PERSPECTIVE

The compulsion of chirality: toward an understanding of left–right asymmetry

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Although it has long been clear that correct development of left–right (LR) asymmetry requires that tissues in the early embryo know whether they lie to the left or right of the midline, the molecular mechanisms that invariantly orient the LR axis have remained obscure. The recent demonstration that the *iv* (*inverted viscerum*) mutation in the mouse may be caused by a mutation in a gene encoding an axonemal dynein heavy chain has been much anticipated (Afzelius 1976; Brown et al. 1991; Levin and Nascone 1997) and sheds light on the earliest steps in the determination of LR asymmetry (Supp et al. 1997). However, many questions are also raised, such as what the roles of axonemal versus cytoplasmic dynein are, and how dynein action is transmitted across fields of cells, a prerequisite to the large-scale asymmetric gene expression known to be involved in determination of body asymmetry (Fujinaga 1996; Levin et al. 1997). In this review we discuss the nature of the information flow from molecular chirality to morphological and behavioral asymmetry as well as some possible molecular candidates for these processes. We also address the timing of initial LR decisions during embryogenesis, and evolutionary aspects of asymmetry.

Most internal organs in the chest and abdomen of all vertebrates lie asymmetrically along the LR body axis despite external bilateral symmetry of the organism itself. In all normal individuals, the LR axis is invariantly oriented such that the apex of the heart points to the left, the aorta loops to the right and the inferior vena cava runs to the left of the spinal column. Similarly, the right lung is divided into three lobes whereas the left has only two. Beneath the diaphragm, the stomach and spleen are on the left and the intestine runs from right to left. Deviation from this normal pattern of asymmetry (*situs solitus*) can lead to complete mirror-image reversals of internal organ placement and anatomy (*situs inversus*) or randomization of organ situs (heterotaxy) as well as some loss of asymmetry (isomerism) (Burn 1991; Winer-Muram 1995). Complete *situs inversus* does not appear to confer any adverse effects on the individual, yet, nonetheless, is estimated to occur in only 1/20,000 humans (although this is commonly thought to under-represent the actual number). Heterotaxia, in contrast, usually results in multiple abnormalities many of which, such as complex heart or vascular defects, are fatal without surgical intervention. Similarly, isomerisms such as Ivemark's sequence (right isomerism, characterized by asplenia) as well as left isomerism (characterized by polysplenia) frequently compromise viability but, in less severe cases, may escape clinical detection (Burn 1991). A hallmark of most sporadic, familial, and experimentally-induced cases of laterality defects is that the organism does not lose its asymmetry; rather, individual organs (separately or together) can exhibit mirror-image asymmetry (Levin et al. 1995; Fujinaga 1996). This has led to the idea that asymmetric development or placement of an individual organ is distinct from the mechanism that orients the LR axis during development. In the absence of LR cues, therefore, the individual organs often become unbiased and develop with either normal or inverted asymmetry.

The search for genes that control the overall pattern of asymmetry has provided some insight into early events leading to LR specification. The first demonstrations of asymmetric gene expression preceding organogenesis was made in chick embryos (Levin et al. 1995). Subsequent studies using mouse, *Xenopus*, and zebrafish embryos suggests that details of the left and right cascades of gene activation may not be conserved (Matzuk et al. 1995; Chiang et al. 1996; Collignon et al. 1996; Lowe et al. 1996); however, in all species examined, it appears that the left-sided gene cascade culminates in expression of *nodal*, which encodes a TGFβ family member (Fig. 1, 2). An important aspect of the these studies is that misexpression of either left- or right-sided genes unbiases organ situs and leads to heterotaxia (Levin et al. 1997; Sampath et al. 1997). This, combined with the expression of the cascades prior to organogenesis, suggests that *nodal* and other downstream genes, such as *lefty-1* and *lefty-2* [also encoding TGFβ family members (Meno et al. 1996)], provide LR cues to the developing organs. An important implication of this work is that mutations in these genes are likely to underlie both familial and sporadic cases of laterality defects in humans. This is likely to be the case for a familial X-linked situs abnormality that results from mutations in *Zic3* (Gebbia et al. 1997), a gene encoding a zinc finger transcription factor. Interestingly, Zic3 shares structural similarity with the product of the *Drosophila* pair–rule gene *odd paired* (*opa*) and

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Developmental Task	Likely Mechanisms	Genes/Factors Involved	References
Differentiate L from R by orienting 3rd axis with respect to AP & DV	*F-shaped molecule, dynein, cytoskeleton, cleavage planes, chirality, etc.*	*inv, iv* dextral snail factor	Brown and Wolpert, 1990; Hutter and Schnabel, 1994; Wood, 1991; Freeman and Lundelius, 1982; Frankel, 1991; Yokoyama et al., 1993; Singh et al., 1991
↓ Elaborate this information into multicellular fields - asymmetric gene expression	*pathway of asymmetrically-expressed genes*	*activin, Shh, nodal, lefty -1, -2 cPTC, cSnR, etc.*	Levin et al., 1995; Collignon et al., 1996; Lowe et al., 1996; Isaac et al., 1997; Levin et al., 1997; Meno et al., 1996
↓ Orient and shape morphogenesis of individual organs (heart, gut, lungs, gall bladder, etc.)	*differential cell proliferation, migration, and cytoskeletal organization*	flectin, JB3, hLAMP-1 *eHAND, dHAND*	Smith et al., 1997; Tsuda et al., 1996; Itasaki et al., 1989; Itasaki et al., 1991; Biben and Harvey, 1997

Figure 1. Development of LR asymmetry in animal development. The development of LR asymmetry in animal morphology can be divided into three phases. In the first phase, the LR axis is first oriented with respect to the AP and DV axes. This process is likely to involve the cytoskeleton and genes such as *iv* and *inv*. In the second phase, this information is magnified into multicellular fields of asymmetric gene expression. The third phase consists of asymmetric morphogenesis of various organs by differential tissue behavior, driven by the asymmetric gene expression.

with the *Drosophila* gene *cubitus interruptus* (*ci*). *opa* is involved in maintaining expression of the *Drosophila* homolog of the mammalian *Wnt* genes, *wingless* (*wg*), whereas *ci* is homologous to the vertebrate *gli* genes that encode a family of factors best known for their involvement in mediating signaling in response to proteins encoded by the hedgehog genes, including *Sonic hedgehog* (*Shh*), suggesting its involvement in the left-sided cascade (Fig. 1). An added benefit of characterizing human mutations in proteins involved in LR signaling cascades

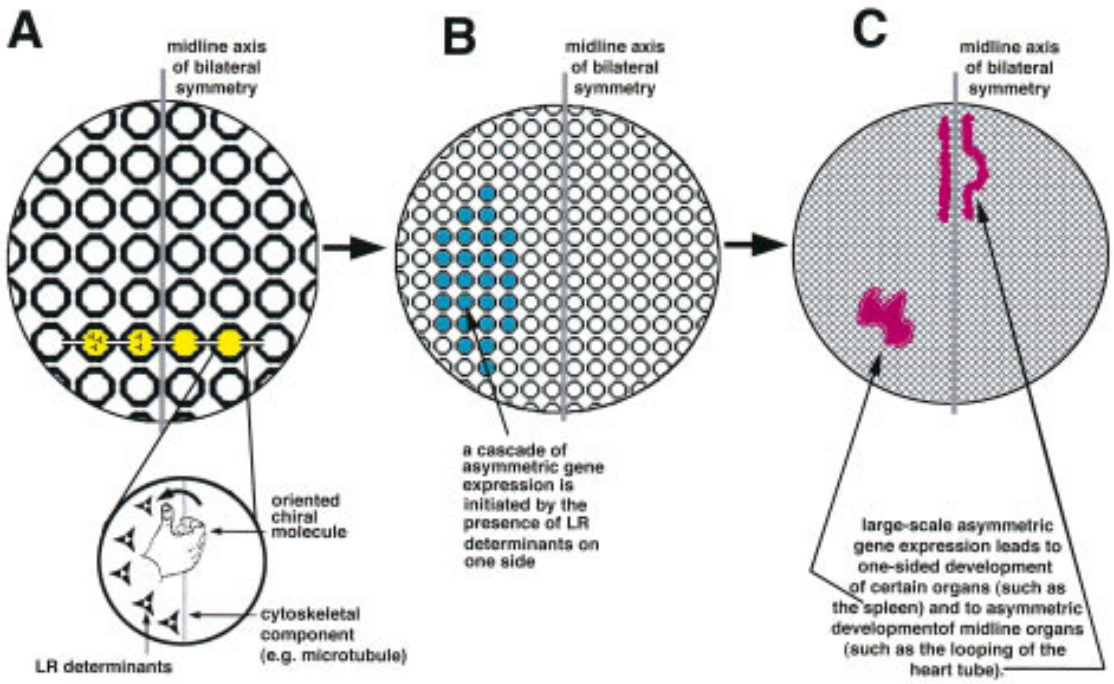

Figure 2. Three phases of elaboration of LR asymmetry. The first step consists of differentiating the left and right sides on the cellular level. This probably takes place by means of a chiral molecule (shown in detail in Fig. 3). A subset of the cells (yellow) of the fairly early embryo undergo this process (*A*). Localized cellular asymmetry is propagated between cells to cause LR determinants to accumulate on one side of the embryonic midline, possibly by a process involving transport through gap junctions. These determinants would then induce cascades of factors in multicellular fields of the embryo (*B*). Finally, the asymmetric presence of these factors induces or suppresses asymmetrically located organs such as the spleen and regulates asymmetric morphogenesis of other organs such as the heart tube (*C*).

will be the important structural information that will be revealed, not the least of which will be alterations yielding potent dominant negative factors which may prove useful for misexpression studies in experimentally accessible species.

Although studies of the genetic cascades involved in LR axis orientation are certainly an important advance, they do not explain how LR asymmetry is oriented with respect to the anteroposterior (AP) and dorsoventral (DV) axes because sided gene expression most certainly reflects some earlier asymmetry in the embryo. It is in this regard that the finding that a gene encoding an axonemal dynein heavy chain is mutated in the murine *iv* locus is particularly fascinating. The absence of ciliary dynein arms is thought to be responsible for the immotile cilia and *situs inversus* characteristic of Kartagener's syndrome (Afzelius 1976, 1985). The similarity between the manifestations of Kartagener's syndrome and the *iv/iv* phenotype have led to the anticipation that *iv* might encode a component of the dynein motor complex and that oriented microtubule arrays may, in some way, provide a cue for LR pattern (Levin and Nascone 1997).

Dyneins are microtubule-based motor proteins that have been traditionally classified as either axonemal or cytoplasmic (for review, see Holzbaur and Vallee 1994). Axonemal dyneins coordinate sliding between adjacent microtubules and provide the motive force for beating of cilia and flagella. Cytoplasmic dyneins transport cellular cargo towards the minus ends of microtubules and mediate numerous processes including retrograde axonal transport, nucleus-directed transport of lysosomes, endosomes, and the Golgi apparatus, as well as chromosome movement during cell division. Dyneins function as large multisubunit complexes containing a mixture of heavy chains responsible for force production, intermediate chains likely involved in subcellular localization, light intermediate chains (absent in axonemal dyneins) and light chains. The complex also interacts with dynactin, itself a large multisubunit complex that appears important for attachment to cellular cargo. At least 15 distinct dynein heavy chain genes are known which, for the most part, have been classified as either axonemal or cytoplasmic based on sequence analysis including a diagnostic alanine (cytoplasmic) or aspartate (axonemal) residue downstream from the highly conserved first P-loop.

Aided by the observation that *legless* (*lgl*), created by insertion of a transgene, is allelic with *iv* (Singh et al. 1991), Supp et al. (1997) cloned a novel dynein heavy chain that is mutated in both *iv* and *lgl*. As expected, transcripts for the gene, termed *left-right dynein* (*lrd*), are normally present in a range of ciliated epidermis in newborn and adult mice. In the early postimplantation embryo, however, expression was detected only in the ventral cells of the node by in situ hybridization. Transcripts were visible as early as day 7.5, prior to the appearance of *nodal* and *lefty-1* and *lefty-2* mRNAs, which lose their normal-sided expression in *iv/iv* mice (Lowe et al. 1996; Meno et al. 1996). The node lacks beating cilia [but does have immotile monocilia which lack dynein arms (Bellomo et al. 1996)]; therefore, it appears unlikely that ciliary beating is the mechanism by which *lrd* is involved in orienting LR asymmetry. Even though the predicted protein sequence of LRD resembles a canonical axonemal dynein, it is not unprecedented for expression of the axonemal class in nonciliated cells, raising the possibility that they operate inside the cell (Vaisberg et al. 1996). Because the node is likely to be involved in early LR patterning (see below), the proposal, favored by Supp et al., that a dynein motor complex acts within cells of the node to polarize it along the LR axis is particularly attractive.

How might the dynein motor complex polarize node cells? When the chick node is ablated prior to stage 4, it is regenerated such that the embryo develops with normal AP and LR asymmetry (Yuan et al. 1995; Psychoyos and Stern 1996). In addition to defining a window of developmental plasticity, this experiment also suggests that the node might be sensitive to signals from surrounding tissue. Acquisition of LR pattern within node cells can be visualized by *Shh* expression, which first occurs at low levels uniformly throughout the node but becomes stronger and left-sided at stage 4^+ (Levin et al. 1995). Thus, the emerging picture is that the chick node becomes oriented between stages 4 to 5 and, in turn, programs sided gene expression in lateral plate mesoderm shortly thereafter (see Fig. 2). One hypothesis is that LRD may function to polarize the node in response to external cues and, thus, would limit *Shh* expression to the left side. Unfortunately, *Shh* transcripts are not detected asymmetrically in the mouse node (Collignon et al. 1996), so this hypothesis cannot be tested by studying *Shh* expression in nodes of *iv/iv* embryos. The answer will await defining either the mouse node equivalent of *Shh* or developing the means to disrupt LRD function in the chick node.

Assuming a cytoplasmic dynein complex is involved in patterning LR asymmetry, how might arrays of microtubules become oriented with respect to DV and AP axes? *lrd* transcripts were noted (Supp et al. 1997) only in the ventral cells of the node (at the egg cylinder stage, the epiblast and hypoblast of the mouse embryo are shaped like a cup with the inside, epiblast surface being dorsal) and, since these cells are ingressing ventrally towards the extraembryonic, visceral endoderm (hypoblast), it seems likely that they have knowledge of the DV axis. Presumably, this information is inherent in the epiblast prior to node formation, possibly provided by association with the visceral endoderm or contact with extracellular matrix. Thus, assuming that AP pattern precedes LR pattern (see below), it is possible that the node or the primitive streak calculates LR information from external AP (head process/streak) cues and intrinsic knowledge of DV (epiblast/hypoblast) polarity—the informational equivalent of orienting the ''F'' molecule (see below).

Precisely which tissue derives LR information from AP and DV is unclear. While the expression of *lrd* and many other members of the LR cascade in the node suggest it as the likely candidate, it should be also noted that expression of *lrd* was detected as early as day 3.5 in

mouse (Supp et al. 1997), and chick embryos exhibit asymmetrical expression of several genes along the entire length of the streak just before node formation (Levin et al. 1995, 1997). Based on this we propose that, at least in the chick, it is more likely that the initial LR calculations are done in the base of the streak (Köhler's sickle is a reasonable choice), and propagated through the streak where the information is integrated with the other complex signaling going on in the node as gastrulating cells pass through it. Consistent with this view, *HNF3β* and *activin receptor IIa* are both expressed asymmetrically in the primitive ridges at stages 3–4$^-$, prior to known asymmetry in the node (Levin et al. 1995, 1997). Thus, it is possible that the node receives LR cues from the primitive streak immediately adjacent to it. One possibility therefore is that the node functions as a discrete relay station in the passage of LR signals from early events at Köhler's Sickle (e.g.), to the latter events initiated by *nodal* expression.

Wherever the derivation of LR cues from DV and AP information takes place, it is unclear how the three cardinal body axes are integrated at a molecular level. Much of the thinking regarding coordination of the three cardinal body axes has been influenced by Brown and Wolpert who proposed the involvement of a chiral molecule, termed the ''F'' molecule, that would recognize the polarity of two fixed axes to orient the third (Brown and Wolpert 1990; see Fig. 3). An attractive possibility is that microtubules are fundamentally involved. Microtubules may be involved as part of the input or the output of the calculation that combines LR, AP, and DV information. In one model (Brown and Wolpert 1990; Brown et al. 1991), microtubules may be oriented with respect to either AP or DV, and permit binding of a chiral F molecule. Additional input from the remaining unaligned axis (DV or AP) would fix the direction of the F molecule and thus orient the LR axis. Alternatively, in the output model (Levin and Nascone 1997), a chiral F molecule that is oriented with respect to the AP and DV axes could initiate microtubule nucleation along the LR axis (e.g., minus end to the left), allowing the easy unidirectional transport of LR determinants by proteins such as dynein (as in Fig. 3). Examination of cytoskeletal components in the various mouse mutants may differentiate between these two models. In either case, the node, streak, or Köhler's Sickle may turn out to be the crucial site(s) for the integration of AP and DV that results in the first determination of LR pattern.

An additional mechanism for integrating DV and LR information, as well as for asserting cellular LR differ-

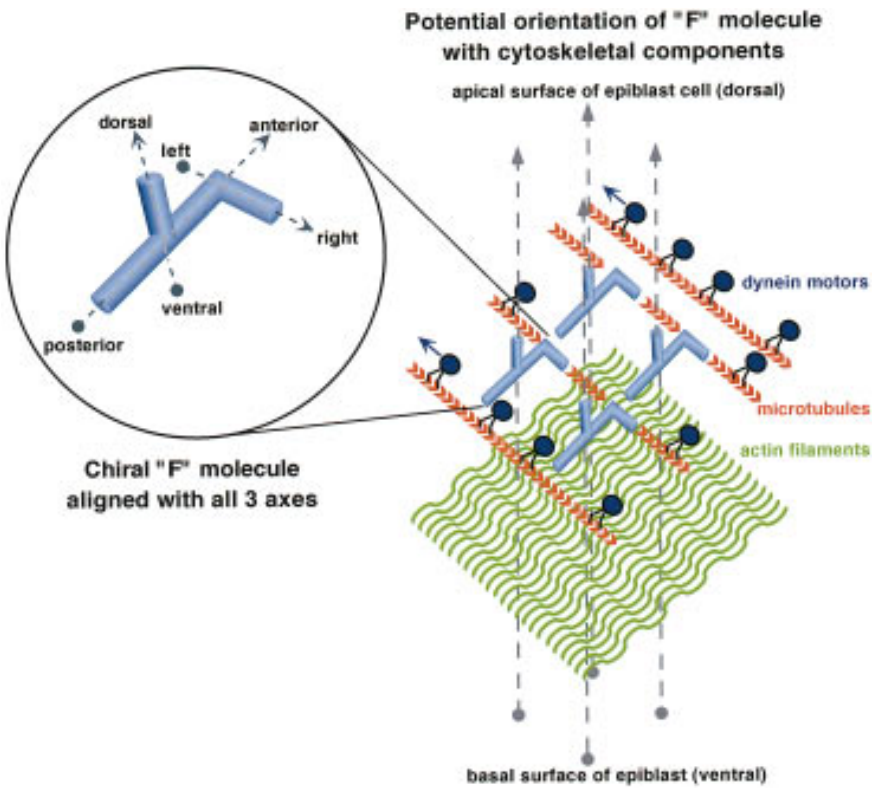

Figure 3. Model for determination of LR asymmetry at the cellular level: involvement of a chiral molecule. Brown and Wolpert (1990) first proposed the existence of a chiral F molecule that would align the three cardinal body axes. Thus, the direction of the LR axis could be derived from the orientation of the F molecule with respect to the DV and AP axes. We suspect that this derivation occurs in cells residing within an early organizing center of the embryo (possibly Köhler's sickle in the chick, or the Nieuwkoop or Spemann centers in the frog; see text). As such, these cells would have inherent knowledge of future DV and AP polarity. In the case of the chick, DV polarity in these cells would be reflected by their apical and basal surfaces and relationship to the hypoblast, whereas anterior and posterior polarity would be by position with respect to the periphery or marginal zone and center of the blastodisc (the axis that defines the growth of the primitive streak). The organization of cytoskeletal constituents, such as actin filaments on the basal surface, would serve to align the F molecule. Taking a cue from the orientation of the F molecule, other cellular proteins, such as microtubules (orange chevrons) may become polarized along the LR axis. LR determinants may be shuttled to one side of the cell or the other by dynein motors, possibly contributing to their intercellular transport to propagate LR information to neighboring cells (as in Fig. 2). Note that nothing is currently known about the nature of the hypothetical F molecule nor the cellular components with which it may interact. Thus, while the orientation of microtubules are diagrammed as the output of the calculation performed by aligning the F molecule, it is equally probable that microtubule orientation serves to align the F molecule with respect to the DV or AP axes.

ences across multicellular fields of cells, may involve gap junctions (Levin and Nascone 1997). Gap junctions between cells allow the passage of small signaling molecules (Bruzzone et al. 1996; Goodenough et al. 1996) and are thought to be involved in a variety of key developmental events (Fraser et al. 1987; Guthrie and Gilula 1989; Lo 1996). Interestingly, early *Xenopus* embryos exhibit dorsoventral differences in gap-junctional communication (GJC) (Guthrie 1984; Guthrie et al. 1988; Olson and Moon 1992). By regulating the flow of small LR morphogens, differential dorsoventral patterns of GJC can result in a LR asymmetric distribution of such molecules on an embryo-wide scale (as in Fig. 2). Consistent with this hypothesis, manipulations of GJC in early *Xenopus* embryos lead to specific laterality defects (M. Levin and M. Mercola, in prep.). We are currently pursuing the role of gap junctions in coordinating the LR and DV axes.

Recent studies of twinned chick (Levin et al. 1997) and *Xenopus* (Hyatt et al. 1996; Nascone and Mercola 1997) embryos have also been interpreted as evidence that the initial orientation of LR asymmetry is first determined within the streak or node [or their amphibian counterpart(s)]. During normal development, the presumed radial symmetry of the blastoderm (chicks) or fertilized egg (*Xenopus*) is first broken when future streak or organizer tissue acquires the ability to organize the embryo's DV and AP axes (for review, see Slack and Tannahill 1992; Sive 1993). Ectopic AP/DV organizing centers either occur spontaneously or can be induced readily in *Xenopus* by microinjection of molecules that signal through the Wnt/β-catenin/Lef–Tcf/Siamois pathway (Moon et al. 1997). Spontaneous head-to-head twins in chick embryos each develop normal LR asymmetry (Levin et al. 1997) unless the two body axes are closely juxtaposed such that interference between the left and right programs of gene expression can occur (Levin et al. 1996). In one such scenario, right-sided activin would suppress the left-sided program initiated by *Shh* and might account for the loss of LR asymmetry frequently seen in the right sibling of human twins joined at the trunk. Similarly, in *Xenopus* embryos, induced secondary body axes that form the left sibling of side-by-side twins also exhibit normal LR asymmetry (Nascone and Mercola 1997). Because, in chick and *Xenopus* twins, each body axis is initiated by separate organizing centers, it has been argued that no LR pattern exists in the embryo prior to the induction of the organizing centers themselves, which would then locally orient LR asymmetry. This conclusion is consistent with temporal data from Danos and Yost (1996) suggesting that LR asymmetry is patterned after gastrulation. However, the data cannot rule out the possibility that induced (and primary) axes may take LR cues from a circumferential pattern that might exist in the embryo, much as is thought to occur in ciliates (Frankel 1991b). In either model, all the experiments point to the embryo's organizing centers as the likely source of de novo LR calculation or integration of circumferential pattern with DV and AP.

Several difficult but important mechanistic questions remain. Where and when is the LR axis actually oriented with respect to the DV and AP? Is the location of this center the same in all species? Do microtubules become arrayed in these cells following programming by surrounding tissues? What is the nature of the signals from surrounding cells that pattern them? Perhaps the greatest insight will be gained by understanding the nature of the F molecule (indeed whether it really exists), the elucidation of whether it functions within the node, and, if so, whether it acts upstream or downstream of *lrd*.

Even more elusive are the questions of why and how LR asymmetry arose evolutionarily. It is unclear whether asymmetry (or more likely, chirality) is basic to the animal body plan, and the seeming outward symmetry of most animals a later modification, or whether asymmetry is the later tweak that is imposed on a basically symmetrical system (Jefferies 1991). Likewise, it is entirely unclear why consistent asymmetry is so prevalent. One could argue that some asymmetry is necessary in organs such as the gut and heart, for physiological reasons. Mechanisms for generating asymmetry between two sides are also easy to imagine, given gene networks and magnification of small stochastic differences, such as in the Notch–Delta system (Artavanis-Tsakonas et al. 1995). Given the ease of generating such random asymmetry, and given that animals with full *situs inversus* appear phenotypically unimpaired, why are not all animal populations a racemic mixture of opposite enantiomers in a 1:1 ratio? The ubiquity of consistently biased, not simply asymmetric, species suggests that either the biasing component is an extremely old vestige of our evolution, or that for some unknown reason it is not possible to produce offspring with a pure 50:50 incidence of *situs inversus totalis* and *situs solitus*. This impossibility is consistent with the observation that the *iv* mouse, usually thought of as instantiating this possibility, actually has significant incidence of heterotaxia, and is thus phenotypically impaired (Layton 1978). Interestingly, the sinistral forms of certain chiral snail shells are seen, upon close inspection, to also imply consequences for shell form aside from chirality (Gould and Young 1985).

Another interesting issue concerns the degree of linkage of visceral and neurological asymmetry. Brain lateralization and hand preference are popular examples of LR asymmetry (Harnad 1977). Amazingly, patients with *situs inversus* exhibit the same low incidence of left-handedness as is found in the general population (Cockayne 1938; Torgersen 1950). The fact that developmental processes can be perturbed in such a way as to fully reverse morphological asymmetry of the viscera but leave brain asymmetry in its normal bias suggests either that the mechanisms controlling neurological asymmetry comprise a completely separate pathway from those controlling body *situs*, or that they are linked, but that mutations giving rise to human laterality defects have so far occurred at points downstream of the divergence of the two pathways. Given that most manipulations studied to date involve all visceral organs, the latter possibility would imply that neurological asymmetry is calculated and set apart from body *situs* quite early in development.

Whether neurological asymmetry represents an early

branch of the general LR system or a completely different pathway has significant bearing on an issue that has been crucial at all points in the study of LR determination: the timing when left is first distinguished from right in development. Different times can be plausibly suggested as the earliest possible step, for different animals. Ciliates are perpetually chiral and inherit their asymmetry directly from the parent (Nelsen et al. 1989; Frankel 1991a). Snails occur in dextral and sinistral forms, and the first signs of this show up in the chirality of radial cleavage at the first few cell divisions (van-den-Biggelaar 1991). The same is true of *Caenorhabditis elegans*, whose asymmetry stems from asymmetric early cell divisions and the mechanical forces of the egg shell (Wood 1991, 1997; Hutter and Schnabel 1994). In more complex organisms such as the frog and chick, the situation is more complex, and it is unlikely that LR decisions are permanently made until at least blastula (frog) or equivalent stage in amniotes. This is consistent with the observation that mice that result from early blastomeres being added, subtracted, and recombined are phenotypically normal with respect to LR asymmetry. Thus, it is commonly thought that, at least in mammals, LR decisions have to be made rather late in development (e.g., after the blastocyst stage).

There is, however, an interesting set of observations that suggest that, even in mammals, chirality is determined as early as the first few cell divisions, and certainly before the streak appears. Nonconjoined monozygotic twins, while not exhibiting the kinds of visceral laterality defects that occur in conjoined twins, do manifest many subtler kinds of mirror-image asymmetry. Pairs of such twins have been noted to present mirror asymmetries in hand preference, hair whorl direction, tooth patterns, unilateral eye and ear defects, and even tumor locations and undescended testicles (Newman et al. 1937; Gedda et al. 1981; Yager 1984; Carton and Rees 1987; Beere et al. 1990; Townsend and Richards 1990; Morison et al. 1994; Cidis et al. 1997). Most healthy, nonconjoined twins presumably result from separation of cleavage, morula, or early blastocyst stage embryos (James 1983): It is much easier to imagine the splitting of a two-cell embryo rather than a complex structure such as the egg cylinder (twinning at that late stage would seem likely to yield conjoined or incompletely patterned twins). Thus, some chiral information may be present in the very early mammalian embryo, manifesting itself in hair whorls, etc., if the cells are separated at an early stage. In contrast, the asymmetry of the major body organs seems to be unspecified (or at least plastic enough to be respecified) at those stages, and is developed correctly for both monozygotic twins. This may be related to the fact that heterotaxic reversals in hair whorls and tooth patterns would not be expected to be disadvantageous, whereas discordant *situs* for internal organs clearly is subject to negative evolutionary pressure. In any case, understanding the evolutionary and developmental origin of LR information in various species is likely to be an extremely important and exciting piece of the puzzle of embryonic development.

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