Differences in Left-Right Axis Pathways in Mouse and Chick: Functions of FGF8 and SHH

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A molecular pathway leading to left-right asymmetry in the chick embryo has been described, in which FGF8 is a right determinant and Sonic Hedgehog a left determinant. Here evidence is presented that the *Fgf8* and Sonic Hedgehog genes are required for left-right axis determination in the mouse embryo, but that they have different functions from those previously reported in the chick. In the mouse FGF8 is a left determinant and Sonic Hedgehog is required to prevent left determinants from being expressed on the right.

Vertebrates appear bilaterally symmetrical, but their internal organs are asymmetric. The molecular pathway that establishes left-right (LR) asymmetry has been studied in several species (*1*, *2*). In chick, LR asymmetries are first detected around Hensen's node. Sonic Hedgehog (SHH), which is expressed to the left of the node, induces Nodal expression to the left of the node and subsequently in the left lateral plate mesoderm (LPM) (*3*). In turn, Nodal induces expression of Pitx2, a transcription factor thought to directly specify left identity during organ development (*4*, *5*). Thus, SHH functions as a "left determinant" in the chick. In contrast, FGF8 is expressed to the right of the node and functions to repress Nodal and Pitx2 on the right (*6*). Thus, FGF8 is a "right determinant" in the chick. Here we show that SHH and FGF8 have different functions in the mouse LR asymmetry pathway from those in the chick.

FGF8 function in mice was analyzed with $Fgf8^{neo/-}$ compound heterozygotes (*7*), which display a variable, hypomorphic phenotype that includes LR asymmetry abnormalities not present in their normal ($Fgf8^{neo/+}$, $Fgf8^{+/-}$, or +/+) littermates (*8*). At embryonic day 9.5 (E9.5) to E11.5 (*9*), heart looping, the earliest morphological marker of LR asymmetry in vertebrate embryos, was abnormal in 49% (21% looping to the left; 28% failure to loop) and normal (to the right) in 51% of the 144 *Fgf8* mutants examined (Fig. 1, A and B) (*10*). Abnormal looping was rarely detected (2%) in 533 normal littermates. Thus, about half of *Fgf8* mutants displayed abnormalities in early development indicative of a defect in LR axis specification.

Lung anatomy is a good indicator of LR identity, because the right lung normally has four lobes and the left lung only one (Fig. 1, C and E). In 49% of 102 mutant embryos examined at E16.5 to birth, the lungs on each side had three to four lobes (Fig. 1, D and F) (right pulmonary isomerism). Some of these embryos also had abnormal abdominal situs (*11*). In an additional 4% of mutants we observed situs inversus totalis (Fig. 1, G and H). In the remaining 47% of mutants there were no obvious lung abnormalities. Heart defects (*11*) very similar to those observed in other embryos with right isomerism (*12*) were detected in 77% of all mutants. No situs or other abnormalities of the organs were detected in 38 normal littermates. The finding that only ~50% of mutants display right isomerism might be explained by dosage sensitivity: The amount of FGF8 produced in $Fgf8^{neo/-}$ compound heterozygotes may be variable and close to threshold levels required for specification of left identity. Consistent with this hypothesis, we detected right pulmonary isomerism in only 8% (2/25) of $Fgf8^{neo}$ homozygotes, in which about twice as much functional *Fgf8* RNA should be produced. Alternatively, factors in the genetic background may influence the frequency of affected individuals (*12*).

We next examined the expression of Nodal, Lefty2, and Pitx2, genes that are normally expressed in the left LPM (*13*) (Fig. 2, A, C, and E) and are thought to function as left determinants in mice (*2*). Nodal expression was detected by assaying for β-galactosidase

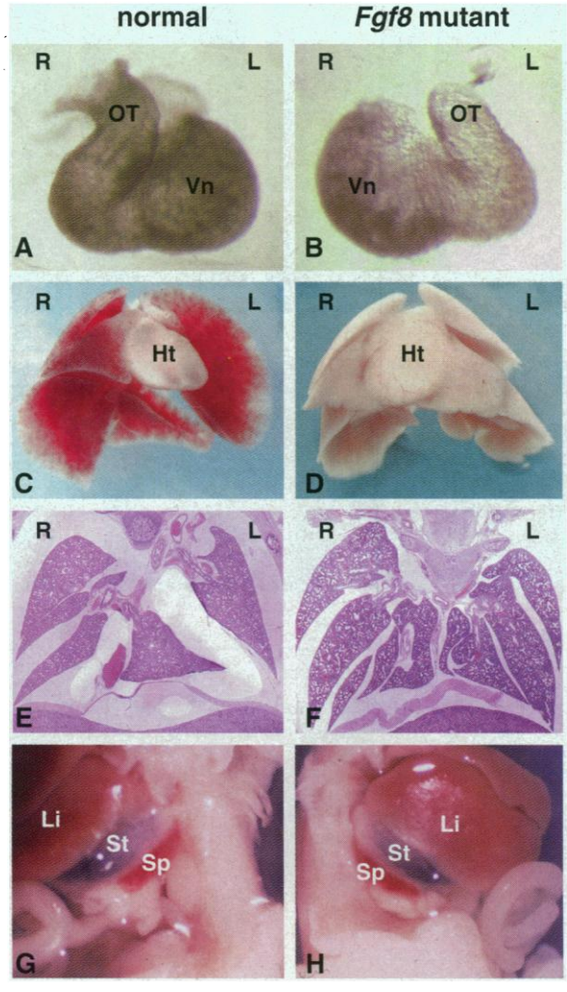

Fig. 1. LR asymmetry abnormalities in *Fgf8* mutant embryos. Organs are from normal ($Fgf8^{+/+}$, $Fgf8^{-/+}$, or $Fgf8^{neo/+}$) and *Fgf8* mutant ($Fgf8^{neo/-}$) mouse embryos. Hearts dissected from (**A**) normal and (**B**) mutant embryos at ~E9.5. The reversed looping of the mutant heart is apparent. Lung morphology at E18.5 (**C** and **D**) in whole mount and (**E** and **F**) in frontal sections. (C and E) In the normal lung, there is one lobe on the left and there are four on the right. (D and F) In the mutant embryos, there are three to four lobes on both sides, demonstrating right pulmonary isomerism. The abdominal contents of (**G**) a normal embryo viewed from the left side and (**H**) a mutant embryo viewed from the right side, illustrating reversal of abdominal situs. Abbreviations: Ht, heart; Li, liver; OT, outflow tract; Sp, spleen; St, stomach; Vn, ventricle.

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(β-Gal) activity in *Fgf8* mutants carrying Ndl^{lacZ} (*14*). In 63% of $Fgf8^{neo/-}$;$Ndl^{lacZ/+}$ mutant embryos, Nodal expression was not detected in the LPM (Fig. 2B and Table 1). In 50% of $Fgf8^{neo/-}$ mutant embryos, Lefty2 RNA (*15*, *16*) was also not detected (Fig. 2D and Table 1). Likewise, Pitx2 expression (*5*, *16*) was not detected in 41% of $Fgf8^{neo/-}$ mutants (Fig. 2F and Table 1). These data correlate well with the observation that 50% of *Fgf8* mutant fetuses display right pulmonary isomerism, and indicate that FGF8 is a left determinant in the mouse embryo.

Nodal is thought to function before the 2 to 3 somite stage in the mouse, because it is detected in an asymmetric domain around the node before it is expressed in the left LPM (*17*). In *Fgf8* mutant embryos at ~E8.0 (0 to 1 somites), the node and its descendants are present, but β-Gal activity was either absent or was detected at a low level in scattered cells in the vicinity of the node in 6 out of 10 cases. In the remaining mutants and 12 normal embryos examined at E7.5 to E8.0, β-Gal activity was detected in its normal domain around the node (Fig. 2, G and H). Thus, loss of *Fgf8* function perturbs Nodal expression around the node.

To determine if FGF8 can directly induce Nodal expression, we implanted a bead soaked in recombinant FGF8 protein (FGF8-bead) in the right LPM of normal ($Ndl^{lacZ/+}$) mouse embryos at the 0 to 2 somite stage (~E8.0). In most cases, ectopic Nodal expression was detected after ~8 hours of culture, in tissue near an FGF8-bead but not a control bead (BSA-bead); in two of these cases Nodal expression was extensive in the right LPM (Fig. 2, I and J). The FGF8-beads had no effect on Nodal expression when they were implanted on the right at later stages (4 to 8 somites) or when implanted on the left (*18*). Thus, FGF8 can rapidly induce Nodal expression at the 0 to 2 somite stage.

Together our data indicate that in mouse FGF8 functions at an early stage in the LR pathway to establish left identity. To perform this function, FGF8 may need to be asymmetrically distributed in the vicinity of the node. However, *Fgf8* gene expression, which is detected in cells within the primitive streak and caudolateral to the node, does not appear to be asymmetric in this domain (*19*). Molecules produced in or near the node may be displaced to the left by a "nodal flow" caused by movement of cilia on cells within the node (*20*). It is tempting to speculate that FGF8, and consequently Nodal expression, becomes asymmetrically distributed as a result of this leftward flow.

Remarkably, FGF8 has been identified as a right determinant in the chick (*6*). In light of the apparent reversal of function of FGF8 in the mouse and chick LR asymmetry pathways, we investigated the function of SHH, a known left determinant in chick (*3*), in the mouse by analyzing *Shh* loss-of-function mutants. Cardiac looping was reversed in 3 out of 30 (10%) $Shh^{-/-}$ embryos at E9.0 to E11.5. In another 9 out of 30 (30%) *Shh* mutant embryos, the cardiac loop was abnormally positioned. In all remaining mutant embryos and all but 1 out of 83 (1.2%) normal littermates, cardiac looping was normal. Surprisingly, similar abnormalities have

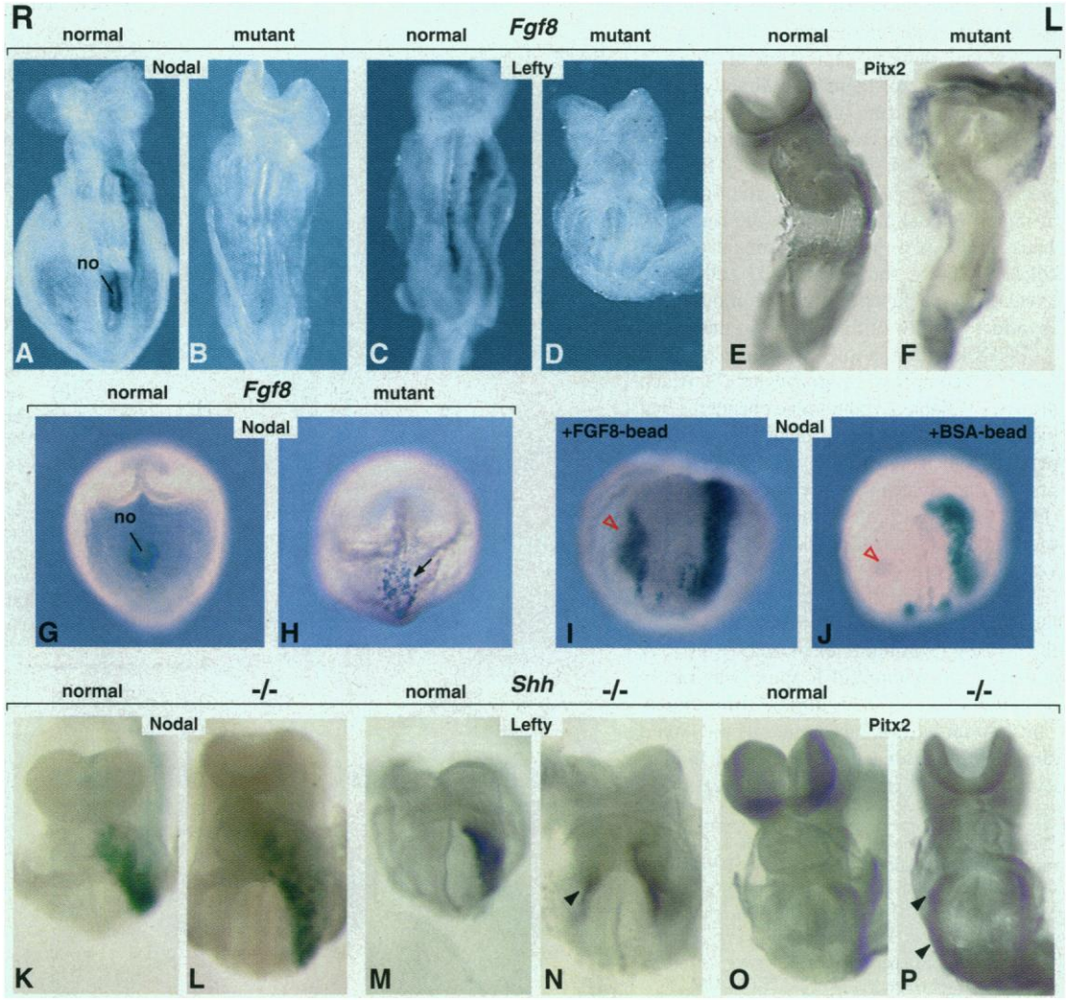

Fig. 2. Expression of Nodal, Lefty2, and Pitx2 in *Fgf8* and *Shh* mutant embryos. Mouse embryos were assayed for gene expression as indicated (*28*) and are viewed in whole mount from the ventral side in all panels. (**A** to **H**) Normal and $Fgf8^{neo/-}$ mutant embryos and (**K** to **P**) normal ($Shh^{+/-}$ or +/+) and $Shh^{-/-}$ mutant embryos at ~E8.25 (2 to 8 somite stage). In normal embryos gene expression is detected on the left, and Nodal expression is also detected around the node (no). The lack of gene expression in *Fgf8* mutant embryos is apparent. Arrowheads (N and P) point to ectopic Lefty2 and Pitx2 gene expression domains in the right LPM of $Shh^{-/-}$ embryos. (G and H) Nodal expression in embryos at ~E8.0 (0 to 1 somite stage), before the onset of LPM expression. The horseshoe-shaped expression domain in the normal embryo, and the scattered Nodal-expressing cells in the vicinity of the node [arrow in (H)] in an *Fgf8* mutant embryo are apparent. (**I** and **J**) Protein-soaked beads (red, open arrowheads) were implanted in the right LPM of mouse embryos at the 0 to 2 somite stage. After ~8 hours of culture, Nodal expression was detected in tissue near an (I) FGF8 bead, but was not detected near a (J) control (BSA) bead.

not previously been reported in *Shh*$^{-/-}$ embryos carrying the same mutation (*21*, *22*). Next we assayed for Nodal, Lefty2, and Pitx2 expression in *Shh*$^{-/-}$ embryos (Table 1). In most mutant embryos, Nodal expression was detected in its normal domain in the left LPM (Fig. 2, K and L), but in one case (Table 1) it was bilateral (*22*). Lefty2 expression was detected in its normal domain on the left in 44% of *Shh*$^{-/-}$ embryos and was bilateral in another 44% (Fig. 2, M and N). Pitx2 expression was bilateral in all *Shh*$^{-/-}$ embryos assayed (Fig. 2, O and P).

The finding that all three genes are expressed in the left LPM of most *Shh*$^{-/-}$ embryos provides conclusive evidence that SHH is not required for their left-sided expression, and therefore it is not a left determinant in mouse, as it is in chick. Instead, the data are consistent with the hypothesis that SHH, a known long-range signaling molecule, is a right determinant that functions to repress expression of left determinants on the right. Alternatively, the data are also consistent with a less direct role for SHH in the LR asymmetry pathway. Studies in *Xenopus* and mice have demonstrated that midline structures, including the notochord and floor plate, are required for normal LR identity (*22*–*26*). This may reflect a requirement for a physical barrier that prevents the spread of left determinants to the right (*27*). Because *Shh* is required for normal development of the floor plate (*21*), as well as for expression of Lefty1 (*27*) in the ventral midline (*22*), it is unclear whether loss of *Shh* function causes the LR defects described here because it functions as a repressor of gene expression or is required to establish a physical barrier in the midline.

The LR asymmetry pathways as they are currently known in chick and mouse are illustrated in Fig. 3. The possibility that the early steps in these pathways differ in the two species has been considered for some time, in part because several genes are asymmetrically expressed around the chick but not the mouse node, and because LR abnormalities had not been detected in *Shh*$^{-/-}$ embryos. Here we provide genetic and experimental evidence that FGF8 has opposite roles in the specification of LR identity in mouse and chick. Moreover, we show that SHH is required for normal LR specification, but its function as an early left determinant in chick has not been conserved in the mouse. Before we can speculate about the evolutionary significance of these findings, it will be necessary to know the full extent to which LR asymmetry pathways differ in chick, mouse, and other vertebrate species, with respect to the functions of specific molecules and the mechanisms by which they are asymmetrically distributed.

Table 1. Gene expression in *Fgf8* and *Shh* mutant embryos.

Gene	Expression domain				Total no. of mutants	No. of normal littermates*
	None (%)	Right side only (%)	Bilateral (%)	Left side only (%)		
			Fgf8$^{neo/-}$			
Nodal	17 (63)	0	0	10 (37)	27	28
Lefty2	12 (50)	2 (8)	0	10 (42)†	24	18
Pitx2	9 (41)	1 (4)	3 (14)‡	9 (41)	22	22
			Shh$^{-/-}$			
Nodal§	3 (21)	0	0	11 (79)	14	29
	1 (20)	0	1 (20)	3 (60)	5	6
Lefty2	1 (12)	0	4 (44)	4 (44)	9	13
Pitx2	0	0	9 (100)	0	9	16

*Expression detected in the left LPM only. †In two embryos, expression was detected in the caudal but not the rostral LPM. ‡Bilateral expression was detected only in the caudal LPM: In two embryos no expression was detected rostrally; in one embryo expression was detected only in the right rostral LPM. §Nodal expression was studied either by assaying for β-Gal activity in *Shh*$^{-/-}$;*Ndl*$^{lacZ/+}$ embryos (upper row) or by RNA in situ hybridization in *Shh*$^{-/-}$ embryos (lower row).

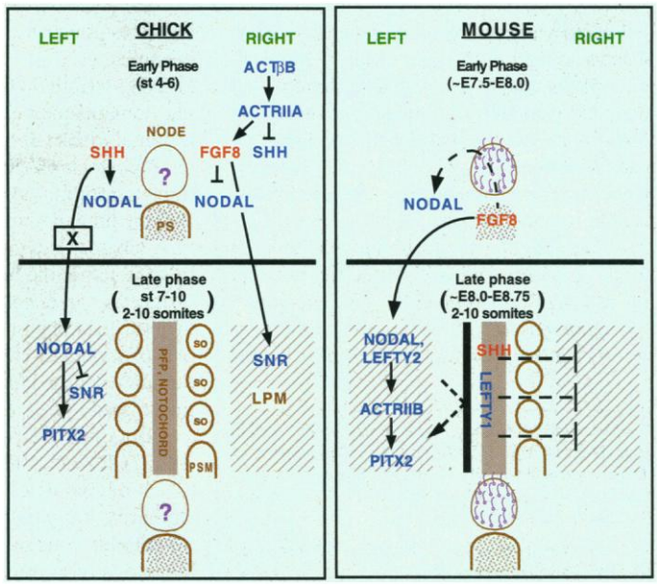

Fig. 3. Comparison of LR asymmetry pathways in mouse and chick. Schematic diagrams summarizing molecular pathways involved in determining LR identity in chick and mouse embryos [reviewed in (*1*, *2*)]. The solid horizontal lines separate the pathways into two phases. Cells in the mouse node have motile cilia, which may displace molecules functioning as left determinants to the left of the node. The question mark indicates that it is not yet known whether there are motile cilia in the chick node. FGF8 is expressed to the right of the chick node and functions to inhibit Nodal expression on the right. In contrast, our data indicate that in the mouse FGF8 is required for Nodal expression around the node during the early phase, and in the left LPM during the late phase. The dashed curved arrow indicates that FGF8 may be distributed to the left by the motion of the cilia in the node. The somites and presomitic mesoderm to the left of the mouse midline structures (prospective floor plate and notochord) have been omitted to make room for a bar and dashed arrow representing a possible physical barrier in the midline that prevents spread of left determinants to the right side. Dashed symbols (- - -|) indicate the possible production by midline structures of an active repressor of left determinants on the right. Our data indicate that in the mouse SHH is not required to induce Nodal expression, but is required to provide midline barrier or repressor functions (or both) that prevent the expression of left determinants on the right. Abbreviations: PFP, prospective floor plate; PS, primitive streak; PSM, presomitic mesoderm; so, somites; X, a molecule required for induction of Nodal expression by SHH.

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8. Mutant embryos were identified by phenotypic characteristics after E8.5. At earlier stages they were genotyped by a polymerase chain reaction analysis with standard protocols in which primer pairs were used to amplify a DNA fragment unique to each allele: 5′-CTTAGGGCTATCCAACCCATC-3′ and 5′-AGCTCCCGCTGGATTCCTC-3′ for *Fgf8*$^{\Delta 2,3}$ (*Fgf8*$^{-}$); and 5′-GTTCTAAGTACTGTGGTTTCC-3′ and 5′-GGTCTCCACAATGAGCTTC-3′ for *Fgf8*^{neo}. The annealing temperature was 54°C.

9. Noon of the day on which the vaginal plug was detected was considered as E0.5 in the timing of embryo collection.
10. E. N. Meyers and G. R. Martin, data not shown.
11. In 11 of these 50 embryos the liver was abnormally symmetrical, and the spleen, which is normally found on the left, was absent or small. In 2 of these 11 embryos the direction of bowel rotation was reversed. In another 4 of these 50 embryos the liver and spleen appeared morphologically normal, but stomach and spleen situs were reversed. In all embryos with right pulmonary isomerism, and 28 others, a variety of heart abnormalities were detected, including malposition of the cardiac outflow tracts and other vascular vessels.
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13. In normal embryos, Nodal expression is transiently detected in the left LPM beginning at the 2 to 3 somite stage (*17*). Lefty2 RNA is also detected in the left LPM of normal mouse embryos at early somite stages (*15*). Pitx2 RNA is normally detected in the left LPM, but beginning slightly later and persisting longer than Nodal or Lefty2 expression (*5*).
14. Ndl^{lacZ} is an allele in which a *lacZ* gene disrupts the Nodal gene and functions as a reporter for Nodal expression (*17*). $Fgf8^{-/+}$;$Ndl^{lacZ/+}$ animals have normal situs, indicating that double heterozygosity for loss-of-function alleles of *Fgf8* and *Ndl* does not cause any abnormalities in LR asymmetry. These results contrast with the finding of genetic interactions in double heterozygotes for Ndl^{lacZ} and a null allele of either *Smad2* [M. Nomura and E. Li, *Nature* **393**, 786 (1998)] or *Hnf3β* (*25*).
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18. Embryos were isolated at the 0 to 2 somite stage (~E8.0), and a heparin bead (H5263, Sigma) soaked in phosphate-buffered saline (PBS) containing bovine serum albumin (BSA, 1 mg/ml) (control BSA-bead) or in PBS-BSA with FGF8 (1 mg/ml) (b isoform; R&D Systems, Minneapolis, MN) (FGF8-bead) was inserted into the right LPM lateral to the node. After 1 hour in stationary culture, the embryos were incubated for 6 to 8 hours in a rotary culture apparatus, as described by K. Sturm and P. Tam [*Methods Enzymol.* **225**, 164 (1993)]. During the culture period the embryos developed to the 4 to 8 somite stage. After culture, the embryos were washed in PBS, fixed, and assayed for β-Gal activity as described (*17*). Ectopic nodal expression was detected in tissue near the bead in 11 out of 18 embryos with FGF8-beads, and in 1 out of 7 embryos with control beads. The Nodal expression domain appeared normal in two embryos with FGF8-beads implanted in the left LPM. No ectopic Nodal expression was detected in 22 embryos isolated at the 4 to 8 somite stage, after implantation of an FGF8-bead in the right LPM and culture as described above.
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28. Nodal expression was detected by X-Gal staining for β-Gal activity (*17*). Lefty and Pitx2 expression were detected by RNA in situ hybridzation (*16*). The probe that was used to detect Lefty expression (*15*) hybridizes to Lefty2 RNA, which is detected in the lateral plate mesoderm, and to Lefty1 RNA, which is detected in the prospective floor plate (ventral midline). Because Lefty1 RNA was not consistently detected in normal embryos, its absence in the mutant embryos is not considered significant.
29. We are grateful to E. Robertson and C. Chiang for $Ndl^{lacZ/+}$ and $Shh^{-/+}$ mice, respectively. We thank M. Kuehn, H. Hamada, and M. Blum for Nodal, Lefty, and Pitx2 probes, respectively, and J. Bristow and D. Goldman for helpful advice. We thank M. Embry and A. Gannon for technical assistance. We are also grateful to S. Strickland and our laboratory colleagues for critical readings of the manuscript. E.N.M. was supported by a Mentored Clinical Scientist Development award HD01216 from the NIH. This work was supported by NIH grant RO1 HD34380 (G.R.M.).

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Bacterial Photoreceptor with Similarity to Photoactive Yellow Protein and Plant Phytochromes

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A phytochrome-like protein called Ppr was discovered in the purple photosynthetic bacterium *Rhodospirillum centenum*. Ppr has a photoactive yellow protein (PYP) amino-terminal domain, a central domain with similarity to phytochrome, and a carboxyl-terminal histidine kinase domain. Reconstitution experiments demonstrate that Ppr covalently attaches the blue light–absorbing chromophore *p*-hydroxycinnamic acid and that it has a photocycle that is spectrally similar to, but kinetically slower than, that of PYP. Ppr also regulates chalcone synthase gene expression in response to blue light with autophosphorylation inhibited in vitro by blue light. Phylogenetic analysis demonstrates that *R. centenum* Ppr may be ancestral to cyanobacterial and plant phytochromes.

All photosynthetic organisms respond in some manner to light quality and quantity. Multicellular plants control development, floral induction, and phototropism through photoreceptors that absorb specific wavelengths of light. Algae, cyanobacteria, and anoxygenic photosynthetic bacteria control motility and gene expression in response to light.

Until recently, phytochrome was thought to be a plant- and algal-specific red and far-red light photoreceptor (*1*). However, the cyanobacteria *Synechocystis* and *Fremyella diplosiphon* have proteins with similarity to plant phytochromes (*2*). Plant and cyanobacterial phytochromes contain similar NH_2-terminal chromophore (bilin) binding domains as well as one or two COOH-terminal kinase domains (Fig. 1). Cyanobacterial phytochromes exhibit similarity to histidine sensor kinases (*2*). Plant phytochromes contain limited sequence similarity to histidine kinases, lacking some critical sequence motifs such as the highly conserved histidine residue of autophosphorylation (*3*). Nevertheless, plant phytochromes do undergo autophosphorylation, suggesting that they function as a sensor kinase in a signal transduction cascade (*4*).

Plant blue-light receptors cryptochrome CRY1, CRY2 (*5*), and NPH1 (*6*) contain flavin as chromophores. Cryptochromes exhibit similarity to photolyases, whereas NPH1 has similarity to serine kinases. Prokaryotic homologs of cryptochrome and NPH1 have

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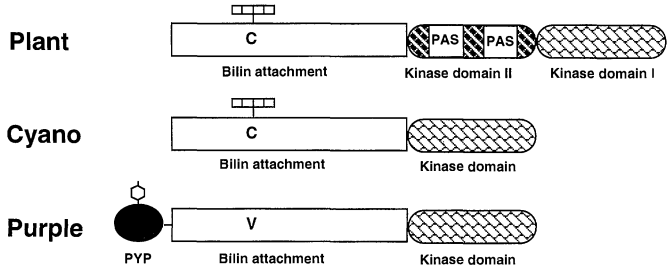

Fig. 1. Domains conserved among various phytochrome-like sequences.