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<td></td>
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<td>163</td>
<td>83</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>pXatv 20 pg</td>
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<td>L</td>
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<td>127</td>
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<td>6</td>
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</table>

Embryos injected (doses indicated) with pXEX/Xatv or pCSKA/Xatv on the left or right were scored at stage 43-45 for heart and visceral orientation (data pooled from six experiments, presented as percentages).

‡Alterations caused by Xatv injections are statistically significant compared to vector-injected controls (P < 0.001, Fisher’s exact test). Notations as in Table 1 also apply. indet, indeterminate situs.
inconclusive indicated; see Table 4 for details).

In three experiments, the percentage of embryos bilaterally scored (Tables 2-4).

The different stages of analysis for Xnr1 or X Pitx2 expression, or organ situs, preclude comparison of all three characteristics within the same embryos. To correlate as directly as possible the Xatv-mediated suppression of Xnr1/X Pitx2 expression with the situs abnormalities, we carried out experiments (not shown) in which one-third each of a batch of left-side Xnr1-injected embryos were analyzed for Xnr1, or X Pitx2, or allowed to develop further for situs scoring. In three experiments, the percentage of embryos bilaterally lacking Xnr1 or X Pitx2 correlated well with the incidence of indeterminate situs/malformations of either the heart or gut, or both organs, and the overall results were completely consistent with the experiments in which these features were individually scored (Tables 2-4).

The complex spatiotemporal expression profile of Xatv during embryogenesis indicates a role in several developmental processes. Our functional data imply that Xatv acts as a feedback antagonist of Nodal signaling in two of these: mesendoderm induction and L-R axis specification. The induction of Xatv by Xnr signaling in animal caps is consistent with the temporal appearance of Xatv after Xnr in normal embryogenesis. Our results, in addition to those from other laboratories, imply that mesendoderm induction in vivo is the result of a spatiotemporally coordinated balance between cell-autonomously activated inducers (e.g. Zorn et al., 1999), positive feedback Xnr relays (Osada and Wright, 1999), and negative feedback via Xnr-induced factors such as Xatv. Another general conclusion is that, in addition to Nodal signaling playing an essential role in mesendoderm induction in Xenopus, zebrafish and mice (Collignon et al., 1996; Osada and Wright, 1999; Piccolo et al., 1999; Zhou et al., 1993), the lefty/antivin factors seem to be conserved negative regulatory influences on this pathway (Bisgrove et al., 1999; Meno et al., 1999; Thissie and Thissie, 1999).

Asymmetric Xnr1 expression during tailbud tadpole stages induces asymmetric Xatv expression, and a negative feedback loop through Xatv-mediated antagonism is again indicated by our observation that Xatv overexpression suppresses Xnr1/X Pitx2 expression in the left LPM. Overall, we suggest that induction of Xatv is important to limit the amount of mesoderm induction at gastrulation, and ensure the transient nature of ‘left signals’ within tailbud stage LPM that are essential for asymmetric morphogenesis.

**Xatv – a lefty ortholog**

The structure, function and expression pattern of Xatv argue that it is a true lefty ortholog. The level of identity between Xatv and mouse lefty1/lefty2 (approx. 35% over the whole protein) is lower than for other TGFβ-like factors such as BMP4, but similar to that between mammalian and frog nodal orthologs (approx. 36% overall). Two zebrafish lefty-like
genes, zlf1 (which probably corresponds to antivin) and zlf2, were recently described (Bisgrove et al., 1999). From considerations of sequence and expression patterns, it is unclear whether mouse lefty1 and lefty2 are homologous to zebrafish atv/zlf1 and zlf2, respectively, or if both pairs arose by species-specific gene duplication. It is similarly difficult to make definitive homology arguments for Xatv; while the Xenopus genome may contain additional Xatv/lefty-related genes, we only isolated the A/B pseudotetraploid copies of the same gene.

A general conclusion from our studies is that the Xatv expression pattern seems to comprise aspects of those of lefty1/lefty2 and zlf1/zlf2, with additional species-specific domains. Like lefty2 (Meno et al., 1997), Xatv is expressed during gastrulation in the area of mesoderm formation. Xatv becomes progressively restricted to the embryonic midline during neurulation and, following neural tube formation, axial Xatv expression marks the neural tube floorplate, notochord anlage, hypochord and dorsal endoderm. The symmetric expression of Xatv in the floorplate differs from the left floorplate expression of lefty1 in mouse (Meno et al., 1997), but species-specific anatomical differences in this structure may contribute to this disparity. A requirement for full axial midline specification in the development of normal visceral asymmetry (Yost, 1998) may be connected to the role of lefty ligands in preventing ‘left signals’ crossing to the embryo’s right side (Meno et al., 1998). Xatv expression within the axial midline may serve a similar function in Xenopus.

Two Xatv expression domains not noted for lefty1/atv genes in fish or mouse are the left dorsal endoderm in the anterior of tailbud Xenopus embryos, and the hypochord. Both tissues have been linked to normal cardiovascular development in vertebrates. For example, extirpation of anterior endoderm in frogs decreases the frequency of beating heart tissue (Nascone and Mercola, 1995), and in mouse, it has been shown that GATA4-expressing endoderm is required for normal heart morphogenesis (Narita et al., 1997). An inductive role for the hypochord as a VEGF source has been proposed in dorsal aorta formation (Cleaver and Krieg, 1998). Xatv may modulate the function of signaling molecules involved in these activities, or additional inductive influences. In particular, we speculate that the left-sided dorsal endoderm expression in anterior regions may be connected to the heart and/or gut looping process.

Later in development, Xatv is asymmetrically expressed in the left LPM, like atv/zlf1, zlf2, and mouse lefty2 (Bisgrove et al., 1999; Meno et al., 1997; Thisse and Thisse, 1999). Zebrafish atv/zlf1 is also expressed very transiently in the left dorsal diencephalon; further examination will be necessary to determine if corresponding expression occurs in Xenopus.

**Xatv and mesendodermal patterning**

The ability of Xatv to suppress mesoderm induction by activin or Xnr2 in animal caps mimics the activities of zebrafish atv and mouse lefty in zebrafish embryos (Thisse and Thisse, 1999). Xatv is induced by Xnr signaling, and Xatv blocks induction by Xnr. In the left LPM, Xatv can suppress Xnr1 expression. These results are consistent with concurrent studies carried out in mouse and zebrafish (Meno et al., 1999). A parsimonious model to explain these observations is that negative feedback modulation by Xatv of the level of Xnr expression acts to regulate the amount of mesendoderm induced by Nodal/Xnr signaling, and/or affect its dorsoventral/anteroposterior character. This hypothesis is concordant with the expanded nodal expression and overproduction of nascent mesoderm in lefty2 homozygous null mouse embryos. A good example of the modification of mesodermal character by Xatv is the reduction of organizer markers and appearance of globin expression induced by Xnr/Xatv coexpression (Fig. 4). Presumably, the precise regulation of the expression levels of these dose-dependent inducers and feedback antagonists is tightly linked to cell determination during gastrulation. It would thus be useful to understand the spatiotemporal distributions of these factors at the protein level in relation to high resolution embryonic fate maps.

Our observation that Xatv cannot neutralize ectoderm implies that it selectively antagonizes inducers like activin/Xnr, and not BMPs. In fact, Xatv might upregulate BMP activity in vivo, without necessarily affecting BMP transcription, if it were to reduce the Xnr-mediated induction of BMP inhibitors such as chordin and noggin (e.g. Osada and Wright, 1999; this paper). Future experiments will address whether Xatv antagonizes other mesendoderm inducers (e.g. derrière; Sun et al., 1999).

Regarding Xatv expression in the anterior mesendoderm of neurula stage embryos (e.g. Fig. 2E), Xnr4 is also expressed in this tissue (Joseph and Melton, 1997), suggesting a potential functional interaction between these two factors in anterior tissue specification.

As already suggested for zebrafish atv/zlf1 (Bisgrove et al., 1999; Thisse and Thisse, 1999), Xatv might antagonize Xnr2 or activin in several ways: direct binding to the inducing ligand, stimulating an Xatv receptor followed by intracellular suppression, or by binding Xnr/activin receptors thereby preventing ligand access. Previous data are consistent with the latter mechanism (Meno et al., 1999; Thisse and Thisse, 1999). Our experiments suggest that there are differences in the ability of Xatv to suppress activin and Xnr2. For example, while 100 pg of Xatv RNA could completely suppress activin-mediated mesendodermal induction, the same dose reproducibly suppressed only the induction of organizer-specific markers by Xnr2 (Fig. 4). The relevance of this observation is currently unclear. Based upon work in fish and mouse, however, signaling by nodal (unlike activin) seems to require the presence of facilitating factors in the EGF-CFC family (Gritsman et al., 1999; Shen et al., 1997). Thus, the interactions of Xnr/Xatv with the receptor signaling system may be significantly different from those for activin/Xatv.

**Absence of ‘left’ signals disrupts asymmetry**

Right-sided Xnr1 misexpression induces a mirror-image duplication of the left expression pattern of Xatv (Fig. 5; Table 1). This effect reiterates the inductive relationship between Xnr and Xatv in animal caps, and suggests a level of commonality between the processes of mesoderm induction and L-R specification. Conversely, left-sided Xatv overexpression downregulates or abrogates the endogenous asymmetric expression of Xnr1 and XPitx2 (Fig. 6; Tables 2, 3).

Our data indicating that Xatv acts to suppress ‘left’ pathways in Xenopus are largely concordant with functional studies of mouse lefty1 in chick embryos (Yoshioka et al., 1998). Implantation of cells producing lefty1 or lefty2 on either side of the node in stage 5 chick embryos led to the suppression of
left-sided nodal and Pitx2 expression in approx. 50% of the embryos. In our case, in addition to the highly efficient abrogation of endogenous Xnr1/XPitx2 expression caused by left-sided Xatv misexpression, a lower percentage (approx. 20%) of frog embryos showed bilateral absence of Xnr1/XPitx2 expression in response to right-sided Xatv delivery. This variability between chick and frog may reflect the different misexpression methods used. In both species, the ability of right-sided delivery to suppress ‘left’ gene programs contralaterally may represent secretion across the midline, or an effect on tissues other than the LPM. The observation that placing lefty-expressing cells in the right LPM of older chick embryos (stage 7+) induced right-sided Pitx2 expression (Yoshioka et al., 1998) is difficult to understand, since we did not detect this inductive property for Xatv in frog embryos. It is possible that this observation in chick represents a species-specific outcome, an indirect effect on the axial midline/node, or a non-physiological response related to the dose and/or timing of lefty misexpression.

**L-R morphogenesis and left signals**

The current explanation for the way that bilateral LPM expression of nodal/Xnr1 and Pitx2/XPitx2 leads to situs randomization involves a hypothetical ‘stochastic selection step’, at the global or organ-specific level, in which either the endogenous or the ectopic left signal becomes dominant and triggers normal or reversed asymmetric morphogenesis (e.g. Sampath et al., 1997).

The absence of ‘left’ Xnr1/XPitx2 signals seen in Xatv-injected Xenopus embryos may lead to organ dysmorphogenesis via an intermediate bilateral ‘right’ state (right isomerism), and addressing this point will require further study, including the development of additional molecular markers and higher resolution morphological criteria for organ situs in Xenopus. In mouse, specific anatomical criteria (some of which, e.g. lung lobation, are not found in frogs) allow fairly detailed characterization of non-global situs alterations, for example, pulmonary or thoracic isomerisms, and more complex cardiovascular system alterations such as those seen in lefty1 mutants (Meno et al., 1998). In contrast, here we have only broadly categorized the cardiac and visceral orientation in Xenopus as normal, reversed, or indeterminate. Despite this caveat, the ability of left-sided Xatv overexpression to suppress Xnr1/XPitx2 expression in the left LPM, and the inability of these embryos to undergo normal L-R asymmetric morphogenesis, suggests that Xatv functions in vivo to downregulate the expression and activity of ‘left’ signals (Xnr1, XPitx2) that are essential for L-R axis development.

One important possibility that is hard to rule out is that the dysmorphogenesis seen in a low percentage of embryos receiving Xatv on the right side reflects interference with a right side gene cascade (see Introduction), rather than contralateral secretion and interference with ‘left pathways’. While the lack of suitable markers is again a hindrance, the failure of right-sided Xatv expression to induce Xnr1 or XPitx2 on the right, or to generate a high incidence of bilaterally absent Xnr1/XPitx2 expression, does suggest that such right side interference cannot produce left isomerism.

Thus, our data overall fit better with the hypothesis that ‘left’ signals (involving Xnr1/XPitx2), which can be blocked by Xatv misexpression, are essential for asymmetric morphogenesis. The right pulmonary isomerism observed in Pitx2−/− mice (Lin et al., 1999) supports this idea. On the other hand, treatment of mouse embryos with retinoic acid antagonists, which apparently blocks lefty, nodal and Pitx2 expression, causes situs randomization (Chazaud et al., 1999). An important experiment will be to test the effect on L-R morphogenesis of selectively removing left-sided nodal expression from mouse embryos, perhaps by conditional gene inactivation.

Although we cannot determine when the Xatv-mediated block to the left gene cascade occurs, when assayed at stages corresponding to the earliest phase of asymmetric Xnr1 expression, left-sided Xatv delivery causes bilateral absence of Xnr1 at an approx. 70% frequency (similar to the incidence in Table 3; data not shown). It will therefore be important to determine how Xnr1-Xatv function is linked to earlier steps in L-R axis specification, including the actions of the putative L-R coordinator, Vg1 (Hyatt and Yost, 1998). Additional outstanding issues include whether Xatv affects only the LPM, and/or organizer-proximate L-R specification processes occurring around gastrulation, and if it affects Xnr1 alone and/or additional signals.

**Xatv/lefty function in asymmetric morphogenesis**

We present a model for Xatv function in L-R axis determination in frog embryos (Fig. 8). The expression domain of Xnr1 in the left LPM changes rapidly with time. It is first activated posteriorly, then rapidly expands anteriorly through the left LPM and begins to undergo retraction posteriorly. In anterior regions, expression shifts forward to abut the heart anlage (Lowe et al., 1996). The mechanisms regulating this dynamic movement, which runs against the rostral-caudal direction of tissue specification, are unknown, but the degree of shifting is too large to be accounted for by cell migration. Primarily, there must be spreading of Xnr1 expression anteriorly and progressive downregulation posteriorly. In our model, the inducer/inhibitor relationship between Xnr1/Xatv constitutes a self-regulating negative feedback loop (Fig. 8).

First, Xnr1 induces Xatv and XPitx2 expression in a spatiotemporal pattern following that of Xnr1. With a built-in delay, the induction of Xatv then inactivates Xnr1 expression in the same spatiotemporal manner, beginning posteriorly. This feedback loop would serve to prevent the inappropriate spread of Xnr signals through autoactivation (Osada and Wright, 1999; Sajioh et al., 2000; S. Osada and C. V. E. W., unpublished data), and ensure transient Xnr1 expression as a trigger of asymmetric morphogenesis. Since XPitx2 expression is prolonged compared to Xnr1, it presumably escapes Xatv-mediated inactivation. As a transcription factor, XPitx2 then begins to stabilize the initially labile L-R asymmetries by activating, together with other factors, the downstream effector programs that directly drive asymmetric morphogenesis within the organ anlagen. At later stages, the Xatv expression within the developing heart, after the termination of asymmetric Xnr1 transcription, suggests that Xatv modulates inductive influences of perdurant Xnrs, or that Xatv expression is also induced or maintained by other signaling molecules involved in later aspects of L-R differential morphogenesis.

Aspects of this model may be conserved in other vertebrates. In chick and mouse, examination of the published literature indicates that nodal expression also spreads in a stereotypical fashion through the LPM (Collignon et al., 1996; Logan et al., 1999).
REFERENCES


Randall, A. M. S. Cheng and others
Feedback inhibition of nodal signaling


