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Characterization and Mutation Analysis of Human *LEFTY A* and *LEFTY B*, Homologues of Murine Genes Implicated in Left-Right Axis Development

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Summary

Members of the transforming growth factor (TGF)–β family of cell-signaling molecules have been implicated recently in mammalian left-right (LR) axis development, the process by which vertebrates lateralize unpaired organs (e.g., heart, stomach, and spleen). Two family members, *Lefty1* and *Lefty2*, are expressed exclusively on the left side of the mouse embryo by 8.0 days post coitum. This asymmetry is lost or reversed in two murine models of abnormal LR-axis specification, *inversus viscerum* (*iv*) and *inversion of embryonic turning* (*inv*). Furthermore, mice homozygous for a *Lefty1* null allele manifest LR malformations and misexpress *Lefty2*. We hypothesized that *Lefty* mutations may be associated with human LR-axis malformations. We now report characterization of two *Lefty* homologues, *LEFTY A* and *LEFTY B*, separated by ~50 kb on chromosome 1q42. Each comprises four exons spliced at identical positions. *LEFTY A* is identical to *ebaf*, a cDNA previously identified in a search for genes expressed in human endometrium. The deduced amino acid sequences of *LEFTY A* and *LEFTY B* are more similar to each other than to *Lefty1* or *Lefty2*. Analysis of 126 human cases of LR-axis malformations showed one nonsense and one missense mutation in *LEFTY A*. Both mutations lie in the cysteine-knot region of the protein LEFTY A, and the phenotype of affected individuals is very similar to that typically seen in *Lefty1*$^{-/-}$ mice with LR-axis malformations.

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Introduction

All vertebrates lateralize unpaired organs of the chest and abdomen during embryogenesis. This process leads, for example, to the characteristic positions of the heart apex, stomach, and spleen to the left of the midline. Studies of vertebrate model systems have begun to uncover the molecular mechanisms controlling this highly conserved process. Several genes are expressed asymmetrically prior to the appearance of anatomic left-right (LR) differences (Harvey 1998). Among these are *Lefty1* and *Lefty2* (Meno et al. 1996, 1997, 1998). Both are expressed on the left side of the embryo in the floor-plate and in the lateral-plate mesoderm; *Lefty2* is expressed much more strongly in lateral-plate mesoderm than in floor-plate, whereas the reverse is true for *Lefty1*.

The Lefty proteins are members of the transforming growth factor (TGF)–β family of cell-signaling molecules, which serve a large variety of functions in growth and development (Kingsley 1994; Masague 1996; Heldin et al. 1997). All family members encode preproproteins that undergo cleavage at a dibasic or RXXR site, releasing the carboxy-terminal portion to be secreted. This portion of the protein is relatively well conserved among family members and includes six invariant cysteines. These residues form intrachain disulfide bonds that stabilize the protein into a conformation called the "cysteine knot," a region essential for ligand binding. An additional cysteine in most, but not all, family members mediates homo- and heterodimerization to form functional ligands.

The deduced amino acid sequences of *Lefty1* and *Lefty2* do not fall into any of the recognized subfamilies of TGF-β ligands. Uniquely, *Lefty1* and *Lefty2* have two, rather than one, putative cleavage sites that release carboxy-terminal mature protein. And, unlike most other members of the TGF-β family, neither *Lefty1* nor *Lefty2* has the seventh conserved cysteine that mediates dimerization.

Aside from asymmetric expression, additional evidence implicates these two genes in mammalian LR-axis

specification. First, the left-sided expression of *Lefty1* becomes bilateral or right-sided in three mouse mutants of LR-axis specification—*inversus viscerum* (*iv*), *inversion of embryonic turning* (*inv*) (Meno et al. 1996, 1997), and no turning (*nt*) (Melloy et al. 1998). Second, ectopic expression of mouse Lefty1 or Lefty2 proteins in the chick results in abnormal expression patterns of other asymmetrically expressed genes (Yoshioka et al. 1998). Third, and most convincingly, mice with *Lefty1* deletions manifest LR-axis malformations, confirming its essential role in LR-axis specification (Meno et al. 1998). *Lefty1*$^{-/-}$ mice typically have left-sided morphology (unilobar) of both lungs, cardiac malformations, and abnormalities of the inferior vena cava and/or azygous veins. Notably, *Lefty2* expression in the *Lefty1* null background becomes bilateral or right-sided, as does *nodal,* another asymmetrically-expressed member of the TGF-β family. Thus, *Lefty1* appears to regulate *Lefty2,* indicating that these molecules are part of the same genetic pathway but do not function identically.

As in mice, LR-axis malformations can also be found in humans (Casey 1998). Mirror-image reversal (*situs inversus*) occurs in ~1/10,000 live births and by itself causes no harm to the individual. *Situs ambiguus* describes LR anatomy that is neither normal (*solitus*) nor *inversus*. This occurs with a frequency similar to that of *inversus* but is much more deleterious. Usually present are severe and often fatal heart malformations. Intestinal malrotation and spleen abnormalities (e.g., asplenia or polysplenia) are the rule rather than the exception. We have identified an X-linked transcription factor, *ZIC3,* that is mutated in a small number of sporadic and familial cases (Gebbia et al. 1997), but the majority of the underlying molecular genetics of human LR-axis malformations remain unexplained.

Given the data implicating *Lefty1* and *Lefty2* in murine LR-axis determination, we hypothesized that the human homologue(s) of these genes may be mutated in some cases of LR-axis malformations. Herein we describe our characterization of two human *Lefty*-related genes and our search for mutations in a large group of individuals with abnormal LR-axis development.

Subjects and Methods

Subjects

Clinical information and material for analysis were provided by clinicians throughout North America and Europe. Informed consent was obtained from patients participating in this study, which was approved by the institutional review board at Baylor College of Medicine. Genomic DNA was extracted from whole blood or cell lines (lymphoblast or fibroblast) with the Puregene DNA isolation kit (Gentra Systems) according to the manufacturer's protocol.

Isolation of LEFTY A *and* LEFTY B

Using the mouse *Lefty1* cDNA sequence (GenBank D83921) as a query sequence to search the expressed sequence tag (EST) database resulted in the identification of multiple homologous human EST sequences, including T25016 (clone *21D8*). The PCR product, amplified from clone *21D8* by a pair of primers (LF1: 5′-CTGCCCATGATCGTCAGCATC-3′; LF2: 5′-CTCCTTGGCACGAGCGCACCAT-3′), was used as a hybridization probe to screen two human cDNA libraries. Two unique clones were isolated and sequenced: *PLC1,* from a placental library (Stratagene, number 937225), and *DNT2,* from a differentiated-teratocarcinoma library (Stratagene, number 937231).

A total human genomic library in the lambda DASH phage was screened by hybridization with a probe generated by primers LF1 and LF2, resulting in the isolation of clones *lg1, lg2,* and *lg3*. A human PAC genomic library (Ioannou et al. 1994) (*RPCI-1,* Roswell Park Cancer Institute) was screened by PCR with LF1/LF2 primers, leading to isolation of clone *73A4*. Comparison of the sequences of the cDNA clones and genomic clones showed that there are two homologues of murine *Lefty* genes in the human genome.

The location and sequence of all flanking introns were determined by means of direct sequencing of PCR products obtained from amplification by use of cDNA-specific primers. Sequences of the primers used for exon-exon PCR will be provided by the corresponding author (B. C.) upon request.

Mapping of LEFTY A *and* LEFTY B

DNA from the PAC clone (*73A4*) that contains both *LEFTY A* and *LEFTY B* was digested with multiple rare-cutter enzymes, electrophoresed in a 0.3% agarose gel (Seakam Gold, FMC Bioproducts), and blotted onto a charged nylon filter. This filter was hybridized with probes specific to the 5′ and 3′ UTRs of *LEFTY A* and *LEFTY B,* to generate a long-range restriction map.

DNA from phage genomic clone *lg1* was used as a probe for fluorescent in situ hybridization. Images were obtained with an epifluorescence microscope equipped with a cooled-charge coupled device camera (Photometrics) and were recorded as gray-scale images (Baldini and Lindsay 1994). Chromosomes were counterstained with 4,6-diamidine-2-phenylindole (DAPI). Pseudocoloring and merging of images were performed with Photoshop software (Adobe).

Sequence Analysis

PCR products and plasmids were sequenced by the Dye Terminator cycle sequencing kit (Perkin-Elmer) according to the manufacturer's instructions, and reaction products were analyzed by an ABI373A sequencer (Applied Biosystems). Nucleotide sequences of the cDNA clones and genomic clones were aligned by the Sequencher software (Gene Codes). Putative amino acid sequences of human *LEFTY A*/*LEFTY B* and mouse *Lefty1*/*Lefty2* were aligned by CLUSTAL W (Thompson et al. 1994).

Mutation Analysis

Primer pairs for exon amplification were designed from the sequences adjacent to the exon-intron boundaries (table 1). The relative locations of each primer are shown in figure 1. SSCP analysis was performed as described by Orita et al. (1989), except that PCR products were labeled with $\alpha[^{32}P]$-dCTP. PCR products were separated on native 6% acrylamide gels containing 10% glycerol at 20 W at 4°C for 14 h or on MDE gels (FMC Bioproducts) at 6 W at 22°C for 14 h. Autoradiography was performed at −80°C for 8–24 h. Any band shifts detected by SSCP analysis were analyzed further by sequencing PCR products generated in two or more independent amplifications.

Results

Identification and Mapping of LEFTY A *and* LEFTY B

Two human cDNA clones, *PLC1* and *DNT2*, were obtained by screening a placental library and a teratocarcinoma library, respectively. Three phage genomic clones—*lg1*, *lg2*, and *lg3*—were obtained through genomic library screening. Sequence comparison of the cDNA clones and the genomic phage clones led to the identification of two highly homologous but distinctive genes. We designated the gene encoded by the *PLC1* cDNA clone and the *lg1* and *lg3* genomic clones as *LEFTY A*, and the one encoded by the *DNT2* cDNA clone and the *lg2* genomic clone as *LEFTY B* (fig. 2).

A subsequent BLAST search using the deduced amino acid sequence from the open reading frames of these genes identified a protein sequence with very high homology, *ebaf* (Kothapalli et al. 1997). This gene was isolated through a differential-display approach designed to identify genes associated with menstrual-phase endometrium (hence the original gene name of *ebaf*, for "endometrial bleeding–associated factor"). The deduced amino acid sequence showed the greatest homology to *Lefty* (now called *Lefty1*), at 77% identity and 83% similarity. Comparison of the *LEFTY A* cDNA with the previously characterized *ebaf* sequence showed several mismatches, some of which resulted in differences in the deduced amino acid sequence. The 3′ UTRs of the cDNAs, however, were essentially identical, suggesting that the sequence differences in the coding region could have resulted from technical artifacts. Resequencing of the cDNA clone originally described as *ebaf* resolved these differences, confirming that the two genes are identical. *LEFTY A* and *LEFTY B* have identical exon-intron organization (table 1). Both genes comprise four exons interrupted at the same positions of the coding sequences. This organization is comparable to that of

Table 1

PCR Primer Pairs for SSCP Analysis of *LEFTY A* and *LEFTY B*

Primer Pair (5′→3′)[a]				Product Size (bp)
Forward		Reverse		
Number	Sequence	Number	Sequence	
2LF1	CCCTCCTGCAGCCTTCTCAAG	LF13	AGCTCTGGCTGAACCTCTTTC	306
LF31	TGTGGCTCTGCTGGGCACTCT	2LF2	CACCGGAGTGGGCACATCTGA	310
2LF3	CTGCCATCCTCAGAGCTCCC	2LF4	GGCCTAGCAGCGCCTTCCC	353
2LF5	GTAGGGGGAAGGCGCTGCTA	LF32	CGGCTCAGCTGCTGCCAGAA	201
LF19	TGGTGTACCGTCCACGAGAGC	2LF6	TGCCTGTCTCTTTATTCCGGCTTA	290
2LF7	TGAGAGGTGGATCATAAATCTCCA	LF11	AGAAATGGCCAATTGAAGGCC	291
LF1	CTGCCCATGATCGTCAGCATC	LF29	AGACCACCTCTATGCACACGT	186
LF12	CAGCACACACCTGCTGGTGTT	LF21	GAGCAGCCTCCTACTCCTGCC	259
LF19	TGGTGTACCGTCCACGAGAGC	LF27	GTCTGGACCACTCAGTGGCTG	291
LF26	AGGAGTAGGAGGCTGCTCGGC	LF20	TGGTGTACCGTCCACGAGAGC	169
LF23B	TGTCTGGCTGTGAGCTCCCAG	LF14	AGAGTGCCCAGCAGAGCCACA	234
LF28	CAGGCCTTCTGACTTCAGCCT	LF11	AGAAATGGCCAATTGAAGGCC	255
LF8B	TGACCGAGGAGCAGCTCCTGG	LF25	CTTCGACACCTCCAGAGTGGG	264
LF31	TGTGGCTCTGCTGGGCACTCT	LF13	AGCTCTGGCTGAACCTCTTTC	234
LF16	AGATGTACATTGACCTGCAGG	LF2	CTCCTTGCACGAGCGCACCAT	133

[a] Positions of primers are shown in figure 1.

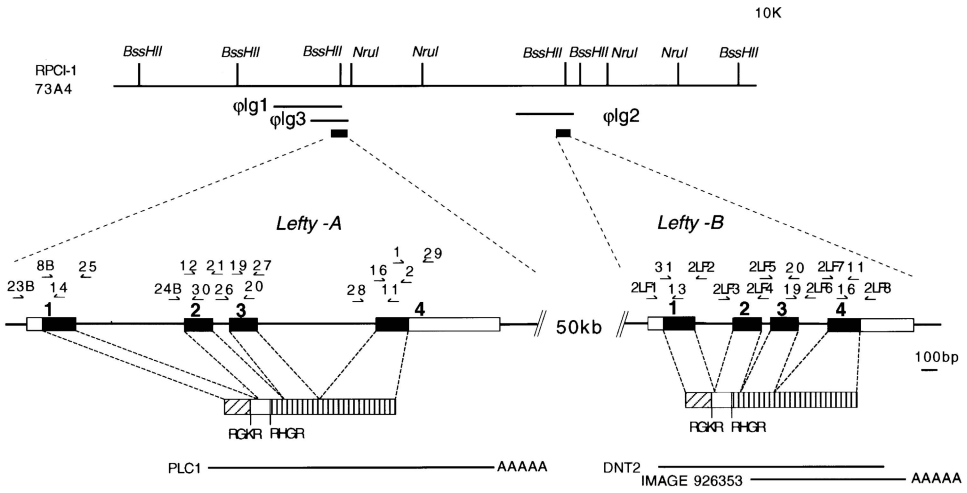


Figure 1 Genomic organization of human *LEFTY A* and *LEFTY B*. *Top,* long-range restriction map of PAC clone *RPCI-73A4,* which contains both genes. *Center,* intron-exon organization. Coding regions of exons are indicated by darkened boxes and untranslated regions by open boxes. Numbered arrows correspond to primers used in SSCP analysis (see table 1). Hatched lines show location of amino acids encoded by each exon within the final protein products. *Bottom,* Corresponding cDNA clones .

mouse *Lefty1* (GenBank AJ000083), and all splice sites conform to the *5′*/gt … ag/*3′* consensus (table 2).

The predicted protein sequences of *LEFTY A* and *LEFTY B* are quite similar, with amino acid identity of 96% (fig. 3). Nucleotide identity within the coding sequence is 97%. Both *LEFTY A* and *LEFTY B* protein contain 366 amino acids, compared with 368 in mouse Lefty1 and Lefty2. Two putative proteolytic cleavage sites—RGKR at amino acid residues 74–77 and RFER at amino acid residues 132–135—are present both in LEFTY A and LEFTY B and at the corresponding positions of mouse Lefty1 and Lefty2. The amino terminal cleavage sequence RGKR is conserved among the four proteins, whereas the carboxy terminal cleavage sequence is RHGR in *LEFTY A* and *LEFTY B,* RQKR in *Lefty1,* and RFER in *Lefty2*. Comparison of human *LEFTY A/LEFTY B* and mouse *Lefty1/Lefty2* shows significantly higher identity within rather than between species (fig. 3*b*).

PCR screening of a PAC genomic library (*RPCI-1*) identified a clone, *73A4,* that contained both the *LEFTY A* and *LEFTY B* genes. Restriction mapping with *Bss*HII and *Nru*I showed that *LEFTY A* and *LEFTY B* are separated by ~50 kb and are oriented in tandem (fig. 1). The two genes were localized by FISH to chromosome 1q42 (data not shown), a region syntenic to the location to which the mouse *Lefty* genes have been mapped at chromosome 1H5 (Meno et al. 1997). The mapping results in humans are identical to those obtained previously for *ebaf* (Kothapalli et al. 1997).

Mutation Analysis

In a panel of 112 sporadic and 14 familial cases of LR-axis malformations, two mutations in *LEFTY A* were detected in sporadic cases (fig. 3*a* and table 3). Patient 143 had a CGA→TGA change leading to premature stop at codon 314, whereas patient 137 had an AGC→AAC change at codon 342, resulting in a serine-to-lysine change. This serine is conserved among human *LEFTY A/LEFTY B* and mouse *Lefty1/Lefty2*. Both mutations are located in exon 4, which encodes the cysteine knot. Neither of these changes was present in 200 ethnically matched control chromosomes. Analysis of parental DNA samples detected maternal and paternal heterozygosity for the mutations in patients 137 and 143, respectively. Neither of the carrier parents manifests any known anatomic abnormalities.

Table 3 summarizes the abnormal anatomy of these two mutation carriers. The findings were based on echocardiography, abdominal ultrasound, and autopsy (complete for patient 137, thorax only for patient 143). The patients were quite similar in their cardiac anatomy: d-looping of the ventricles with normally related great arteries, left ventricle hypoplasia, and complete atrioventricular canal defect with a common atrioventricular valve. Both also had lesions resulting in left ventricular outflow-tract obstruction and inferior vena caval drainage to the left of the descending aorta, in conjunction with the presence of a left-sided superior vena cava. Each patient had left pulmonary isomerism, that is, both lungs

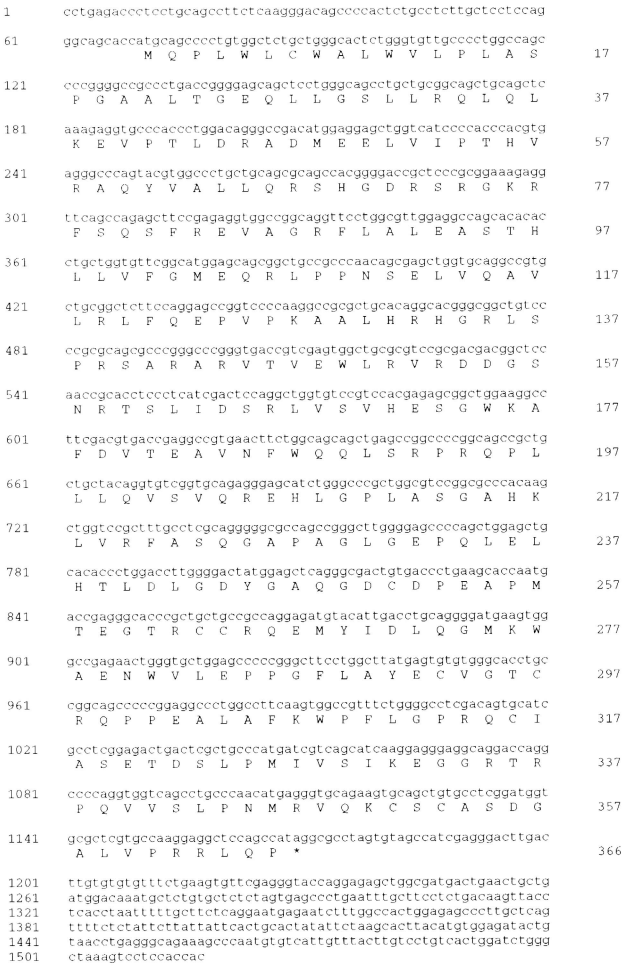


Figure 2 cDNA sequence and deduced amino acid sequence of *LEFTY B*.

had the lobar and hilar anatomy characteristic of a normal left lung.

Discussion

We have isolated the cDNAs and characterized the genomic structure for two genes highly related to mouse *Lefty1* and *Lefty2*. These two members of the TGF-β family of cell-signaling molecules have been implicated in mammalian LR-axis development by their asymmetric expression pattern and by the abnormal phenotype of *Lefty1* mutants (Meno et al. 1996, 1997, 1998). The two murine *Lefty* genes are separated by ~30 kb on mouse chromosome 1H2 (Meno et al. 1997). The human syntenic region is 1q42, where the two human *LEFTY* genes map, separated by ~50 kb. All four genes comprise four exons with identical junctions. Overall, their amino acid sequences are 78% identical. These four genes appear to form a distinct subfamily of TGF-β–related proteins, since the TGF-β family member that is next most similar to *Lefty1* is TGF-β4, at 37% identity.

As shown in figure 3*b*, human *LEFTY A* and *LEFTY B* are more closely related to each other than to either of the mouse homologues. The sequence comparison suggests that human *LEFTY A* and *LEFTY B* may have duplicated relatively recently after the divergence of the mouse and human common ancestor. Alternatively, a common ancestor of the mouse and human genes may have duplicated prior to divergence of the two evolutionary lines, with subsequent homogenization between the two tightly linked genes of each species through concerted evolution. The mechanisms of concerted evolution (either gene conversion or unequal crossing-over) could have affected regulatory sequences as well, such that the human and mouse genes may not be regulated in precisely the same manner. Sequence analysis alone, therefore, precludes determination of specific orthologous relationships (for example, whether *LEFTY A* is the functional equivalent of *Lefty1*).

Identification of a second human gene highly similar to that originally described as *ebaf* has important implications. The full-length cDNA clone of *ebaf* was used in Northern blot analyses of normal adult human tissues and several malignant tumors (Kothapalli et al. 1997). Comparison of the cDNA sequences of *LEFTY A/ebaf* and *LEFTY B* shows that the coding sequences are 97% identical, suggesting that the previously reported hybridization data may reflect expression of both genes (Kothapalli et al. 1997). The results reported here will facilitate further study of the role of human *LEFTY*-related proteins in normal and abnormal endometrial function.

Both *LEFTY A* and *LEFTY B* apparently contain retinoic-acid response elements (RAREs) nearly identical to that identified in *Lefty1* (fig. 4) (Oulad-Abdelghani et al. 1998). This element in *Lefty1* comprises an imperfect inverted repeat of two hexamers, AGGTCC and TGACCT, separated by 8 bp. This complete 20-bp element bound a purified retinoic-acid receptor, RARα1, and it could drive CAT expression when cotransfected into COS-1 cells with an RARα1 expression vector. Furthermore, the *Lefty1* expression pattern is altered in gastrulation-stage embryos of mice treated with retinoic acid.

Conservation of this RARE in each of the human genes lends further support to its functional significance in controlling expression of *Lefty*-related genes and suggests that mouse *Lefty2* may also be responsive to retinoic acid. Retinoic-acid exposure in both humans and rodents has been associated with a variety of congenital malformations, including those of LR-axis specification (Kim et al. 1995; Smith et al. 1997; Yasui et al. 1998). It is an intriguing possibility that part of the teratogenic

Table 2

Intron-Exon Organization of *LEFTY A* and *LEFTY B*

Exon	Size (bp)	3′ Acceptor	5′ Donor
1	5′ UTR+250		AGCTTCCGAGgtgag[a/g]ccct
2	247	ttgtccccagAGGTGGCCGG	TCGACTCCAGgtgggggtcg
3	240	catgtctcagGCTGGTGTCC	GGGACTATGGgtaggtgc[a/g]g
4	361+3′ UTR	gcccacacagAGCTCAGGGC	

NOTE.—Where two nucleotides appear underlined and in brackets, the left nucleotide is *LEFTY A* and the right, *LEFTY B*.

effect of retinoic acid may be mediated through misexpression of the *Lefty*-related genes.

Two mutations in *LEFTY A* have been identified in human cases of LR-axis malformations. Clearly, the nonsense mutation is of functional significance for that particular allele. The S342K missense substitution occurs in the cysteine-knot region, a portion of the secreted protein thought to be directly involved in receptor binding. The mutation is nonconservative with respect to charge, and the serine at this position is present among all four of the Lefty proteins identified to date. Strikingly, the phenotypes of the two individuals with *LEFTY A* mutations are similar to those seen in the *Lefty1*-deficient mice—left pulmonary isomerism, cardiac malformations characterized by complete atrioventricular canal defect and hypoplastic left ventricle, and interrupted inferior vena cava. This constellation of findings is most typically associated with polysplenia, which, along with a right-sided stomach, was present in patient 137 (the autopsy was limited to the chest in patient 143). No spleen anomalies were reported in *Lefty1*$^{-/-}$ mice, and only one homozygote had a right-sided stomach. The relative paucity of abdominal defects in the mice has been attributed to the absence of ectopic, right-sided *Lefty2* expression in the more posterior regions of the embryo.

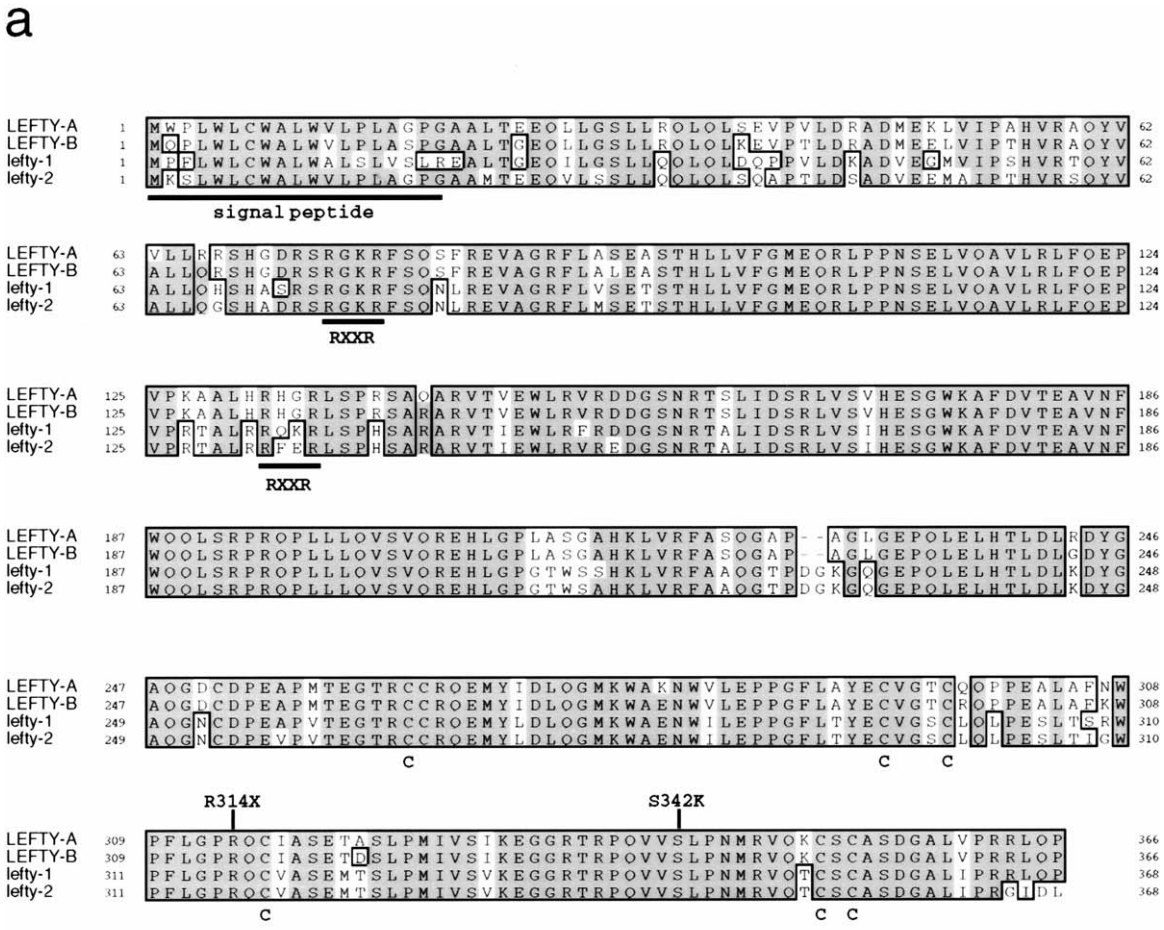
Although each mutation was found to be carried by one of the parents, the alleles may still be contributory to the phenotype in the affected individuals because of incomplete penetrance or failure to identify deleterious alleles in other LR genes present in the offspring. Incomplete penetrance is well documented in this disorder, both in humans and in animal models. Indeed, no abnormalities were identified in 14 of 40 *Lefty*$^{-/-}$ mice (Meno et al. 1998), and penetrance is less than 100%, for example, in the spontaneous mutant *iv* (Icardo and Sanchez de Vega 1991) and in the targeted deletion of *ActRIIb* (Oh and Li 1997). The normal handedness of asymmetric gene expression in LR development appears to be randomized. Thus, chance alone may sometimes lead to a normal phenotype in these murine models.

Other experiments in mice suggest that the cumulative effects of mutations in more than one gene may play a role in LR-axis malformations. *Nodal,* another TGF-β–related family member, is asymmetrically expressed in left lateral-plate mesoderm (Collignon et al. 1996; Lowe et al. 1996), apparently under the control of *Lefty1* (Meno et al. 1998). Mice homozygous for null alleles of *nodal* do not develop normally past 7.5 days post coitum, prior to the appearance of anatomic LR asymmetry (Conlon et al. 1991; Iannaccone et al. 1992). LR malformations are seen, however, in double heterozygotes of *nodal* and either *HNF3β* (Collignon et al. 1996) or *Smad2* (Nomura and Li 1998), the latter a member of the Smad family of proteins mediating TGF-β signaling. Although none of the mutations alone leads to a disturbance in LR-axis development, the reverse is not true. Some of the *nodal*$^{+/-}$;*HNF3β*$^{+/-}$ and *nodal*$^{+/-}$;*Smad2*$^{+/-}$ double heterozygotes apparently had normal LR anatomy. Again, chance may be the proximal cause.

The *LEFTY A* mutant alleles, therefore, may be necessary, but not sufficient, to give an LR phenotype in these affected individuals, and the remaining allele(s) from other LR gene(s) have yet to be discovered. Further study of the two *LEFTY A* patients, however, failed to uncover mutations in other known mammalian LR genes, including *ZIC3, nodal, HNF3β,* and *ActRIIb*. Unfortunately, this negative result does not negate the hypothesis of multiple heterozygosity for deleterious alleles, since the number of candidate genes for human LR-axis malformations appears to be quite large (Casey 1998).

Mechanisms more subtle than multiple heterozygosity for null alleles may also underlie some cases of LR-axis malformations. In the spontaneous mutant *iv* (Layton et al. 1993) and the targeted loss-of-function allele of *ActRIIB* (Oh and Li 1997), the background strain in which the mutations appear affects the penetrance of the phenotype among homozygotes. This same effect of background strain on penetrance is also seen in the mouse mutant *NOD,* a model of gestational diabetes with a high incidence of LR-axis malformations among offspring of diabetic dams (Morishima et al. 1996). All of these examples suggest the presence of modifying loci that by themselves are not deleterious.

Study of vertebrate-model systems suggests that many genes mediate the development of anatomic LR asym-



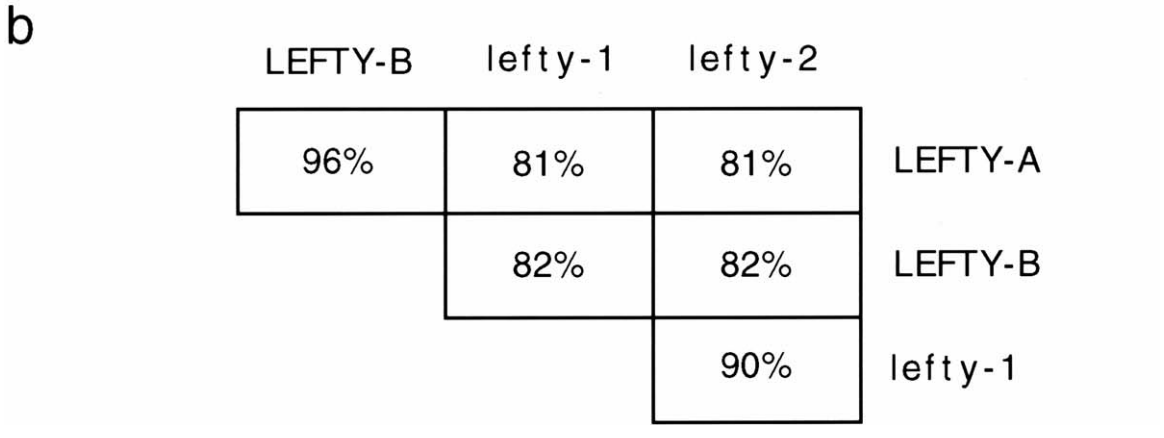


Figure 3 *a*, Protein sequence alignment of the *Lefty* homologues. Numbers on the right refer to amino acid position. Putative signal peptide and RXXR (proteolytic cleavage) sites are underlined. The six cysteine residues conserved among TGF-*β* family members are highlighted. The R314X and S342K mutations identified in *LEFTY A* are located in the region that forms the cysteine knot (see text). *b*, Pairwise comparison of amino acid identity among Lefty-related proteins.

Table 3

Phenotypes of Patients with *LEFTY A* Mutations

		Phenotype				
		Cardiac				
Patient	Mutation	Segmental Diagnosis[a]	Ventricles[b]	Great Arteries	Veins[c]	Other
137	1025 (g→a) S342K	A, D, S	HLHS; CAVC	Normal relationship; aortic atresia	Left SVC to coronary sinus, right SVC to right atrium; inter-left IVC to left atrium, right IVC to right atrium; pulmonary veins return to left atrium	Bilateral left lungs; polysplenia; midline liver; right stomach; small intestine malposition
143	989 (c→t) R314X	I, D, S	RV-dominant CAVC; hypoplastic LV; dextrocardia	Normal relationship; subaortic obstruction; coarcation	Left SVC to left-sided right atrium; inter-left IVC to left atrium; no right SVC; inverted atrial appendages	Bilateral left lungs; right liver; left stomach

[a] Segmental anatomy of atria, ventricles, and great arteries (Van Praagh 1972).
[b] HLHS = hypoplastic left heart syndrome; CAVC = complete atrioventricular canal; RV = right ventricle; LV = left ventricle.
[c] SVC = superior vena cava; IVC = inferior vena cava.

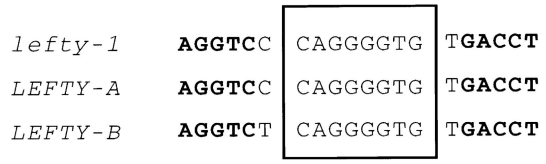


Figure 4 RARE of *Lefty1* (Oulad-Abdelghani et al. 1998), *LEFTY A,* and *LEFTY B,* identified in the 5′ flanking region of exon 1. Two hexamers (palindrome position in boldface) flank a conserved 8-bp spacer (boxed).

metry. The process can be sensitive to dosage (as seen in the double heterozygotes for loss-of-function alleles of *nodal, HNF3β,* and *Smad2*) and to maternal environment (as seen in studies of the *NOD* mouse). Furthermore, some of the genes involved, including *Lefty1,* appear, in large measure, to be tethering the handedness of subsequent asymmetric gene expression. Loss of these tethering genes, therefore, leaves to chance the development of normal LR anatomy (sometimes the organism guesses correctly, sometimes not). All of these studies suggest that the molecular genetics of human LR-axis malformations will be characterized by genetic heterogeneity further modulated by chance and environment. Characterization of the *LEFTY* genes and the identification of these unusual mutations are important steps in piecing together this complex biological process.

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