channel-targeted excitotoxins[8–14] analogous to those used by *C. purpurascens*. □

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Left–right asymmetric expression of the TGFβ-family member *lefty* in mouse embryos

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Examples of lateral asymmetry are often found in vertebrates, such as the heart being on the left side, but the molecular mechanism governing the establishment of this left–right (L–R) handedness is unknown[1]. A diffusible morphogen may determine L–R polarity[2], but a likely molecule has not so far been identified. Here we report on the gene *lefty*, a member of the transforming growth factor-β family, which may encode a morphogen for L–R determination. Lefty protein contains the cysteine-knot motif[3] characteristic of this superfamily[4,5] and is secreted as a processed form of relative molecular mass 25K–32K. Surprisingly, *lefty* is expressed in the left half of gastrulating mouse embryos. This asymmetric expression is very transient and occurs just before the first sign of lateral asymmetry appears. In the mouse mutants *iv* and *inv*, which cause *situs inversus*, the sites of *lefty* expression are inverted, indicating that *lefty* is downstream of *iv* and *inv*. These results suggest that *lefty* may be involved in setting up L–R asymmetry in the organ systems of mammals.

By applying a systematic subtraction procedure to the P19 embryonal carcinoma cells[6], we have recently isolated a number of undifferentiated cell-specific complementary DNA clones[7]. One such clone (549) was a new gene related to transforming growth factor-β (TGF-β) (Fig. 1), which we call *lefty*. The six cysteine residues that are conserved among TGFβ-related proteins and which are necessary to form the cysteine-knot structure[3], are also found in Lefty at the predicted positions (Fig. 1*c*). However, the entire sequence of Lefty shares only 20–25% similarity with known members of the TGF-β superfamily.

The *lefty* gene product has unique structural features. First, whereas the carboxy terminus of the TGFβ-related proteins is usually CX_1CX_1, Lefty has a longer C-terminal sequence, CX_1CX_{13}. Second, Lefty lacks the cysteine residue required for dimer formation through the disulphide linkage (Fig. 1*b*, *c*). Finally, TGFβ-related members are initially synthesized as a prepro-protein, which is subsequently cleaved at an RXXR site to release a mature form of 110–140 amino-acid residues. In Lefty, RGKR and RQKR are found at amino-acid residues 74–77 and 132–135, respectively (Fig. 1*a*, *b*). If one of these sequences is a cleavage site, a mature protein of 291 or 233 amino acids long should be produced, which is much larger than mature forms of other TFGβ-related proteins.

We have analysed the Lefty protein using the anti-Lefty antibodies αL1 and αL2 (Fig. 2). First, we transfected 293T cells[8] with a *lefty* expression vector. Using western blot analysis, we detected a 42K protein in the transfected cells (Fig. 2). The size of this protein is consistent with that of the prepro-protein (or pro-protein). However, Lefty protein was not detected in conditioned medium. The *lefty* gene was next expressed in BALB/3T3 cells. The principal form of the Lefty protein in these cells had a relative molecular mass of 32K (Fig. 2). This value corresponds to cleavage at RGKR (amino-acid residues 74–77). On the other hand, 25K–27K as well as 32K proteins were detected in conditioned medium (Fig. 2). The size of 25K–27K corresponds to a form cleaved at RQKR (residues 132–135). Apparently, BALB/3T3 cells could secrete these processed forms. These results indicate that the Lefty prepro-protein can be differentially processed depending on the cells in which it is synthesized.

The expression of *lefty* in post-implantation mouse embryos was examined by whole-mount *in situ* hybridization (Fig. 3). In pre-primitive streak embryos at 6.0 days post-coitum (d.p.c.), *lefty* messenger RNA was undetectable (data not shown). When the primitive steak appeared (6.5 d.p.c.), *lefty* mRNA was detected in the anterior half of the streak (Fig. 3*a*). At 7.0 d.p.c., *lefty* is expressed along the streak except at its caudal and rostral extremities (Fig. 3*b*). Transverse sections made form the same embryo showed *lefty* expression in mesoderm (Fig. 3*c*). At this stage, there was no apparent sign of asymmetry in the expression. On 7.5 d.p.c., *lefty* expression decreased; only a low level was detectable in some mesoderm cells (data not shown). *lefty* mRNA then disappeared at the presomite headfold stage (7.75 d.p.c.; data not shown).

In embryos with a few pairs of somites (8.0 d.p.c.), *lefty* was highly expressed in two regions: the lateral region and the region near the ventral midline (Fig. 3*d*, *e*). The former region extended laterally along the anteroposterior axis, with its anterior margin caudal to the primordial heart and its posterior margin near the allantois (Fig. 3*e*). Surprisingly, however, expression was observed only on the left side (Fig. 3*d*). Transverse sections prepared from

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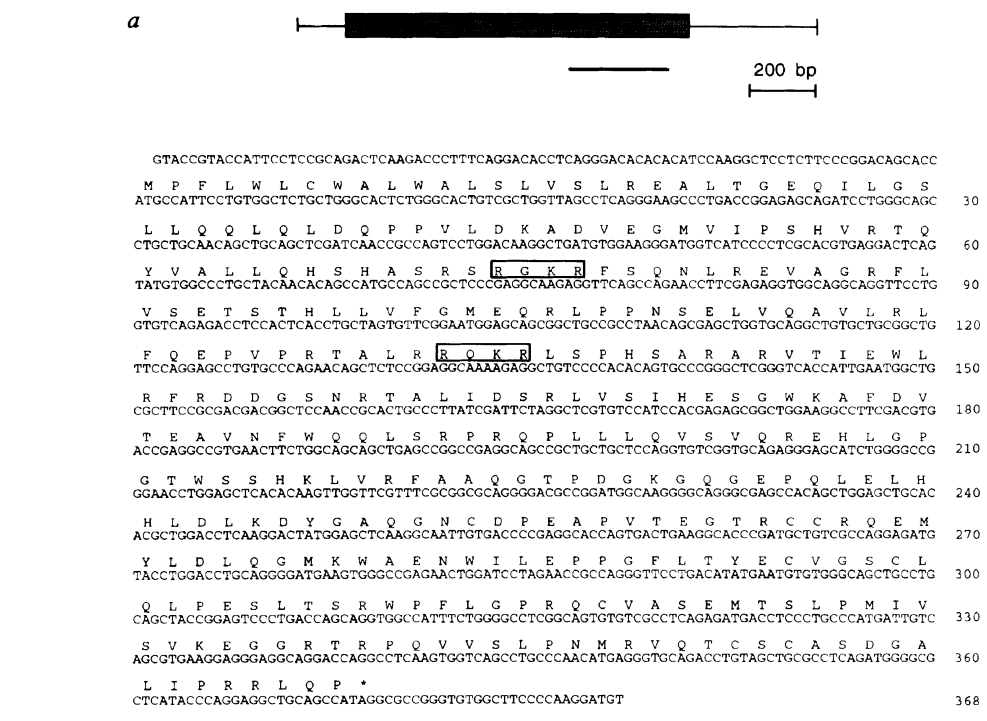
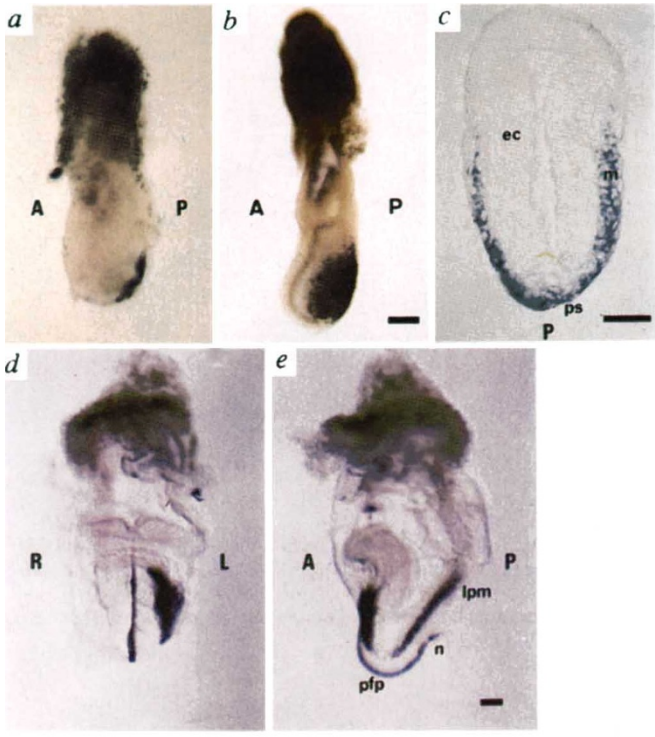


FIG. 1 *lefty* is a novel member of TGFβ superfamily. *a*, Structure of a full-length *lefty* cDNA clone (1.6 kb) is shown at the top, the filled box indicating a protein-coding region and the bar the *Aat*I 0.5-kb fragment used as a probe for whole-mount *in situ* hybridization. The nucleotide sequence of *lefty* mRNA and the predicted amino-acid sequence are shown below. Two putative proteolytic cleavage sites, RGKR and RQKR, are boxed. *b*, Comparison of overall structures of TGF-β and Lefty prepro-proteins. Hatched region, signal sequence; black, proprotein region; white, mature protein. *c*, Amino-acid sequence homology between Lefty and several other TGFβ-related proteins. Highly conserved cysteine residues are boxed.

METHODS. A derived cDNA library was constructed from undifferentiated P19 cells. This cDNA library was subtracted with mRNA from retinoic-acid-induced differentiated P19 cells (mainly neurons and astrocytes). A partial cDNA clone for *lefty* (clone no. 549) was obtained as one of the undifferentiated cell-specific clones[7]. Using clone 549, several full-length cDNA clones were isolated from the same cDNA library.

FIG. 3 Transient and asymmetrical expression of *lefty* in post-implantation embryos. *lefty* expression in various stages of mouse embryos was examined by whole-mount *in situ* hybridization. The developmental stage of each embryo was: *a*, 6.5 d.p.c.; *b* and *c*, 7.0 d.p.c.; *d* and *e*, 8.0 d.p.c., with 3–4 pairs of somites. *c*, Transverse section of a 7.0 d.p.c. embryo. *d*, Ventral view of an 8.0-d.p.c. embryo showing the asymmetrical expression of *lefty*. *e*, Lateral view (from the left) of an 8.0-d.p.c. embryo showing the extension of *lefty* expression along the anteroposterior axis. Anterioposterior (A–P) and R–L axes are indicated. *f–n*, Serial transverse sections made from an 8.0-d.p.c. embryo; seven representative sections (*f–l*) are shown. Note that *lefty* expression in the splanchnopleure is exclusively on the left side. *m*, Magnified view of the area inside the rectangle in panel *i*, emphasizing the asymmetry of *lefty* expression in the prospective floorplate. Approximate position of each section is shown in *n*. In *o*, the sites of *lefty* expression in 8.0-d.p.c. embryos are summarized: *lefty*-expressing areas are shown in red in a ventral view of an 8.0-d.p.c. embryo. ► Abbreviations: al, allantois; ec, embryonic ectoderm; fg, foregut; lpm, lateral plate mesoderm; m, embryonic mesoderm; n, node, nf, neural fold; pfp, prospective floor plate; pmc, promyocardium; ps, primitive streak; sm, somite; sp, splanchnopleure. Scale bars, 100 μm (*b*,*e*) and 25μm (*c*).

METHODS. Embryos of ICR mice (Charles River) were prepared for whole-mount *in situ* hybridization according to ref. 17. To generate antisense and sense probes, the *Aat*I 0.5-kb fragment from a cDNA clone (thick bar in Fig. 1*a*) was subcloned in Bluescript. Antisense and sense probes were produced with T3 and T7 RNA polymerases, respectively. The sense probe was the control in most experiments, giving no detectable staining in 6.0–9.5-d.p.c. embryos. Developmental stages were confirmed by their morphology[18]. In the case of embryos later than 8.0 d.p.c., the number of somites were counted. Paraffin sections were prepared (10 μm thick) after whole-mount *in situ* hybridization.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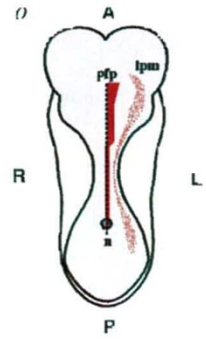
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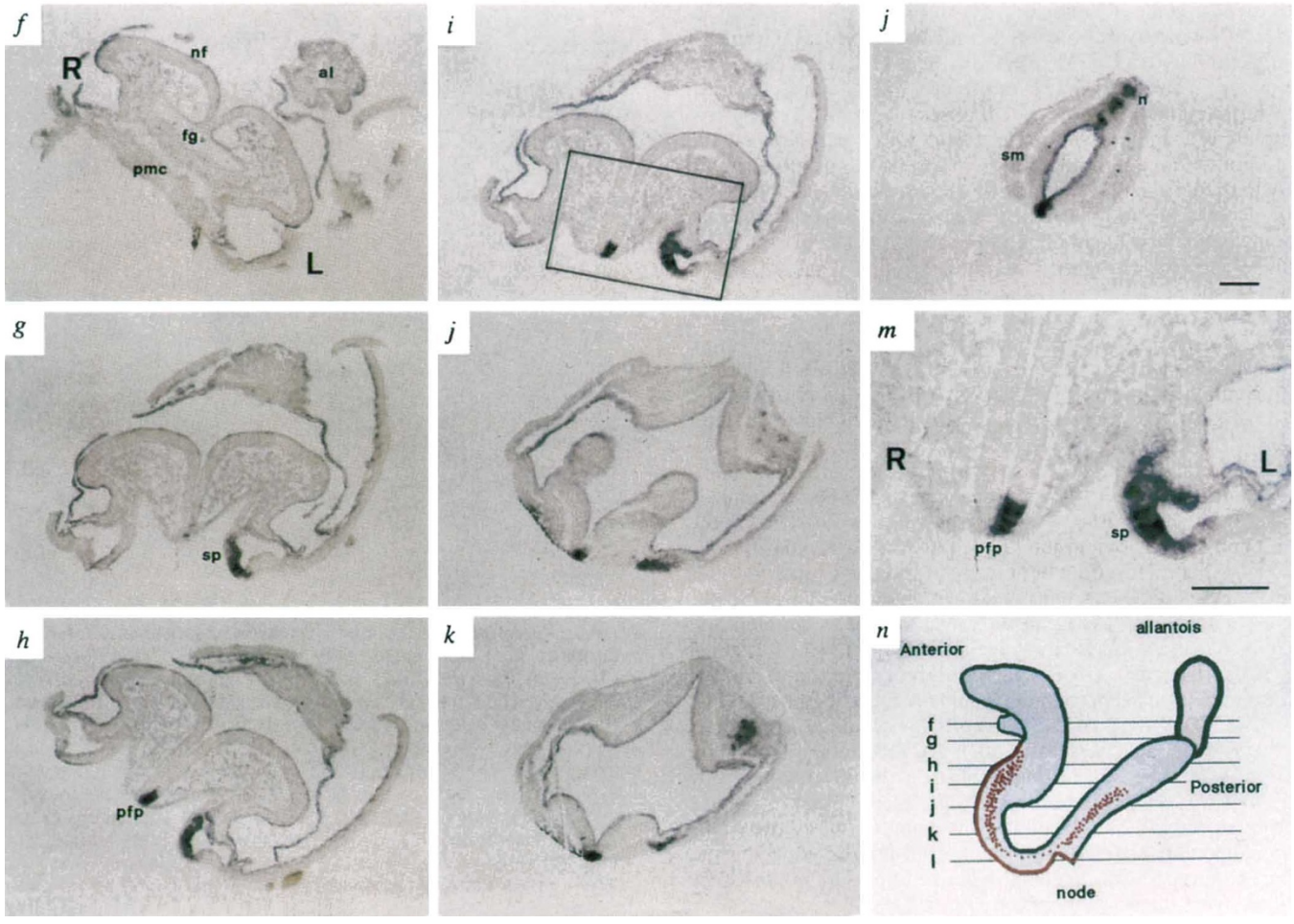
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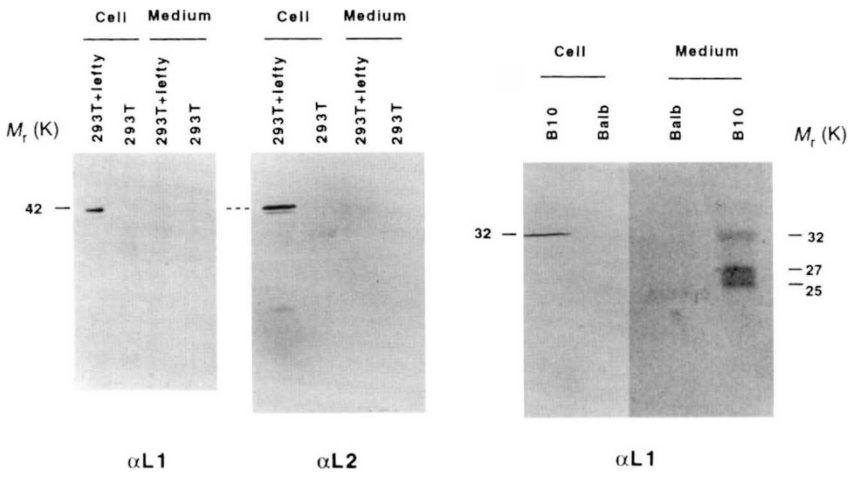

FIG. 2 Lefty protein is differentially processed according to cell type. Cell lysates and conditioned medium were prepared from the following cells: 293T cells alone, 293T cells transfected with a *lefty* expression vector (lanes 293T+*lefty*), BALB/3T3 cells (Balb) and a BALB/3T3 cell line stably transformed with a *lefty* expression vector (lanes B10). Lefty protein present in each sample was analysed by western blot with anti-Lefty antibodies, αL1 and αL2. METHODS. Two oligopeptides, ASDGALIPRRLQP (for αL1) and LSPHSARARVTIE (for αL2), corresponding to amino-acid residues 356–368 and 136–148, respectively, were used to immunize rabbits. Antibodies were affinity-purified and used for immunology. For ectopic expression of *lefty*, a full-length cDNA was subcloned in expression vectors pEFBOS[16] and pEFSAneo (the resulting plasmids are referred to as pEFBOS-*lefty* and pEFSAneo-*lefty*, respectively). 293T cells[8] were transfected with pEFBOS-*lefty*. 36 h after transfection, culture medium was replaced with medium containing 1% FCS. 24 h later, cells and conditioned medium were recovered for western blot analysis. Cells were suspended in PBS, sonicated, and an equal volume of 2 × SDS sampling buffer added to the sonicated solution. Proteins in conditioned medium were precipitated with trichloroacetic acid (TCA), washed with acetone and dissolved in 1 × SDS sampling buffer. *lefty*-expressing BALB/3T3 cell lines were obtained by transfecting BALB/3T3 cells with 2.5 μg pEFSAneo-*lefty* and 25 μg pEFBOS-*lefty*, followed by selection with 400 μg ml^{-1} G418. B10, one of the stable transformants expressing a high level of Lefty protein, was mainly used here. Cell lysates and conditioned medium were prepared from BALB/3T3 or B10 cells as described for 293T cells.

8.0-d.p.c. embryos were examined (Fig. 3*f*–*n*): *lefty* was expressed in the lateral plate mesoderm (mainly splanchnopleure rather than somatopleure), but not in the promyocardium or endocardium (Fig. 3*f*). Therefore, *lefty*-expressing cells did not correspond to those destined to form heart.

The *lefty* gene was also expressed near the ventral midline of 8.0-d.p.c. embryos (Fig. 3*d*, *e*). Transverse sections located *lefty* expression in the prospective floorplate, but not in notochord/notochordal plate (Fig. 3*f*–*n*). Expression in the prospective floorplate was again asymmetric. In the anterior region (at the level of midbrain and hindbrain), its expression was found exclusively in the left half (Fig. 3*h*, *i*, *m*). The expression site gradually came closer to the midline in the more posterior region (Fig. 3*j*, *k*), and finally reached the midline in the region near the node (Fig. 3*l*). The *lefty*-expressing regions in an 8.0-d.p.c. embryo are all shown in Fig. 3*o*.

The asymmetric expression was surprisingly transient (data not shown): *lefty* mRNA was undetectable at the presomite headfold stage, appeared asymmetrically in embryos with 3–4 pairs of somites, and disappeared in embryos with 6–8 pairs of somites (8.5 d.p.c.); *lefty* expression was no longer detectable at later stages.

Two mutations in mouse, *iv*[9,10] and *inv*[11], are known to cause *situs inversus*. In *iv*/*iv*, the L–R asymmetry seems to be randomized because only 50% of *iv* homozygotes show *situs inversus*[9,10]. On the other hand, all *inv* homozygotes have reversed L–R polarity[11]. To investigate the relationship between *lefty* and these mutations, we recently mapped the chromosomal location of *lefty*. The gene was located on mouse chromosome 1 (Y. Matsuda *et al.*, unpublished data). As *iv* and *inv* are located on chromosome 12 (ref. 12) and 4 (ref. 11), respectively, *lefty* is clearly different from *iv* and *inv*. We next examined *lefty* expression in *iv* and *inv* mutant embryos (Fig. 4): in about half (3/7) of the *iv*/*iv* embryos obtained from intercrossing *iv*/*iv* and *iv*/*iv*, *lefty* expression was inverted and found on the right side (Fig. 4*a*); in these embryos, the inversion of *lefty* expression was seen both in the splanchnopleure and prospective floorplate (Fig. 4*b*). The remaining *iv*/*iv* embryos (4/7) showed *lefty* expression on the left side (Fig. 4*a*, *c*). All *iv* homozygotes showed ectopic *lefty* expression in somatopleure. For *inv*, embryos were obtained from intercrossing *inv*/+ and *inv*/+ as homozygotes are lethal[11]. About 1/4 of the embryos (5/17) showed *lefty* expression

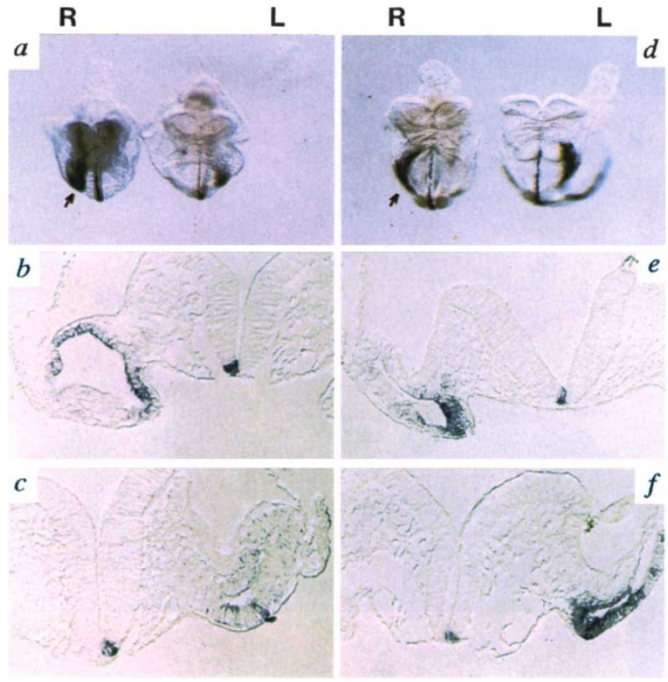

FIG. 4 Expression of *lefty* is inverted in *iv* and *inv* mutant embryos. *a*, *b* and *c*, Embryos from *iv*/*iv* × *iv*/*iv* intercross. *d*, *e* and *f*, Embryos from *inv*/+ × *inv*/+ intercross. The L–R axis of the embryos is indicated. *a*, Of seven embryos from the *iv*/*iv* × *iv*/*iv* intercross, three showed *lefty* expression on the right side (embryo with arrow), and the remaining four had expression on the left side (embryo without arrow). Transverse sections of these embryos are shown in *b* and *c*: *b*, embryo with inverted *lefty* expression; *c*, embryo with normal *lefty* expression. *d*, Of 17 embryos from the *inv*/+ × *inv*/+ intercross, five showed *lefty* expression on the right side (arrow); the remainder showed expression on the left side (no arrow). Transverse sections of these embryos are shown in *e* and *f*: *e*, embryo with inverted *lefty* expression; *f*, embryo with normal *lefty* expression. SI/Col *iv*/*iv* mice (Jackson Labs) and FVB/N *inv*/+ mice were used in this study.

on the right-side (Fig. 4*d*). In these embryos, *lefty* expression was inverted both in the splanchnopleure and prospective floorplate (Fig. 4*e*). The remaining embryos showed *lefty* expression on the left side (Fig. 4*d,f*).The inversion of *lefty* expression was not found in the embryos from the [*inv*/+ × +/+] cross (0/16), so those embryos with inverted *lefty* expression are probably *inv* homozygotes. These results show that the second wave of *lefty* expression is downstream of *iv* and *inv*.

lefty may act at a relatively late stage of L–R determination because L–R polarity in mammals is committed by the presomite stage[13]. Lefty protein in the splanchnopleure may attract primordial cells of heart and induce their asymmetric growth or migration. The significance of the asymmetric expression in the ventral neural tube is not clear, but it may be required for generating certain structural/functional asymmetries of the central nervous system. Asymmetric expression of activin receptor typeIIA, sonic hedgehog and cNR-1 has been reported in chicken embryo and interpreted as representing a hierarchy of expression of these genes. The *cNR-1* gene appears to be a chicken homologue of mouse *nodal*[15], and also belongs to the TGF-β superfamily. However, *lefty* is highly diverged form *cNR-1* and *nodal* according to its structure (Fig. 1*c*). Expression patterns of *cNR-1* and *lefty* appear similar, but there are striking differences: for example, *lefty* is expressed in one half of the prospective floorplate, a feature not shared by *cNR-1*. It is not certain whether *cNR-1* is a functional homologue of *lefty* or whether more than one member of the TGF-β superfamily is involved in L–R asymmetry. □

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Relationship between asymmetric *nodal* expression and the direction of embryonic turning

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GROWTH factors related to TGF-β provide important signals for patterning the vertebrate body plan[1–3]. One such family member, *nodal*, is required for formation of the primitive streak during mouse gastrulation[4–6]. Here we have used a *nodal–lacZ* reporter allele to demonstrate asymmetric *nodal* expression in the mouse node, a structure thought to be the functional equivalent of the frog and chick 'organizer'[7], and in lateral plate mesoderm cells. We have also identified two additional genes acting with *nodal* in a pathway determining the left–right body axis. Thus we observe in *inv* mutant embryos[8] that the sidedness of *nodal* expression correlates with the direction of heart looping and embryonic turning. In contrast, HNF3-β$^{+/-}$ *nodal*$^{lacZ/+}$ double-heterozygous embryos display LacZ staining on both left and right sides, and frequently exhibit defects in body situs. Taken together, these experiments, along with similar findings in chick[9], demonstrate that elements of the genetic pathway that establish the left–right body axis are conserved in vertebrates.

Studies of a retrovirally induced recessive lethal mutation, termed 413.d (ref. 4), have demonstrated that *nodal*, a member of the transforming growth factor (TGF)-β superfamily of secreted growth factors, is essential for the initiation and maintenance of a distinct primitive streak[6,10]. To document precisely the pattern of *nodal* expression, we generated a novel mutant allele (Fig. 1*a*) in which the second exon was replaced by an IRES sequence linked to a LacZ–neo fusion cassette[11] leaving the potential regulatory elements intact. Heterozygous embryos carrying the *nodal*^{lacZ} allele were morphologically normal, whereas homozygous mutants displayed exactly the same abnormalities as those observed with the original 413.d allele (not shown). In heterozygotes, expression of β-galactosidase faithfully recapitulates that described for *nodal* transcripts (Fig. 1*b*). At the headfold stage we observed equivalent numbers of cells expressing *nodal* messenger RNA adjacent to the lateral edges of both sides of the developing node (Fig. 1*c*). In contrast, the pattern of LacZ staining extends more posteriorly, forming a continuous boundary around the caudal notochordal plate (Fig. 1*d*). This difference probably reflects the persistence of β-gal activity, and strongly suggests that some descendants of the *nodal*-expressing cell population are displaced caudally around the periphery of the node. By early somite stages, *nodal* mRNA expression has become markedly asymmetrical, with more intense staining consistently seen on the left (Fig. 1*e*). In tissue sections, approximately twice the number of LacZ-positive cells are present on the left side of the node as against the right (Figs 1*f*, 2*c*). This subtle cellular asymmetry in the mouse node is reminiscent of the transient morphological handedness of Hensen's node in chick[12].

The increased sensitivity afforded by β-gal staining also reveals an asymmetric domain of *nodal* expression beginning at the 3–4-somite stage, confined to a subpopulation of lateral mesoderm cells on the prospective left side of the embryo (Fig. 2). This stripe of cells extends rostrally from the primitive streak to a position coincident with the caudal region of the developing heart tube (Fig. 2*d*). Asymmetric *nodal* expression precedes visible heart looping or axial rotation of the embryo, and persists until approximately the 12–14-somite stage, when the embryo is midway through the morphogenetic turning sequence.

It is well established that prospective lateral plate mesoderm arises from ectodermal cells that ingress at rougly the midpoint along the proximal–distal axis of the streak[13–18]. This cell population contributes mesoderm to both sides of the definitive body axis. In contrast, induction of *nodal–lacZ* expression in lateral mesoderm is strictly confined to the left side of the embryo. We sought to identify additional asymmetrically expressed genes that might regulate *nodal* expression. In chick, asymmetric expression of *Cnr-1* a *nodal* homologue, HNF3-β, and *sonic hedgehog* (*shh*) have been implicated in regulating left–right axis formation[9]. Moreover, ectopic *shh* expression was shown to cause alterations in the sidedness of *Cnr-1* expression and reversal of heart asymmetry[9]. The mouse *shh* gene is known to be expressed in cells of the notochordal plate and notochord[19–21]. We found no evidence for asymmetric *shh* expression in late-streak and early-somite