Antagonistic Signaling by Caronte, a Novel Cerberus-Related Gene, Establishes Left-Right Asymmetric Gene Expression

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Summary

Left-right asymmetry is initiated during chick embryogenesis in small domains near Hensen’s node. Subsequently, broad asymmetric gene expression domains are established in the lateral plate mesoderm, ultimately determining the directionality of morphogenetic events. The transfer of asymmetric information from the node to the lateral plate is mediated by Caronte (Car), a novel member of the Cerberus/Dan gene family, which induces targets by antagonizing symmetrically expressed BMP signals. In addition, BMP antagonism by Car induces asymmetric expression of Lefty in the midline, preventing spread of left-sided signals to the contralateral side.

Introduction

There has been dramatic recent progress in uncovering the genetic pathways responsible for establishing left-right (L-R) asymmetric morphogenesis in vertebrates (reviewed in Harvey, 1998; Vogan and Tabin, 1999). Bilateral asymmetry appears to be first broken at or near the node, possibly by the unidirectional rotation of cilia (reviewed in Vogan and Tabin, 1999), resulting in small asymmetric domains of signaling molecules around the node. In the chick, for instance, an inferred Activin-like signal simultaneously induces a small domain of Fibroblast growth factor 8 (Fgf8) on the right side of the node (Boettger et al., 1999) and limits expression of Sonic hedgehog (Shh) to the left side of the node (Levin et al., 1995). Subsequently, broad asymmetric expression of other genes is seen in the lateral plate mesoderm (LPM). The TGFβ family member Nodal is induced throughout the left LPM (Levin et al., 1995), where it activates left-sided LPM expression of the transcription factor Pitx2 and hence affects asymmetric morphogenesis (reviewed in Harvey, 1998). However, a critical gap in our knowledge remains: it is still not known how the small asymmetries at the node are transmitted over long distances to induce broad patterns of asymmetric gene expression throughout the LPM.

In the chick, production of Shh at the node is both necessary and sufficient for left-sided Nodal expression in the LPM (Levin et al., 1995; Pagán-Westphal and Tabin, 1998), indicating that the early asymmetries at the node and the subsequent asymmetries in the LPM are functionally linked. Nevertheless, examination of the expression domain of Patched, a sensitive marker for Shh signaling (Goodrich et al., 1996; Marigo et al., 1996), suggests that Shh only acts directly over a fairly short distance in cells adjacent to the node (Pagán-Westphal and Tabin, 1998), consistent with the range of hedgehog signaling in other systems (reviewed in Hammerschmidt et al., 1997). Moreover, Shh protein is unable to induce Nodal expression in LPM explant culture unless paraxial mesoderm, the tissue normally adjacent to the node, is included (Pagán-Westphal and Tabin, 1998), suggesting that an unknown secondary signal is produced in the paraxial mesoderm in response to Shh, which in turn induces Nodal throughout the LPM. Finally, parallel experiments have shown that Fgf8 on the right side of the node leads to repression of Nodal in the right LPM (Boettger et al., 1999), suggesting that Fgf8 also acts by regulating this secondary signal in the paraxial mesoderm.

Here, we describe a novel Cerberus-related gene, Caronte (Car), expressed asymmetrically in the left paraxial mesoderm. Car is a secondary signal induced by Shh and repressed by Fgf8, and Car misexpression is sufficient to activate Nodal in the LPM. Moreover, unlike other positively acting factors in the L-R cascade, Car functions antagonistically, blocking the repressive activity of bilaterally expressed BMPs. Thus, Car acts as a key long-range signal mediating the transfer of L-R positional information from the node to the periphery, bridging the crucial gap between local asymmetric signaling at the node and broad asymmetric gene expression in the LPM.

The existence of long-range asymmetric signals creates a potential problem, as it is important to confine the activation of target genes to one side. Several lines of evidence suggest that the midline functions as a barrier to prevent the contralateral spread of asymmetric signals (Levin et al., 1996; Loehr et al., 1997). At least part of this barrier function is played by Lefty-1, a TGFβ-related protein expressed asymmetrically in the developing midline (Meno et al., 1998). In mice carrying a targeted deletion of Lefty-1, Nodal is expressed in the right LPM in addition to the left (Meno et al., 1998). Moreover, in the chick, application of Lefty-1 protein between the LPM and the domain of Shh expression blocks activation of Nodal, consistent with Lefty-1 acting as a barrier to signaling downstream of Shh (Yoshikawa et al., 1998). However, the factors responsible for the asymmetric expression of Lefty-1 in the midline and the mechanism by which Lefty-1 mediates its barrier function remain unknown. We find that, in addition to antagonizing BMP signaling in the LPM, antagonism of BMPs by Car induces the expression of a chick Lefty gene in the midline. Since Lefty is itself a member of the TGFβ family, we suggest that this is a negative feedback
loop and that Lefty acts to sequester Car activity, preventing its spread to the contralateral side.

Results
Isolation of Caronte, a Novel Cerberus Family Member
Modulation of the activity of inductive factors by antagonists is an important regulatory mechanism used during embryogenesis (reviewed in Perrimon and McMahon, 1999). The Cerberus/Dan family is an important group of genes encoding secreted antagonists of BMPs and other factors (Bouwmeester et al., 1996; Belo et al., 1997; Biben et al., 1998; Pearce et al., 1999).

Because a TGFβ family member, Lefty, is involved in establishing L-R asymmetry, our attention was drawn to antagonists of TGFβ superfamily signaling. A novel chick Cerberus family member was identified by degenerate PCR based on sequences conserved between the Xenopus and mouse Cerberus genes, and a full-length cDNA was isolated. This gene is predicted to encode a 272-amino acid protein with a hydrophobic signal sequence at its amino terminus and a cysteine knot domain close to its carboxyl terminus (Figure 1A). Homology between this deduced chicken protein, Xenopus Cerberus, and the five known members of the mouse Cerberus/Dan gene family is restricted mainly to the cysteine knot domain (Figure 1B). Across this region, the chick protein is 65% identical to mouse Cer1 and 61% identical to Xenopus Cer. A similar level of identity is shared with the other family members. The spacing of the nine cysteines is also conserved among all of these proteins. However, across the whole protein, the sequences are less than 30% identical to each other, suggesting that the chick gene is a novel member of the Cerberus gene family, and not the homolog of Xcer or of any of the five mouse genes. We have named this gene Caronte (Car) after the mythological boatman who shuttles souls to the underworld, where the dog Cerberus dwells.

Expression Pattern of Car Suggests a Role in L-R Asymmetry
To investigate the roles Car might play during development, its expression pattern was analyzed by whole-mount in situ hybridization. At early stage 7, Car is detected in the lateral portion of the PAM (J). Figure 1. Sequence and Expression Analysis of Car
(A) Predicted translation product of chick Car. The hydrophobic stretch at the amino terminus is underlined, the cysteine knot domain is boxed, and two potential N-linked glycosylation sites are overlined.
(B) Sequence comparison between chick Car, mouse Cer1, and Xenopus Cerberus. Shaded regions represent residues conserved in at least two out of the three proteins shown. Numbers denote the amino acid positions in the corresponding proteins. In both (A) and (B), asterisks indicate the conserved cysteine residues in the cysteine knot domain.
(C-G) Whole-mount in situ hybridization with a Car probe showing the expression of Car at stages 7 through 81 (dorsal view). At early stage 7, Car transcripts can be seen on the left side of the paraxial mesoderm (PAM) and in the LPM (C). Between stages 7 and 8, the expression domain of Car expands both anteriorly and posteriorly (D-F) and begins to be downregulated at stage 81 (G).

(H-J) Cross sections of the stage 8 embryo shown in (F). In the head region, Car is expressed in the left side of the cephalic mesenchyme (H). In the somite region, Car is expressed in the medial part of the LPM and in the lateral portion of the somite (I). In the newly forming somite, the expression of Car is detected throughout the entire left PAM (J). (K-N) Comparison of the expression patterns of Nodal (K and M) and Car (L and N) between stages 51 and 71. At stage 51, while Nodal expression has already been initiated on the left side of the node (K), Car transcripts have not yet appeared (L). At stage 6, Car is detected in the lateral portion of the PAM (N). Slightly later (stage 71), Nodal expression is initiated in the LPM (M). Red arrows show the boundaries between the PAM and the LPM. cm, cephalic mesoderm; lpm, lateral plate mesoderm; n, node; nc, notochord; nf, neural folds; pam, paraxial mesoderm; so, somite.
Caronte Mediates L-R Asymmetric Gene Expression

Expressed in a restricted domain in the left paraxial mesoderm near Hensen’s node and in the adjacent medial portion of the left LPM. Car is not expressed in equivalent tissues on the right side (Figures 1C and 1D). During stage 7–8, while the asymmetric expression in the left paraxial mesoderm continues to be restricted to newly forming somites, expression in the left LPM expands anteriorly to the head region (Figures 1E and 1F) and posteriorly as the node regresses. Both the paraxial and lateral plate domains of Car are restricted to the mesodermal layer (Figures 1I and 1J). At stage 8, expression in the paraxial tissue disappears, and Car is also downregulated in the lateral part of the LPM expression domain (Figure 1G). By stage 9, Car expression is no longer detectable, and we do not detect Car expression later in stage 18 or stage 26 embryos (data not shown).

Car expression is suggestive of a possible relationship with several molecules in the L-R signaling cascade. Shh is expressed starting at stage 5, prior to Car expression, and both are lost at stage 8, consistent with Shh acting upstream of Car (Levin et al., 1995; Pagán-Westphal and Tabin, 1998). Nodal is expressed in two discontinuous asymmetric domains. It is initiated in a smaller domain directly adjacent to Shh at stage 6, and subsequently in a much larger domain in the left LPM at late stage 7 (Levin et al., 1995). Both of these domains are downstream of Shh signaling, and the smaller medial Nodal domain may be a direct Shh target (Levin et al., 1995). However, the small medial domain does not appear to be involved in activating downstream asymmetric genes in the LPM (Levin, 1996). Car expression in the paraxial mesoderm is initiated at stage 6 (Figure 1N) after the onset of the medial domain of Nodal expression (Figures 1K and 1L). Slightly after the appearance of Car, Nodal is first detected in the LPM (Figure 1M). These results suggest that Car may mediate the Shh-induced activation of Nodal in the LPM, and/or that the medial domain of Nodal expression could act to induce the expression of Car.

Regulation of Car Expression

To test whether Shh is necessary for Car expression, we made use of a monoclonal antibody that interferes with Shh signaling (Ericson et al., 1996; Pagán-Westphal and Tabin, 1998). Beads soaked in the antibody and placed on the left side of Hensen’s node at stage 4–5 (Figure 2D) dramatically repressed Car expression (67%, n = 15, Figure 2F). Control antibody applied in the same manner had no effect on Car expression (n = 7, Figure 2E). To test whether Shh signaling is sufficient to induce
Car expression, a bead soaked in Shh protein was placed on the right side of Hensen’s node at stage 5 (Figure 2A), resulting in bilateral exposure to Shh. This results in bilateral Car expression (100%, n = 14, Figure 2C). Control beads had no effect on Car expression (n = 7, Figure 2B). Thus, Shh signaling is both necessary and sufficient for Car induction.

The medial domain of Nodal is induced by Shh prior to Car expression, suggesting that Nodal could act upstream of Car. To test this, cell pellets expressing Nodal were implanted on the right side of Hensen’s node at stage 5 (Figure 2G), resulting in bilateral exposure to Nodal. Cell pellets expressing Nodal are able to induce expression of Pitx2 (50%, n = 8, Figure 2I). The medial domain of Nodal is induced by Shh prior to Car expression. Control beads had no effect on Nodal expression (100%, n = 18, Figure 2I).

Fgf8 antagonizes the pathway induced by Shh signaling (Boettger et al., 1999), preventing the induction of Nodal in the right LPM. If Car is a secondary signal in this process, Fgf8 could act upstream of Car or via a parallel pathway. To test this, beads soaked in FGF4 (which like Fgf8 represses Nodal; Boettger et al., 1999) were placed on the left side of Hensen’s node at stages 4 and 6 (Figure 2J). As a result, the expression of Car was dramatically decreased (90%, n = 14, Figure 2N). Control beads had no effect on Car expression (100%, n = 12, Figure 2M). Thus, Car is induced by Shh and repressed by Fgf8, and as a result, its expression is limited to the left paraxial mesoderm.

Car Regulates Asymmetric Gene Expression and Morphogenesis

These results suggested that Car could act upstream of the LPM expression of Nodal and its target Pitx2. To test this, we implanted cell pellets expressing Car on the right side of the node at stage 6 (Figure 3A). This resulted in ectopic induction of Nodal in the right LPM at stage 9 (67%, n = 15, Figures 3E and 3F). Cell pellets expressing Car implanted on the left side, where endogenous Car is expressed, had no effect on Nodal expression (Figure 3G). Pitx2 expression is induced in the LPM by Nodal, and as expected, following implantation of Car-expressing cell pellets on the right side at stage 6, ectopic expression of Pitx2 was also observed in the right LPM at stage 9 (48%, n = 21, Figure 3I). The Car-induced expression domain of Pitx2 was broader than that of Nodal. These results suggest that Car regulates the left-sided LPM expression of Nodal and Pitx2.

In previous studies, we inferred the existence of a secondary signal because Shh was not sufficient to induce Nodal in the LPM in explant culture unless paraxial tissue was also included (Pagan-Westphal and Tabin, 1998). We attempted to similarly test whether Car requires an additional signal to induce targets. Car can induce Nodal in LPM explants in the absence of paraxial tissue (data not shown). This observation is consistent with Car directly inducing Nodal expression; however, we cannot rule out the possibility that Nodal induction requires an additional intermediate signal produced in the LPM in response to Car. In either case, the findings are consistent with the in vivo experiments affecting Nodal and Pitx2 expression.

To verify that alterations in Nodal and Pitx2 expression...
in response to Car would also affect morphological asymmetry, Car-expressing cells were implanted on the right side of Hensen’s node, and embryos were allowed to develop to heart looping stages. When harvested at stage 13, all the hearts had successfully undergone looping; however, the side to which they looped was randomized (50% left, n = 14, Figures 3B and 3C).

**Car Acts by Antagonizing BMP Signaling in the LPM**

The results presented thus far suggest that Car is a secondary signal produced in response to Shh that affects asymmetric morphogenesis by activating transcription of downstream targets in the left LPM. Several members of the Cerberus family function as BMP antagonists. This suggests that Car might induce Nodal by interfering with the activity of endogenous BMPs that would otherwise act to repress Nodal expression. We therefore examined the expression of a number of BMP genes in the LPM of stage 7-8 chick embryos. We indeed observed strong bilateral expression of Bmp2, Bmp4, and Bmp7 in the LPM (Figure 4). Bmp2 was expressed in the head fold and in the anterior LPM (Figures 4B and 4F), Bmp4 was expressed in the edges of the neural plate and in the posterior LPM (Figures 4C and 4G), and Bmp7 was also expressed in the posterior LPM (Figures 4D and 4H). All three genes were additionally expressed along the primitive streak.

If Car acts by binding to BMP proteins and preventing them from signaling through their receptors, we reasoned that high levels of BMP protein ectopically applied to the left LPM should exceed the endogenous concentration of Car and thereby repress Nodal. To test this, we introduced beads soaked in 1 mg/ml BMP2 into the left LPM of stage 6 chick embryos (Figure 5A). This soaking concentration results in approximately 2.35 pg of BMP protein per bead, which we hoped might be sufficient to titrate the endogenous Caronte produced throughout the LPM (one bead was introduced per embryo). This resulted in a clear repression of Nodal expression (50%, n = 10, Figure 5C), while control beads had no effect (n = 10, Figure 5B). Thus, BMPs can act to repress the expression of Nodal in the LPM, consistent with the model that Car induces asymmetric LPM gene expression by interfering on the left side with bilateral BMP signals.

All members of the Cerberus family tested thus far, including Cerberus, Dan, and Drm/Gremlin, can interact physically with BMP proteins (Hsu et al., 1998; Piccolo et al., 1999). To directly test whether Car can interact with BMP proteins, we produced epitope-tagged versions of these proteins in 293T cells and performed coimmunoprecipitation assays. Western blot analysis showed that high levels of HA-tagged BMP4 and Myc-tagged Car were present in the media overlying the transfected cells (Figure 5G). Under nonreducing conditions, the apparent molecular weight of HA-BMP4 was roughly double that seen under reducing conditions, suggesting that this molecule is correctly processed by Dorsal views of stage 7 (A-D) and stage 8 (E-H) embryos stained by whole-mount in situ hybridization, showing expression of Car (A and E), Bmp2 (B and F), Bmp4 (C and G), and Bmp7 (D and H). n, Hensen’s node; ps, primitive streak.
Figure 5. Car Activates Nodal Expression by Antagonizing BMP Signaling

(A–C) High concentrations of BMP2 can counteract the activity of endogenous Car. BSA- or BMP2-soaked beads (green circle, 1 mg/ml) were implanted in the left LPM at stage 6 (A). BMP2-soaked beads suppressed the endogenous expression of Nodal in the left LPM at stage 7 (C, green arrowhead), while BSA-soaked beads had no effect on Nodal expression (B, white arrowhead).

(D–F) The BMP antagonist Noggin can mimic the effect of Car in vivo. GFP- or Noggin-expressing cell pellets (pink circle) were implanted in the right LPM at stage 6 (D). Noggin-expressing cell pellets induce the ectopic expression of Nodal in the right LPM at stage 8 (F, pink arrowhead), while GFP-expressing cells have no effect on Nodal expression (E, white arrowhead).

(G) Western blot analysis of conditioned media from 293T cells transfected with HA-tagged BMP4 and Myc-tagged Car expression plasmids. The apparent molecular weight of HA-BMP4 under nonreducing conditions (39 kDa) is roughly twice that seen under reducing conditions (21 kDa). Under reducing conditions, the Myc-Car produced by 293T cells appears as three distinct bands, the largest and most prominent of which migrates at approximately 42 kDa (black arrowhead).

(H) COO-immunoprecipitation of Myc-Car and HA-BMP4 with anti-HA antibodies. Myc-Car from 293T cells was incubated with conditioned media from either mock or HA-BMP4 transfected 293T cells and immunoprecipitated with rabbit polyclonal anti-HA antibody and protein A/G beads. Bound Myc-Car was detected by immunoblotting with mouse monoclonal anti-Myc antibody. The Myc-tagged Car product coimmunoprecipitated specifically in the presence of HA-BMP4 (black arrowhead). Higher molecular weight bands are nonspecific background bands that appear in all immunoprecipitation lanes probed with the anti-Myc antibody.

(I) Western blot analysis of conditioned media from 293T and COS cells transfected with Myc- and Flag-tagged expression plasmids, respectively. Cells transfected with expression vectors for Myc-BMP4, Myc-Activin A (Act A), and Flag-Car produced protein products that migrate as single bands, whereas Myc-BMP7 protein from 293T cells migrates as a sharp 20 kDa band plus a slower migrating, more diffuse band of approximately 25 kDa.
same Flag-Car construct (data not shown). In coimmunoprecipitation assays using a polyclonal anti-Flag antibody, Flag-tagged Car was able to selectively pull down Myc-tagged BMP4 and BMP7, but not Activin A (Figure 5J).

Although these results provide strong biochemical support to the model that Car acts as an endogenous BMP antagonist, at least one member of the Cerberus/Dan family, Xenopus Cerberus, is a multifunctional antagonist that can also bind to Wnt proteins (Piccolo et al., 1999), suggesting that Car might also interact with other physiologically relevant signals. To test whether the ability of Car to antagonize BMP signaling was sufficient to explain Nodal induction, we took advantage of Noggin, a specific BMP antagonist. Noggin-producing cell pellets were implanted on the right side of stage 6 chick embryos, and Nodal expression at stage 8 was examined by in situ hybridization (Figure 5D). Like Car, Noggin application results in strong induction of Nodal in the right LPM (Figure 5F). Taken together, these results strongly suggest that Car functions in the LPM as a BMP antagonist, inducing Nodal and downstream genes by interfering with the repressive activity of endogenous BMPs.

**Car Regulates Lefty in the Midline**

Long-range unilateral signals such as Car need to be restricted to one side of the embryo to regulate asymmetric morphogenesis. In the mouse, Lefty-1 is predominantly expressed in the left half of the ventral neural tube (Meno et al., 1997) and is critical for insuring that targets of left-sided signals are only induced on the left side (Meno et al., 1998). A probe for a chick Lefty gene (a generous gift of Juan-Carlos Izpisua-Belmonte) also reveals left-sided expression of Lefty in the chick midline at stages 7 and 8, although the chick Lefty is expressed in the left half of the notochord rather than the floorplate (Figures 6A-6C and 6F). The limited sequence available for the chick gene does not allow it to be unambiguously identified as the direct homolog of Lefty-1 or Lefty-2. It is therefore referred to here simply as chick Lefty. The confinement of chick Lefty expression to the left half of the midline suggests that this putative barrier may be induced by the same left-sided signals whose activity it subsequently restricts. To test whether the midline expression of chick Lefty is regulated by Car, we implanted Car-expressing cells to the right side of the node at stage 6. When examined at stage 7, Car-treated embryos displayed bilateral Lefty expression in the notochord adjacent to the cell implant (42%, n = 12, Figures 6E and 6G).

Since Car appears to function as an antagonist and not as a direct signal itself, it presumably induces Lefty in the midline by interfering with an endogenous repressor of Lefty. Since BMP2, BMP4, and BMP7 are all expressed in the midline as well as the LPM (Figure 4) and since Car acts as a BMP antagonist, it seemed likely that Car might regulate Lefty expression by antagonizing BMP activity. To test whether BMPs can indeed repress Lefty in the midline, we implanted BMP4-expressing cell pellets to the left of the midline in stage 6 chick embryos. This resulted in repression of the endogenous Lefty expression (75%, n = 8, Figure 6I). To determine whether interfering with BMP activity is sufficient to induce expression of Lefty in the midline, Noggin-expressing cell pellets were implanted to the right of the midline in stage 6 chick embryos. Like Car, Noggin application is sufficient to induce expression of Lefty in the right side of the notochord (60%, n = 10, Figure 6H). Thus, in addition to inducing Nodal in the LPM, Car also functions to induce Lefty expression in the midline by antagonizing BMP signaling.

**Discussion**

The establishment of L-R asymmetry is an important, fundamental feature of the ontogeny of the vertebrate body plan. Although some molecular players involved in this process differ between species, the general outline seems to be conserved (see reviews by Harvey, 1998; Vogan and Tabin, 1999). Early on, a distinction is made between cells on the left and right sides of the node, perhaps on the basis of directional extracellular flow generated by rotation of cilia. This results in differential gene activation in small domains adjacent to the node, including the production of localized signals. Subsequently, broad asymmetric gene expression patterns are established in the LPM that direct L-R-specific morphogenesis. Prior to this study, there was a gap in our understanding of how asymmetries at the node are transferred to the LPM. We have now identified Caronte, a member of the Cerberus/Dan family of BMP antagonists, as the molecule responsible for transducing left-sided positional information from the node to the LPM (Figure 7). The same gene was independently identified by two other groups (Rodriguez-Estevez et al., 1999; Zhu et al., 1999). In contrast to all previously described members of the L-R cascade, which appear to act as simple inductive factors, Car mediates its effects by antagonizing the repressive activities of bilaterally expressed BMP signals.

**Regulation of Asymmetric Car Expression**

In situ hybridization revealed that Car is expressed in a unique pattern on the left side of the chick embryo, first appearing in the paraxial mesoderm and then extending through the left LPM. We find that Shh induces and Fgf protein represses Car expression. In principle, if a small amount of the inductive Shh protein inadvertently crossed the midline, it would fail to induce inappropriate right-sided Car expression because of the repressive activity of the more highly abundant Fgf8 protein. Similarly, if a small amount of Fgf8 were to diffuse across the midline, it would not interfere with Car expression because of the excess of Shh protein on the left side. Thus, the combined activities of Shh on the left and
Figure 7. The Role of Caronte in Mediating Left-Right Signaling in the Chick Embryo

(A) Regulation of Car. In the stage 4 embryo, an Activin-like signal is localized to the right side of Hensen’s node where it induces Fgf8 and represses Shh, resulting in right-sided Fgf8 and left-sided Shh. Fgf8 subsequently represses Car expression in the right paraxial mesoderm, while Shh induces Car expression in the left paraxial mesoderm.

(B) Car-mediated induction of Nodal in the left LPM. Between stages 6 and 7, Car protein is produced exclusively on the left side of the embryo where it antagonizes BMP signaling. Nodal expression is repressed by BMP activity in the right LPM, but due to the BMP-antagonistic effects of Car, Nodal is expressed in the left LPM.

(C) Car-mediated induction of Lefty in the left side of the midline. Car is expressed in tissue adjacent to the left side of the midline where it antagonizes BMP signaling. On the right side, BMPs repress the expression of Lefty. However, due to the BMP-antagonistic activity of Car, Lefty expression is induced in the left side of the notochord. The mechanism by which Lefty acts is not known, but one possibility is that Lefty acts as a sink, directly binding to Car protein and thereby preventing Car activity from reaching the right LPM.

Figure 6. Car Misexpression Can Induce Ectopic Expression of Lefty on the Right Side of the Notochord

(A-C) Normal expression pattern of chick Lefty from stages 7 to 8’ (dorsal view). Lefty is expressed in the left half of the notochord at these stages.

(D-G) Car can induce ectopic Lefty. As shown in dorsal views (D and E) and in cross section (F and G), Car-expressing cells were implanted into the right side of the node at stage 6, and the expression pattern of Lefty was analyzed 4 hr later. While control cells (white arrowhead) had no effect (n = 7) on the expression of Lefty (D and F), Car-expressing cells (red arrowhead) induced ectopic, bilateral expression of Lefty in the notochord (E and G). Red lines demarcate the midline of each embryo, and blue lines indicate the position of the cross sections shown in (F) and (G).

(H) Right-sided Noggin can induce ectopic Lefty. Noggin-expressing cells were implanted into the right side of the node at stage 6. Noggin-expressing cells (pink arrowhead) induced bilateral expression of Lefty in the notochord at stage 7’.

(I) BMP4 can repress Lefty in the midline. BMP4-expressing cells were implanted into the left side of the node at stage 6. The cell pellets (green arrowhead) repressed the expression of Lefty in the left side of the notochord at stage 7’. n, node; nc, notochord; L, left; R, right.

Fgf8 on the right provide tight regulation, resulting in exclusively left-sided Car expression (Figure 7A).

Car Acts Downstream of Shh to Establish Left-Specific Signaling in the Lateral Plate

In the chick, Shh is both necessary and sufficient to indirectly trigger expression of left-sided LPM genes such as Nodal and Pitx2; however, it has been shown that an intermediate signal produced in the paraxial mesoderm is required for this induction (Pagan-Westphal and Tabin, 1998). Both the timing and localization of Car expression made it a strong candidate for such a signal. Car transcripts first appear in the paraxial mesoderm shortly after Shh becomes asymmetrically expressed in the left side of the node, and just prior to induction of Nodal in the left LPM. Moreover, the onset
of Car expression at stage 6 coincides with the time at which a source of Shh is no longer required for explanted left-side mesodermal tissue to activate Nodal expression (Pagan-Westphal and Tabin, 1998).

Unlike Shh, which is tethered to the cell surface by cholesterol and likely travels over a limited number of cell diameters (reviewed in Tabin and McMahon, 1997), the Cerberus/Dan family proteins are freely secreted both in vivo and in vitro (Belo et al., 1997; Biben et al., 1998; Pearce et al., 1999). Car is therefore a much better candidate than Shh for a long-range signal activating gene transcription throughout the left LPM. Consistent with Car acting at a distance, when we applied Car locally the induced pattern of gene expression was very broad.

**Car Acts by Antagonizing BMP Signaling**

Members of the Cerberus/Dan family are antagonists of BMPs and other secreted signals. BMP antagonism, in particular, appears to be a consistent feature of this family (Pearce et al., 1999), and the conserved BMP-binding domain is present in Car. Commmunoprecipitation experiments verified that Car specifically binds to BMPs. The model that Car acts through inhibition of BMP signaling requires that there be a source of BMPs in the LPM. Indeed, we find that Bmp2, Bmp4, and Bmp7 are all expressed bilaterally, and that together they expose the entire LPM to BMP signaling. Furthermore, high concentrations of BMPs result in repression of Nodal in the left LPM. Therefore, it seems likely that the role of Car is to act as a BMP antagonist to block the repression of Nodal on the left side. A BMP pathway, mediated by the BMP receptor ALK2, has been shown in an independent study to be responsible for repressing Nodal in the right LPM in Xenopus (Ramsdell and Yost, 1999). Application of the specific BMP antagonist Noggin verified that BMP antagonism is sufficient to induce Nodal expression. Thus, the transfer of left-sided positional information to the LPM appears to be accomplished by localized inhibition of an otherwise ubiquitous opposing signal (Figure 7B)—a paradigm first introduced to explain the axis-inducing function of BMP antagonists in Xenopus (reviewed in Thomsen, 1997).

In addition to the genes expressed in the left LPM analyzed here, it has been reported that the transcription factor cSnR1 (Isaac et al., 1997). cSnR1 expression can be repressed on the right by ectopic application of Shh (Isaac et al., 1997), and, conversely, cSnR1 expression can be induced in the left LPM by application of Fgf protein (Boettger et al., 1999). Since the signaling downstream of Shh is mediated by Car antagonism of BMP proteins, it seems likely that BMP activity is responsible for activating cSnR1 expression in the right LPM. In principle, this could be achieved directly via BMP-mediated upregulation of cSnR1 transcription or indirectly via BMP-mediated repression of Nodal, which has been suggested to function to repress cSnR1 expression (Isaac et al., 1997).

**Car Induces Lefty in the Midline**

In addition to transducing asymmetric information laterally to the left LPM, Car also acts medially to induce expression of Lefty in the midline. Application of Noggin also induces Lefty expression in the midline, while ectopic BMP protein represses endogenous Lefty, indicating that Car acts in the midline by a mechanism similar to its action in the LPM: antagonizing the activity of endogenous BMPs (Figure 7C). Thus, Car, a long-range left-sided signal, induces expression of Lefty, a presumed barrier to long-range left-sided signaling (Meno et al., 1998). The mechanism by which Lefty achieves its barrier function is currently unknown. However, since Lefty is itself a member of the TGFβ superfamily, and since Car binds to some TGFβ family members, an intriguing (albeit speculative) hypothesis is that Lefty acts as a sink, binding to Car and thereby preventing Car activity from reaching the right LPM. In this model, Car induces the expression of a molecule that then limits the range of its own action, much the way that Shh diffusion is limited by the induction of the Shh targets Ptc and Hip (reviewed in Perrimon and McMahon, 1999). Testing this model will require the future isolation of a full-length chick Lefty clone to address potential biochemical interactions between Car and Lefty.

In summary, by inducing Lefty, Car limits its own activity to the left side of the embryo. There it functions, as shown in our experiments and independently by others (Rodríguez-Esteban et al., 1999; Zhu et al., 1999), as a key secondary signal transferring asymmetric signaling in and around the node to a broad domain in the LPM, such that cells throughout the left side of the embryo can be instructed as to their left-specific developmental fates.

**Experimental Procedures**

**RT-PCR Cloning and cDNA Library Screening**

To isolate Cerberus-related genes from chick, we performed reverse transcription-polymerase chain reaction (RT-PCR) using degenerate primers. Based on the sequences of Xenopus Cerberus (Piccolo et al., 1999) and the murine Cerberus homolog cer/cerberus-like/mCer1 (Belo et al., 1997; Thomas et al., 1997; Biben et al., 1998), we designed three degenerated primers: CerS1, CCGAATTCATAG(AAG)AACT(AAG)CTCTTGTGCTTTCTG(AAA)AGAATGT; CerA1, GCTTCTAGAAGAAGAATGT; and CerA2, GCCTCTAGATCACTAC(ACT)TCATCATGCTATGCAATCTG. Using Long PCR, we amplified a PCR fragment using the following primers with cSnR1 being expressed exclusively in the right LPM: Ptc and Hip (reviewed in Perrimon and McMahon, 1999). Testing this model will require the future isolation of a full-length chick Lefty clone to address potential bio-chemical interactions between Car and Lefty.

In summary, by inducing Lefty, Car limits its own activity to the left side of the embryo. There it functions, as shown in our experiments and independently by others (Rodríguez-Esteban et al., 1999; Zhu et al., 1999), as a key secondary signal transferring asymmetric signaling in and around the node to a broad domain in the LPM, such that cells throughout the left side of the embryo can be instructed as to their left-specific developmental fates.

**Virus Construction and Targeting**

The entire open reading frame of Car was amplified by high-fidelity PCR (Pfu polymerase, NEB) and cloned into a version of pSlaX21 containing an internal ribosome entry site (IRES; Amy Chen, Harvard Medical School). The coding region of the PCR-generated insert was confirmed by sequencing. A ClaI fragment containing the IRES-Car sequence was cloned into the RCAS(BP) retroviral vector (Hughes et al., 1987). To achieve restricted misexpression of Car,
chick DF-1 cells were infected with this construct and pelleted, as described previously (Riddle et al., 1993). Pellets were implanted between the epiblast and the hypoblast on the right side of the node of stage 5/6 embryos in New culture (New, 1955). RCAS virus vector carrying ckgFP (Ed Laufer, Columbia University) was used in control experiments. To misexpress Nodal, Noggin, and BMP4, we used RCAS-Nodal, RCAS-Noggin, and RCAS-BMP4 viral constructs, as described previously (Duprez et al., 1996; Levin et al., 1997; Capdevila and Johnson, 1998).

**Bead Implants**

Stage 4–6 embryos were explanted in New culture, and Affigel-Blue (Bio-Rad) beads soaked in Shh protein or anti-Shh antibody were prepared and implanted as described (Pagan-Westphal and Tabin, 1998). As a control in the anti-Shh antibody experiment, beads soaked in a control anti-VSVG-G antibody (1 μg/ml) were used. Beads soaked in FGFP (1 mg/ml; R&D Systems) or BMP2 (1 mg/ml; R&D Systems) were prepared as described (Boettger et al. 1999). As a control in the FGFP and BMP experiments, beads soaked in 0.1% BSA-PBS were used.

**Whole-Mount In Situ Hybridization**

Embryos were fixed in 4% paraformaldehyde/PBS and processed essentially as described (Levin et al., 1995). The Nodal probe was prepared as described in Levin et al. (1995), and the Pitx2 probe was prepared as described in Logan et al. (1998). The probe for Car was prepared by digesting the template DNA (CarPCR) with EcoRI and transcribing with T3 RNA polymerase. The probe for chick Lefty was prepared by digesting the template with XhoI and transcribing with SP6 RNA polymerase.

**Transient Transfections and Coimmunoprecipitations**

To generate epitope-tagged versions of Car, the stop codon of Car was replaced with a PstI site, and this modified full-length Car was prepared by digesting the template DNA (CarPCR) with SP6 RNA polymerase.-factor with neutralizing activity expressed in the anterior primitive streak. Dev. Biol. 68, 135–151.


