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A Submicroscopic Deletion in Xq26 Associated with Familial Situs Ambiguus

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Summary

Abnormal left-right–axis formation results in heterotaxy, a multiple-malformation syndrome often characterized by severe heart defects, splenic abnormalities, and gastrointestinal malrotation. Previously we had studied a large family in which a gene for heterotaxy, *HTX1,* was mapped to a 19-cM region in Xq24-q27.1. Further analysis of this family has revealed two recombinations that place *HTX1* between *DXS300* and *DXS1062,* an interval spanning ~1.3 Mb in Xq26.2. In order to provide independent confirmation of *HTX1* localization, a PCR-based search for submicroscopic deletions in this region was performed in unrelated males with sporadic or familial heterotaxy. A cluster of sequence-tagged sites failed to amplify in an individual who also had a deceased, affected brother. FISH identified the mother as a carrier of the deletion, which arose as a new mutation from the maternal grandfather. The deletion interval spans 600–1,100 kb and lies wholly within the 1.3-Mb region identified by recombination. Discovery of this deletion supports localization of *HTX1* to Xq26.2 and reveals the first molecular-genetic abnormality associated with human left-right–asymmetry defects.

Introduction

Left-right–axis formation occurs among all vertebrates during embryogenesis. This developmental process leads to asymmetric positioning of unpaired thoracic and abdominal organs. One striking feature of this anatomic arrangement is its invariant handedness. In humans, for example, the cardiac apex, spleen, and stomach lie to the left of the midline, the right lung has three lobes whereas the left has two, and the large intestine follows a right-to-left course in the shape of an inverted U.

Designated "situs solitus," this normal organ position can be completely reversed (situs inversus) or randomized (situs ambiguus). Situs inversus historically has been considered a medical curiosity and by itself is without functional significance. Situs ambiguus describes an overall anatomic arrangement of left-right asymmetry that is neither situs inversus nor situs solitus. The most common anatomic defects associated with situs ambiguus include severe heart malformations, splenic abnormalities, and malposition of the abdominal viscera (Winer-Muram and Tonkin 1989). Mortality is high because of the overall complexity of malformations, particularly of the heart.

Situs inversus and situs ambiguus usually arise sporadically. Familial cases of situs inversus occur most often as a manifestation of immotile cilia syndrome (ICS), an autosomal recessive disorder characterized by a variable combination of chronic upper-respiratory infections, bronchiectasis, deafness, and infertility (Afzelius and Mossberg 1994). Familial situs ambiguus has been reported with autosomal and X-linked transmission (Mathias et al. 1987; Burn 1991; Mikkila et al. 1994; Alonso et al. 1995; Casey et al. 1996) The most commonly reported cases involve multiple affected siblings, and some of these sibships are the result of consanguineous matings, implying autosomal recessive inheritance (Burn 1991). Midline malformations accompany situs ambiguus in several affected individuals in families with presumed X-linked inheritance (Mathias et al. 1987; Mikkila et al. 1994) and, among sporadic cases, are also seen more commonly in males than in females (Aylsworth 1993). These observations suggest that midline malformations may be associated more often with X-linked forms of situs ambiguus. Finally, situs inversus and fatal situs ambiguus have been described among members of the same family in different generations, suggesting that individuals with asymptomatic situs in-

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versus may be at risk of having offspring or other relatives with situs ambiguus and its accompanying morbidity and mortality (Alonso et al. 1995; Casey et al. 1996).

In order to begin understanding the underlying pathogenesis of human left-right–asymmetry defects, we have studied families and individuals with possible X-linked transmission of heterotaxy (either situs inversus or situs ambiguus). Previously we had mapped *HTX1,* a gene associated with familial situs abnormalities, to a 19-cM region of Xq24-q27.1, on the basis of linkage analysis in a single family, LR1 (Casey et al. 1993). Here we report additional linkage analysis of this family, which narrows the *HTX1* critical region to a 1.3-Mb interval in Xq26.2. We subsequently detected a submicroscopic deletion within that 1.3-Mb interval in an affected male from an unrelated family, LR2. In addition to confirming and further refining the *HTX1* locus, identification of this deletion reveals the first molecular-genetic abnormality associated with human left-right–asymmetry defects.

Subjects and Methods

Patients with Left-Right–Axis Malformations

Clinical information and material for analysis were provided by geneticists, cardiologists, and pathologists from institutions 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. DNA was extracted from whole blood or cell lines (lymphoblast or fibroblast) through the use of the Puregene DNA Isolation Kit (Gentra Systems), according to the manufacturer's protocol.

Genotyping

Primer sequences for polymorphic loci have been deposited in the Genome Database (GDB). One hundred picomoles of the forward primer was end-labeled with [γ-^{32}P]dATP in a reaction catalyzed by 1 U T4 polynucleotide kinase in 1 × T4 PNK buffer (10 mM Tris-HCl pH7.6, 10 mM $MgCl_2$, 5 mM DTT) at 37°C for 1 h. PCR was performed under the following conditions: 40 ng genomic DNA, 1 × Perkin-Elmer PCR buffer, 200 mmol each of dNTPs/liter, 0.4 mmol each of forward and reverse primers/liter, and 0.75 U *Taq* Gold polymerase (Perkin-Elmer) in a 15-μl final volume and amplification by a touchdown protocol consisting of 94°C for 1 min for one cycle; 94°C for 1 min, *T* for 1 min, and 72°C for 1 min, for 30 cycles, where *T* is 60°C–46°C and decreases 0.5°C every cycle; 92°C for 1 min, 45°C for 1 min, and 72°C for 1 min, for 3 cycles; and 72°C for 7 min, for one cycle. Amplified sequences were detected on 6% polyacrylamide–7 M urea gels run at 80 W for 3 h and exposed for 2 h–2 d.

Sequence-Tagged Sites (STSs) and Southern Blot Analysis

STS primer sequences have been deposited in GDB. PCR was performed under the following conditions: 80 ng genomic DNA, 1 × Perkin-Elmer PCR buffer, 200 mmol each of dNTPs/liter, 1 mmol each of forward and reverse primers/liter, 1 U *Taq* Gold polymerase (Perkin-Elmer) in 25-μl final volume and amplification by a touchdown protocol identical to that described above, except that the cycles at the 45°C annealing temperature were done six times. Ten microliters each of the final amplification products were electrophoresed through 2% agarose in Tris-acetate buffer (0.04 mol Tris-acetate/liter, 0.001 mol EDTA/liter) at 300 V/m (3 V/cm), stained with ethidium bromide, and photographed.

Southern blot analysis was performed with 5 mg genomic DNA, as described elsewhere (Maniatis et al. 1989), with transfer to Hybond-N+ (Amersham). The probe, radiolabeled with [α-^{32}P]dCTP, was the 1.4-kb *Eco*RI-*Eco*RI fragment from cDNA clone HFBCB92 obtained from the American Type Culture Collection. Hybridization was performed overnight at 65°C and was washed to a final stringency of 0.1 × SSC at 60°C for 10 min.

Cosmid-Library Screening

The X chromosome–specific cosmid library LLOXNC01 was screened as described elsewhere (Wapenaar et al. 1994), by use of the HFBCB92-derived probe. Two cosmids, U23b3 and U23g3, were hybridized to Southern blots of YACs from the *HTX1* critical region, in order to confirm cosmid localization to the region.

FISH

FISH was performed as described elsewhere (Shaffer et al. 1994). Cosmid U23b3 was labeled with digoxigenin and was detected with anti-digoxigenin conjugated to rhodamine (which fluoresces red). A probe for the alpha-satellite to the X chromosome was obtained prelabeled with biotin (Oncor) and was detected with avidin conjugated to FITC (which fluoresces green). Images were captured by a Zeiss Axiophot fluorescent microscope equipped with a triple-bandpass filter and a PSI Powergene 810 probe system. Images were printed by a Tektronix color/monochrome Phasar II SDX printer.

Results

Refined Linkage Analysis in Family LR1: Narrowing of the HTX1 *Critical Region*

Family LR1 has been studied previously, as described elsewhere (Casey et al. 1993). The original

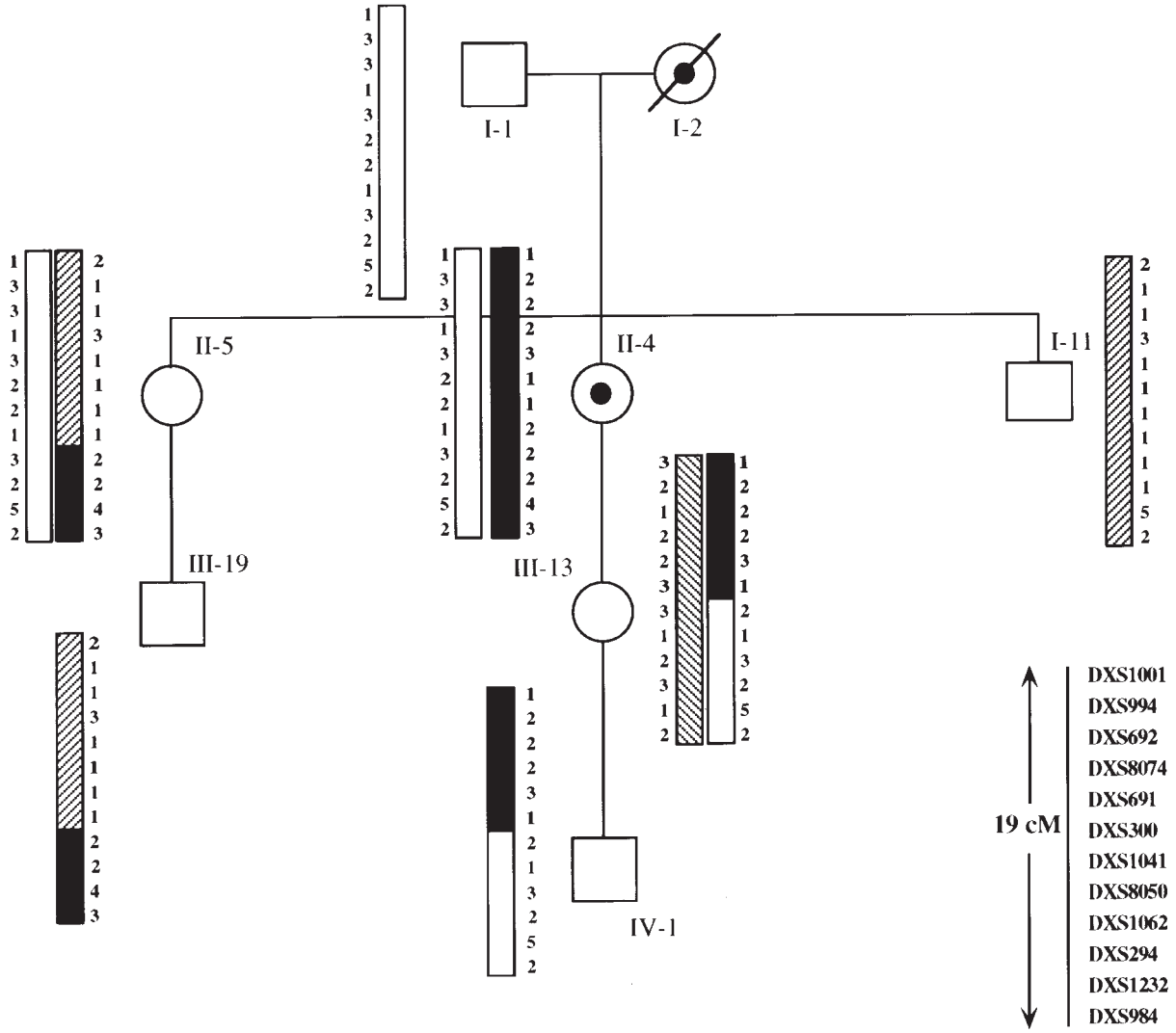

Figure 1 Genotypes for selected members of family LR1. Pedigree positions are according to the previous description of this family (Casey et al. 1993). Obligate carriers are represented by circles containing a black dot. Chromosomal segments between *DXS1001* and *DXS984* are represented by rectangles. Marker order is according to the most recently published consensus map of the X chromosome (Nelson et al. 1995). The blackened chromosome section in II-4 shows the haplotype shared in common by all of the obligate carriers and affected males.

family members, as well as eight additional members, were further analyzed with 10 microsatellite markers localized to Xq24.1-q27 (Nelson et al. 1995). The family members newly tested were all affected or unaffected males (and their mothers, if the latter had not previously been studied) whose lineage could be traced to an obligate-carrier female. The results of a portion of that linkage analysis are illustrated in figure 1. Two daughters of obligate carriers inherited disease chromosomes recombinant between *DXS1001* and *DXS984*. Individual II-5 has inherited the disease haplotype from *DXS1062* to *DXS984*, whereas individual III-13 inherited the disease haplotype between *DXS1001* and *DXS300*. Both individuals have passed the recombinant chromosomes to their sons, III-19 and IV-1, respectively. Neither of these males has any evidence of internal malformations, and both are alive and well at ages 35 and 5 years, respectively.

Inheritance of recombinant chromosomes by these unaffected males suggests that *HTX1* is located in Xq26.2, between *DXS300* and *DXS1062*. This region has been cloned into YACs (Pilia et al. 1996). These YACs were screened by PCR with polymorphic microsatellites previously localized genetically between *DXS1001* and *DXS984* (Gyapay et al. 1994) Two of these markers—*DXS1041* and *DXS8050*—mapped to the region between *DXS300* and *DXS1062* (data not shown). Subsequent analysis of family LR1 by use of these microsatellites showed no recombination between them and the disease phenotype (fig. 1).

Table 1

Summary of Malformations Identified in Affected Males from Family LR2

	Malformation(s)		
Patient	Heart	Abdomen	Other
MB	Atrio-ventricular canal, left superior vena cava	Asplenia, transverse liver, midline stomach, intestinal malrotation	Imperforate anus
KB	Atrio-ventricular canal, transposition of great arteries, pulmonary-valve atresia	Asplenia, transverse liver, midline stomach, intestinal malrotation	

STS-Content Analysis: Submicroscopic Deletion in Family LR2

A search for submicroscopic deletions in 37 males with sporadic or familial laterality defects was performed by use of STSs within the *HTX1* critical region. Among the individuals studied was MB, a male from family LR2 who was situs ambiguus and who also had an affected brother, KB, from whom no material was available for analysis. Complex heart malformations, asplenia, midline liver and stomach, and intestinal malrotation were identified at autopsy in both individuals (see table 1). MB also had an imperforate anus. On the basis of symptoms, physical examination, and chest x-ray, neither parent shows any evidence of internal anomalies. The mother, RB, was also shown to be anatomically normal by echocardiography and abdominal ultrasound. The karyotypes of MB and his mother, RB, were 46,XY and 46,XX, respectively.

No amplification products were detected in MB, with markers *DXS1041, sWXD456, DXS1119E, DXS8050,* and *EF84,* all of which map between *DXS300* and *DXS1062* (fig. 2*A*) (Pilia et al. 1996). The deletion suggested by this failure of amplification was confirmed by Southern blot hybridization using probe HFBCB92, the cDNA clone from which *DXS1119E* was derived (fig. 2*B*) (Adams et al. 1992). The PCR results place the centromeric and telomeric breakpoints of the deletion between *DXS1184* and *DXS1041* and between *EF84* and *DXS1062,* respectively. The size range of the deleted interval is 600–1,100 kb, according to a published physical map of the region (Pilia et al. 1996).

In order to obtain a probe suitable for carrier detection by FISH, a subset of the Lawrence Livermore X chromosome–specific cosmid library was screened with HFBCB92. A single cosmid was identified, and its localization between *DXS300* and *DXS1062* was confirmed by Southern blot hybridization to the YAC contig (data not shown). This cosmid, U23b3, was used as a FISH probe against metaphase spreads prepared from individual RB. As shown in figure 3*A,* the probe hybridizes to only one of the X chromosomes of RB, indicating that she carries the deletion.

Linkage analysis of family LR2 was performed by use of polymorphic markers *DXS1041* and *DXS8050.* For each marker, RB has failed to inherit the obligate paternal allele (fig. 3*B*). The results suggest that she has inherited a new mutation from her father or, alternatively, that he carries the deletion chromosome through gonadal mosaicism.

Discussion

Previously we had mapped a gene associated with familial situs abnormalities, *HTX1,* to a region spanning 19 cM in Xq24-q27.1. We now hypothesize that *HTX1* can be excluded from both the interval between *DXS1001* and *DXS300* and the interval between *DXS1062* and *DXS984,* on the basis of passage of these chromosomal segments from obligate-carrier females to unaffected males. This exclusion requires complete penetrance in family LR1, which we presume on the basis of the presence of a severe, life-threatening phenotype in all affected males during the first few days of life. The genetic evidence therefore favors localization of *HTX1*—or of regions essential for its normal function—to a 1.3-Mb region between *DXS300* and *DXS1062.* Detection of a 600–1,100-kb deletion within this interval in an unrelated family with an essentially identical phenotype supports this conclusion.

This submicroscopic deletion is the first molecular-genetic defect to be identified that is associated definitively with abnormal human left-right asymmetry. Putative mutations in a gap-junction gene, *connexin43* (*cx43*), have been reported in association with situs ambiguus (Britz-Cunningham et al. 1995). Subsequent associations of *cx43* mutations with situs ambiguus have not been reported, and at least two other groups have failed to detect *cx43* mutations in large cohorts of situs ambiguus patients (Gebbia et al. 1996; Penmann-Splitt et al. 1997) With regard to chromosomal anomalies, balanced and unbalanced translocations among a variety of autosomes have been described in sporadic cases of situs ambiguus with or without additional malformations (Freeman et al. 1996). Some of these rearrange-

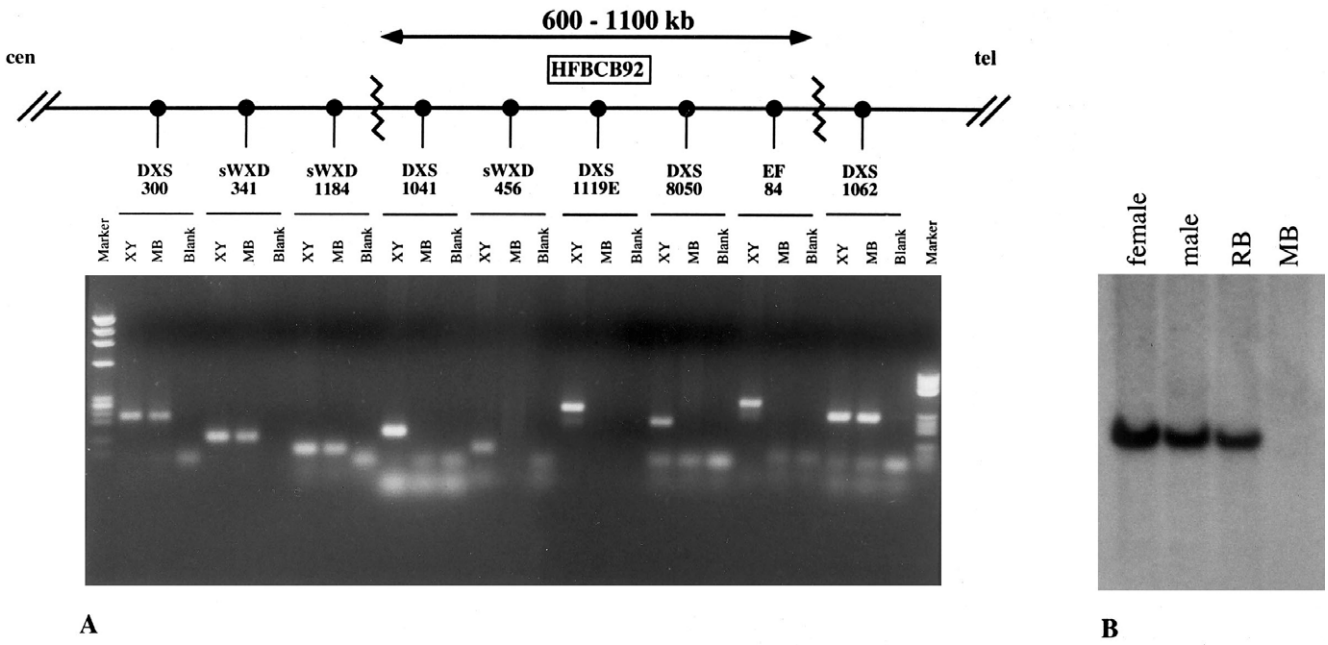

Figure 2 STS content and Southern blot analysis of patient MB from family LR2. *A*, Relative order of STSs between *DXS300* and *DXS1062*, indicated along the unbroken black line. The relative position of the probe used in panel *B* is shown above this unbroken black line, as is the extent of the deletion, inferred from the STS-content results. *B*, Southern blot hybridization of affected male MB, his mother RB, and two controls, by use of probe HBFCB92.

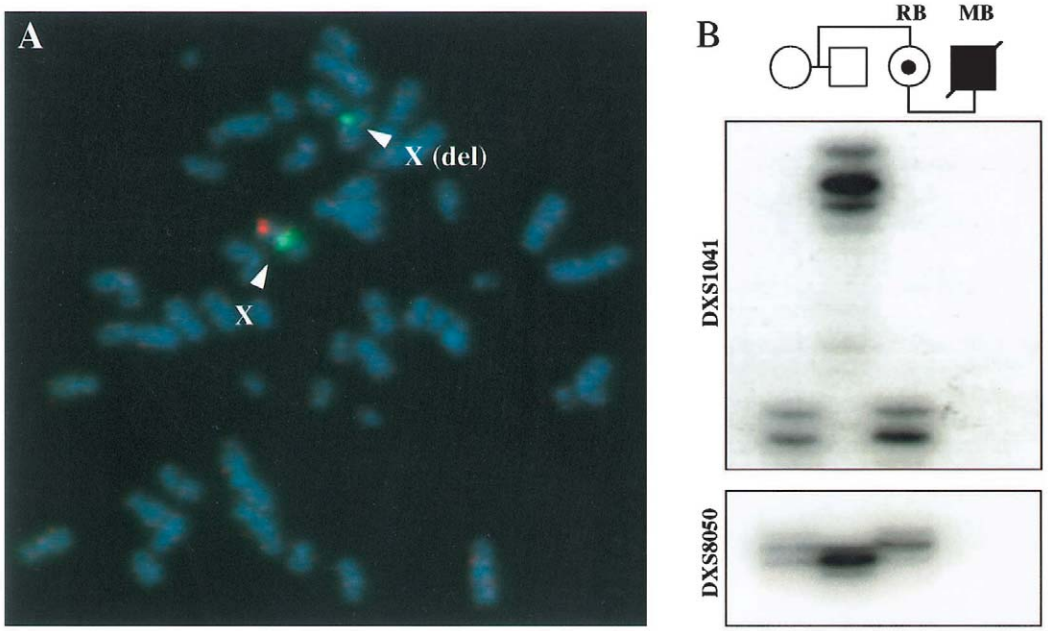

Figure 3 Transmission of the deletion chromosome in family LR2. *A*, FISH using cosmid U23b3 as a probe against metaphase spreads of lymphoblasts from individual RB. *B*, Polymorphic-repeat analysis showing failure of RB to inherit paternal alleles for the indicated markers.

ments, however, have also been detected in unaffected family members. The precise relationship between the chromosomal abnormalities and the accompanying phenotypes has yet to be resolved.

Large deletions may not be a common mechanism of *HTX1* mutation. We have studied 37 males with sporadic or familial heterotaxy and have identified only one large deletion. Failure to detect others, particularly in familial cases with possible X-linked inheritance, suggests that X-linked heterotaxy is more likely to be a single-gene defect than a contiguous-gene syndrome. Whether a single-gene defect accounts for both heterotaxy and imperforate anus in patient MB awaits the cloning of *HTX1* and the identification of intragenic mutations in other individuals harboring both malformations.

A number of molecules likely to play a role in left-right–axis development have been identified in vertebrate model systems. The TGFβ family member *nodal* and its homologues are asymmetrically expressed on the left side of mouse, chick, and frog embryos (Levin et al. 1995; Collignon et al. 1996; Lowe et al. 1996). *Lefty,* another TGFβ family member, also exhibits exclusively left-sided expression in mouse embryos of three to eight somites (Meno et al. 1996) (expression patterns in chick and *Xenopus* have not been reported). Although seen in chick, asymmetric expression of *sonic hedgehog, HNF3*β, and *ActRIIa* homologues in mouse and *Xenopus* has not been identified (Hemmati-Brevanlou et al. 1992; Ang and Rossant 1994; Feijen et al. 1994; Ekker et al. 1995; Collignon et al. 1996; Kondo et al. 1996).

Mice homozygous for null mutations in any one of these genes, including *nodal,* die early in embryonic development or fail to show identifiable defects of left-right–axis formation at birth (Zhou et al 1993; Ang and Rossant 1994; Weinstein et al. 1994; Matzuk et al. 1995; Chiang et al. 1996). It appears, therefore, that some of these genes may not be required for normal left-right–axis development in mammals, whereas other genes may be required but also have additional functions during early embryonic development. Heritable laterality defects in humans, however, imply the presence of mutations in genes whose function may be both essential and specific for developing normal left-right asymmetry. As well as elucidating the causes of situs abnormalities in humans, identification of these genes may provide insight into the general mechanism of left-right–axis formation common to all vertebrates.

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