Distinct transcriptional regulatory mechanisms underlie left-right asymmetric expression of lefty-1 and lefty-2

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Both lefty-1 and lefty-2 genes are expressed on the left side of developing mouse embryos and are implicated in left-right (L–R) axis formation. With the use of transgenic analysis, the transcriptional regulatory regions of these genes responsible for their L–R asymmetric expression have now been investigated. The 9.5-kb upstream region of lefty-1 and the 5.5-kb upstream region of lefty-2 reproduced the expression pattern of the corresponding gene. Examination of deletion constructs revealed the presence of a left side-specific enhancer (ASE) that is essential and sufficient for lefty-2 asymmetric expression. In contrast, the asymmetric expression of lefty-1 was shown to be determined by a combination of bilateral enhancers and a right side-specific silencer (RSS). The 9.5-kb region of lefty-1 and the 5.5-kb region of lefty-2 responded to iv and inv, upstream genes of lefty-1 and lefty-2. The regulation of lefty-2 by iv and inv was mediated by ASE. These results suggest that, in spite of the similarities between lefty-1 and lefty-2, different regulatory mechanisms underlie their asymmetric expression.

[Key Words: Left-right asymmetry; lefty; transcriptional regulation]

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Vertebrates exhibit numerous left-right (L–R) asymmetries, such as the positions of the heart and spleen on the left side of the body. In rodents, L–R asymmetry appears to be determined at the presomite stage (Brown et al. 1991; Fujinaga and Baden 1991a,b). One of the earliest morphological indicators of laterality is the looping of the cardiac tube toward the right side. This event is accompanied by a clockwise axial rotation referred to as embryonic turning, and is followed, much later, by the asymmetric orientation of various visceral organs.

The molecular mechanism that underlies L–R axis formation has remained largely unknown until recently, when insights into this important process have been gained (for review, see King and Brown 1997; Varlet and Robertson 1997; Levin and Mercola 1998). The entire process by which L–R asymmetry is established can be divided into three phases: (1) the initial determination of L–R polarity, (2) L–R asymmetric expression of signaling molecules, and (3) L–R asymmetric morphogenesis induced by these signaling molecules. The mechanism of the initial determination of L–R polarity remains unknown, although several models have been proposed (Brown and Wolpert 1990; Yost 1992). In mice, several mutations are known to affect L–R patterning (Hummel and Chapman 1959; Yokoyama et al. 1991; Heymer et al. 1997; Melloy et al. 1997), two of the more extensively studied of which are iv and inv. L–R specification is randomized in iv/iv mice, whereas it is reversed in inv/inv mice. These phenotypes suggest that the iv and inv genes likely contribute to the initial (early) determination of L–R polarity. The iv and inv genes have been shown to encode a protein related to axonemal dynein (Supp et al. 1997), and a novel protein with ankyrin repeats (Mochizuki et al. 1998), respectively. However, the precise function of these proteins remains unclear.

With regard to the second phase of L–R axis formation, the L–R asymmetric expression of signaling molecules, Levin et al. (1995) have shown that the activin receptor typeIIA, sonic hedgehog, and cNRP-1 genes are expressed asymmetrically in chick embryos, and they have proposed a hierarchy for these genes. cNR-1 is a chick homolog of mouse nodal (Levin et al. 1995), which belongs to the TGFβ superfamily. The pattern of nodal expression is conserved among vertebrates; it is expressed in the left half of mouse, chick and Xenopus embryos (Colignon et al. 1996; Lowe et al. 1996). We have identified previously two highly related TGFβ-related genes, lefty-1 and lefty-2, that are also expressed on the left side of developing mouse embryos (Meno et al. 1996, 1997).
Whereas lefty-1 is expressed predominantly in the prospective floor plate (PFP), lefty-2 is expressed in lateral plate mesoderm (LPM). MISEXPRESSION experiments in chick and Xenopus, as well as analyses of knockout mice support the notion that these genes encode signaling molecules for L–R positional information (Levin et al. 1997; Sampath et al. 1997; Meno et al. 1998). For example, mice deficient in lefty-1 show a variety of positional defects in visceral organs, indicating that lefty-1 is required for the correct positioning of these organs (Meno et al. 1998). For example, mice deficient in lefty-1 show a variety of positional defects in visceral organs, indicating that lefty-1 is required for the correct positioning of these organs (Meno et al. 1998). In lefty-1−/− mice, lefty-2 and nodal are ectopically expressed on the right side, suggesting that Lefty-1 restricts the expression of nodal and lefty-2 to the left side, perhaps by acting as (or inducing) a midline barrier. The observation that expression of lefty-1, lefty-2, and nodal is affected in iv and inv mutant embryos suggests that lefty-1, lefty-2, and nodal all act downstream of the iv and inv genes.

With regard to the third phase of establishment of L–R asymmetry, situs-specific morphogenesis in response to L–R asymmetric signals, genetic evidence in mice (Oh and Li 1997) suggests that type II activin receptors (ActRIIA and ActRIIB) serve as functional receptors for Nodal (and also for Lefty-2). Furthermore, recent studies (Logan et al. 1998; Meno et al. 1998; Piedra et al. 1998; Ryan et al. 1998; Yoshioka et al. 1998) have indicated a role for Pitx2 (also known as Rieg, Otlx2, and Brx1; Mucchielli et al. 1996; Semina et al. 1996; Gage and Camper 1997; Kitamura et al. 1997), a bicoid-type homeobox transcription factor, in mediating transmission of signals from Nodal and Lefty-2. In addition, Snr-1, which encodes a zinc finger-type transcription factor, is expressed on the right side of chick embryos (Isaac et al. 1997) and may be negatively regulated by Nodal.

In spite of the recent progress, our knowledge of L–R determination remains limited. For example, the mechanism by which the initial determination of L–R polarity eventually results in the asymmetric expression of signaling molecules is not known. An understanding of the early events that take place before the asymmetric expression of lefty-1, lefty-2, and nodal will require elucidation of the mechanisms by which these genes are regulated. We have now identified the transcriptional regulatory elements of lefty-1 and lefty-2 that are responsible for the asymmetric expression of these genes. Our results indicate that lefty-2 is regulated by an asymmetric enhancer that is active exclusively on the left side. In contrast, lefty-1 is controlled by an asymmetric silencer that represses expression on the right side. These observations suggest that, although both lefty-1 and lefty-2 are expressed asymmetrically, different regulatory mechanisms underlie the L–R asymmetric transcription of the two genes.

Results

The upstream regions of lefty-1 and lefty-2 determine the expression patterns of these genes lefty-1 and lefty-2 are tightly linked on mouse chromosome 1, being separated by a distance of only 30 kb (Fig. 1A; Meno et al. 1997). Both genes exhibit similar exon-intron structures and possess a putative TATA box at an expected location. We first examined the transcriptional activity of upstream regions of lefty-1 and lefty-2. For lefty-1, the 9.5-kb upstream region containing the TATA box (from −9.5 kb to +80 bp relative to the TATA box) was linked to lacZ, yielding L1-9.5 (Fig. 1A). For lefty-2, the 5.5-kb upstream region including the TATA box (−5.5 kb to +90 bp relative to the TATA box) was fused to lacZ, yielding L2-5.5 (Fig. 1A). L1-9.5 and L2-5.5 were injected separately into the pronuclei of fertilized embryos at the one-cell stage. The embryos were allowed to develop in utero until embryonic day 8.2 (E8.2), at which time they were recovered and the expression of the lacZ transgene was examined by staining with X-gal (5-bromo-4-chloro-3-indoly-β-D-galactoside). Embryos injected with L1-9.5 showed marked X-gal staining in the PFP (Fig. 1B,C). Transverse sections of the embryos indicated that X-gal staining was localized predominantly in the left half of the PFP (Fig. 1D,E). Less marked X-gal staining was also detected in the anterior region of the LPM on the left side (Fig. 1B,E). This pattern of X-gal staining was indistinguishable from that of the distribution of lefty-1 mRNA determined previously by whole mount in situ hybridization (Meno et al. 1997).

Embryos injected with L2-5.5 showed marked X-gal staining in left LPM as well as weaker X-gal staining in left PFP (Fig. 1F–I). At E7.0, X-gal staining was detected in the emerging mesoderm (data not shown). These staining patterns were identical to the distribution profiles of lefty-2 mRNA determined previously by whole mount in situ hybridization (Meno et al. 1997). Thus, L1-9.5 and L2-5.5 were able to recapitulate the expression patterns of lefty-1 and lefty-2, respectively. These results also indicate that lefty-1 and lefty-2 are regulated independently, even though they are tightly linked on the same chromosome.

L–R asymmetric expression of lefty-2 is controlled by a left side-specific enhancer

To locate cis elements responsible for the asymmetric expression of lefty-2, we tested various restriction fragments derived from the 5.5-kb upstream region for the ability to confer asymmetric expression on lacZ (Fig. 2A). Whereas the 5.5-kb upstream fragment fully retained this ability, asymmetric expression was not apparent with L2-3.3 or L2-1.6 (Fig. 2A), suggesting the presence of a left side-specific enhancer in the 2.2-kb region between −5.5 and −3.3 kb. Although L2-3.3 embryos failed to show X-gal staining in LPM, symmetrical staining was detected in the paraxial mesoderm. Because X-gal staining in the paraxial mesoderm was not apparent in L2-1.6 embryos (Fig. 2A), a paraxial mesoderm-specific enhancer (PM; Fig. 2) is likely located between −3.3 and −1.6 kb.

To delineate further the cis elements responsible for asymmetric expression, we prepared two sets of deletion mutants from L2-5.5, all of which contained the 3.3-kb proximal upstream region (Fig. 2B). Among the 5′-dele-
tion mutants, left-sided lacZ expression in LPM and PFP was observed with L2-5.8D25 and L2-5.8D75 but not with L2-5.8D199 (Fig. 2B,C). Of the 38 deletion mutants, L2-5.8D56 showed asymmetric expression, whereas L2-5.8D37 did not (Fig. 2B,C). These results indicated the presence of a left side-specific enhancer referred to as ASE (asymmetric enhancer), in a 380-bp region between −4.1 and −3.7 kb. The observation that X-gal staining in the paraxial mesoderm was often apparent when ASE was deleted (L2-5.8D199 and L2-5.8D37; Fig. 2B) suggest that ASE may be required not only for left-sided expression but also for suppressing the paraxial mesoderm-specific enhancer.

Internal deletion of the 380-bp region containing ASE from L2-5.5, yielding L2D37-199 (Fig. 3A), abolished asymmetric expression (Fig. 3D,G), indicating that ASE is absolutely required for such expression. The ASE–lef2p construct (Fig. 3A), in which the 380-bp ASE fragment was linked to the minimal promoter region of lefty-2 (from −300 to +90 bp), yielded left-sided X-gal staining in left LPM and PFP (Fig. 3B,E). Finally, constructs in which the 380-bp fragment was linked to the hsp68 promoter instead of the lefty-2 promoter [ASE–hsp (R) and ASE–hsp (S); Fig. 3A] gave rise to X-gal staining in left LPM and left PFP (Fig. 3C,F). These results demonstrate that ASE is essential and sufficient for the asymmetric expression of lefty-2.

The left side-specific enhancer of lefty-2 is composed of multiple subdomains

The 380-bp region containing ASE was further dissected by directional and internal deletion analysis (Fig. 4A,B). Serial deletion of the 380-bp region from the 5’ end resulted in the sequential loss of lacZ expression in the PFP and LPM; expression disappeared first from the PFP, then from the posterior LPM, and finally from the anterior LPM (Fig. 4A,C). The L2-5.5Δ1501 construct was expressed in LPM but showed virtually no expression in the PFP. The mutants L2-5.5Δ98, L2-5.5Δ505, and L2-5.5Δ502 showed asymmetric expression in the anterior portion of LPM, but they exhibited a gradual loss of expression in the posterior LPM as the extent of the de-
lefty-1 by bilateral enhancers and a right side-specific silencer

To localize the transcriptional regulatory elements of lefty-1, we tested various restriction fragments derived from the 9.5-kb upstream region for the ability to confer asymmetric expression (Fig. 5A). The 6.0-kb upstream fragment (L1-6.0) fully retained the ability to mediate lacZ expression in the PFP and left LPM (Fig. 5B,E). Whereas L1-3.0 and L1-1.3 retained asymmetric transcriptional activity in the PFP (transverse section indicated that X-gal staining in PFP was left sided [data not shown]), no expression was apparent in LPM (Fig. 5C,D,F,G). These results suggested the presence of an LPM-specific enhancer (LPE) in the 3.0-kb region between −6.0 and −3.0 kb.

The asymmetric expression in the PFP was maintained in L1-3.0 and L1-1.3 (Fig. 5), suggesting the presence of a PFP-specific enhancer(s) within the 1.3-kb upstream region. To delineate further the position of the PFP-specific enhancer, we constructed a series of 5’-deletion mutants from L1-1.3. Because the asymmetric ex-
expression of lacZ in the PFP was not apparent with L1-5'.9 or L1-5'.18, the PFP-specific enhancer referred to as NPE (neural plate enhancer) was localized to a 200-bp region between −1.3 and −1.1 kb.

To examine whether LPE and NPE possess L–R specificity, LPE (the 1.5-kb fragment from −4.5 to −3.0 kb) and NPE (the 0.2-kb fragment from −1.3 to −1.1 kb) were linked to the hsp68 promoter, yielding LPE-hsp and NPE-hsp, respectively (Fig. 6A). Unexpectedly, LPE-hsp gave rise to X-gal staining on both sides of LPM (Fig. 6B). As with L1-9.5 (Fig. 1D,E), the anterior portion, but not the posterior portion, of LPM was positive for X-gal staining. NPE-hsp also lacked L–R specificity, with bilateral X-gal staining apparent in a broad region of the neural plate along the A–P axis (Fig. 6C,F,I,M). Therefore, unlike ASE of lefty-2, neither LPE nor NPE alone confers L–R specificity. It was most likely that LPE and NPE are bilateral enhancers, and that L–R specificity was determined by some other cis element.

Because L1-1.3 showed left-sided X-gal staining in the PFP (Fig. 5A,D,G), a cis element that controls asymmetric expression appeared to reside in the proximal promoter region (between −1.3 and −1.1 kb).
Transcriptional regulatory elements of lefty-1 and lefty-2 respond to iv and inv mutations

Expression of lefty-1 and lefty-2 is affected in situ mutants such as iv/iv and inv/inv mice; the pattern of expression is randomized (left-sided, right-sided, or bilateral) in iv/iv mutants, and reversed in inv/inv mutants (Collignon et al. 1996; Lowe et al. 1996; Meno et al. 1996, 1997). Therefore, lefty-1 and lefty-2 both act downstream of the iv and inv genes. We examined whether expression of the lefty-1-lacZ and lefty-2-lacZ transgenes was affected by the iv and inv mutations, by establishing permanent transgenic lines containing L1-9.5 or L2-5.5 (lines 1-1 and 2-1, respectively). Embryos derived from these permanent lines showed X-gal staining patterns identical to those observed in the transient transgenic embryos; line 1-1 embryos showed X-gal staining in left PFP as well as weaker staining in left LPM, whereas line 2-1 embryos exhibited marked X-gal staining in left LPM and weaker staining in left PFP (data not shown).

Line 2-1 mice were mated with iv/iv mice and the lacZ transgene was transferred to the iv/iv background. The iv/iv embryos harboring the L2-5.5 transgene showed three different patterns of X-gal staining in LPM; left-sided, right-sided, or bilateral (Fig. 7A–C). The L2-5.5 transgene was also transferred to the inv/inv background by mating line 2-1 animals with inv/+ mice (inv/inv homozygotes are lethal). Among the embryos obtained by crossing inv/+; L2-5.5 mice with inv/+ mice, ~25% of lacZ+ embryos (14/46), which were inv/inv homozygotes, showed aberrant X-gal staining pattern in LPM; the staining pattern in such embryos was either right-sided (11/14 embryos; Fig. 7F) or bilateral (3/14 embryos; Fig. 7E). These X-gal staining patterns observed in the iv/iv and inv/inv mice were identical to the patterns of lefty-2 mRNA distribution in these mutants (Meno et al. 1997). Therefore, we concluded that the 5.5-kb upstream region of lefty-2 can reproduce the response to the iv and inv mutations.

To examine whether ASE alone can respond to the iv mutation, we also established a permanent line containing ASE–hsp (line 2-2). Line 2-2 embryos expressed lacZ in left LPM and PFP (data not shown), as observed with the transient transgenic embryos (Fig. 3C,F). Line 2-2 mice were then crossed with iv/iv mice and inv/+ mice. The iv/iv embryos harboring an ASE–hsp transgene showed either left-sided, bilateral, or right-sided X-gal staining both in LPM and PFP (Fig. 7G–I). The inv/inv embryos harboring the ASE–hsp transgene showed either right-sided or bilateral X-gal staining in LPM and PFP (Fig. 7K,L). These results indicate that the regulation of lefty-2 by iv and inv genes is mediated through ASE.
Similarly, the L1-9.5 transgene was transferred to the inv/inv and iv/iv backgrounds. The iv/iv embryos containing the L1-9.5 transgene showed either left-sided (Fig. 7M,P), right-sided (Fig. 7O,R), or bilateral (Fig. 7N,Q) expression in the PFP and LPM. When inv/+; L1-9.5 mice were mated with inv/+ mice, most of the transgene-positive inv/inv embryos showed right-sided X-gal staining in the PFP (Fig. 7U,X), whereas the remainders exhibited bilateral expression in the PFP (Fig. 7T,W). These results indicate that the regulation of lefty-1 by iv and inv genes is mediated by the 9.5-kb upstream region.

**Discussion**

Regulation of lefty-1 and lefty-2 by distinct mechanisms

Lefty-1 and lefty-2 are tightly linked on mouse chromosome 1. However, we have now demonstrated that the two genes are regulated independently and by distinct mechanisms (Fig. 8). The asymmetric expression of lefty-2 appears to be determined solely by a left side-specific enhancer (ASE), given that ASE alone is necessary and sufficient for L–R asymmetric expression. In contrast, L–R asymmetric expression of lefty-1 is determined not by the enhancers (LPE and NPE) but by an RSS.

Despite the similarities in structure and expression patterns between lefty-1 and lefty-2, several differences are apparent between the two genes. First, their major expression domains differ; whereas lefty-1 is expressed predominantly in the PFP, lefty-2 is expressed predominantly in LPM. In addition, lefty-1 expression slightly precedes that of lefty-2; lefty-1 expression begins in the PFP at the two-somite pair stage, whereas the asymmetric expression of lefty-2 begins in left LPM at the three-somite pair stage (Meno et al. 1997; Heymer et al. 1997). Our data suggested that, although both are expressed in...

**Figure 6.** L–R specificity of lefty-1 expression is determined by RSS. (A) Structures and X-gal staining patterns of various lacZ constructs containing portions of the upstream region of lefty-1. (B) X-gal staining patterns of representative embryos transgenic for a set of LPE-containing constructs. X-gal staining in the anterior LPM is bilateral with LPE-hsp and LPEΔ10, but is left-sided with LPEΔ8. (C–O) X-gal staining patterns of representative embryos harboring NPE-containing constructs. (C–E) Anterior views; (F–H), lateral views; (I–O), transverse sections. NPE-hsp shows bilateral staining in the neural plate (C,F,I,M). Addition of the RSS fragment (from −0.9 kb to +70 bp) to NPE-hsp, yielding NPE–RSS–hsp rescued the left-sided staining in the anterior and posterior portions of the PFP (E,H,K,O). Whereas L1-1.3 showed left-sided X-gal staining in the PFP (Fig. 5D,G), deletion of RSS (from −0.9 kb to −70 bp) from L1-1.3, yielding NPEΔ10, resulted in bilateral X-gal staining in the PFP (D,G,J,N) with the exception that left-sided expression was maintained in the anterior end of the PFP. (HG) Hindgut.
left LPM, lefty-1 and lefty-2 are regulated differently. This difference may reflect distinct cell lineages for lefty-1-expressing and lefty-2-expressing mesoderm cells; lefty-2 expression in LPM begins at the left side of the node, whereas lefty-1 expression in LPM begins at the left foregut. Expression of lefty-1 and that of lefty-2 are affected similarly in iv and inv mutants (Meno et al. 1997) but differently in other mutants such as nt and ft. In nt mutant mice, for instance, lefty-1 is not expressed, whereas lefty-2 expression is bilateral (Melloy et al. 1998). A second difference between lefty-1 and lefty-2 is that they likely play distinct roles in L-R determination. Recent analysis of lefty-1−/− mutant mice has suggested that the role of lefty-1 is to restrict the expression of lefty-2 and nodal to the left side of the embryos, whereas lefty-2 and nodal may encode signals for leftness (Meno et al. 1998). Thus, lefty-1 appears to act upstream of lefty-2 and nodal. These differences between the two genes may explain, at least in part, the existence of distinct mechanisms for the regulation of lefty-1 and lefty-2.

The expression patterns of nodal and lefty-2 are highly similar; the timing and sites of their expression are indistinguishable. Furthermore, these two genes may play similar roles in L-R determination; as mentioned above, both Lefty-2 and Nodal may act as determinants for leftness (Meno et al. 1998). In addition, both Lefty-2 and Nodal can induce Pitx2 expression in chick embryos (Logan et al. 1998; Piedra et al. 1998; Yoshioka et al. 1998). Therefore, the asymmetric expression of nodal and lefty-2 may be regulated by similar mechanisms. We and others have recently identified a left side-specific enhancer in the nodal gene (H. Adachi, Y. Saijoh, S. Ohishi, K. Mochida, and H. Hamada unpubl.; E.J. Robertson, pers. comm.).

Our data suggest that ASE is composed of multiple subdomains. Thus, it is likely that different sets of cis-regulatory sequences within the 380-bp region contribute to the regulation of lefty-2 in the PFP, the anterior LPM, and the posterior LPM. This notion is supported by our observations with lefty-1 mutant mice (Meno et al. 1998). In lefty-1−/− embryos, both lefty-2 and nodal are bilaterally expressed in LPM. However, the ectopic expression of lefty-2 in right LPM was always confined to the anterior portion, suggesting that lefty-2 is regulated differently in the anterior and posterior LPM.
Positive signals on the left side or negative signals on the right side?

Theoretically, L–R asymmetric transcription of left side-expressed genes can be achieved by one of the two regulatory mechanisms; transcriptional activation on the left side or transcriptional silencing on the right side. Although our data may appear to support the former mechanism for lefty-2 and the latter mechanism for lefty-1, it is premature to reach such a conclusion. In the case of lefty-2, for instance, the L–R specificity of ASE could be conferred either by the presence of an ASE-activating signal on the left side and its absence on the right side, or by the presence of an ASE-repressing signal on the right and its absence on the left. The same logic applies to RSS of lefty-1. Distinguishing between the two possible mechanisms for the asymmetric expression of lefty-1 and lefty-2 will require identification of the transcription factors that bind to ASE and RSS.

Materials and methods

IacZ constructs

Chromosomal lefty-1 and lefty-2 genes were isolated from a genomic library derived from 129/Sv mice. The 3’ end of the lefty-1-upstream fragment (SalI–HindIII 9.5 kb) is located in the 5’-untranslated region (+80 bp relative to the putative TATA box, −20 bp relative to the translation initiation codon). The 3’ end of the lefty-2 upstream fragment (SalI–HindIII 6 kb) is also located in the 5’-untranslated region (+90 bp relative to the putative TATA box, −10 bp relative to the initiation codon). These upstream fragments were linked to the lacZ fragment (HindIII–BamHI 4 kb) derived from pCH110 (Pharmacia) and were then subcloned in Bluescript or pGEM, yielding L1–9.5 and L2–5.5. In the 5’Δ set of L2–5.5 deletion mutants, deletion proceeded from the SalI site located at the 5’ end of L2–5.5 toward the 3’ end. In the 3’Δ set of L2–5.5 deletion mutants, deletion proceeded from the internal BamHI site (located at −3.3 kb) toward the 5’ end (3’-deletion mutants were constructed by adding each of 3’-deleted fragments to L2–3.3 in a correct orientation). For generation of lacZ constructs containing the hsp68 promoter, various DNA fragments were rendered blunt ended and subcloned at the Smal site of hsp68 lacZpa [Kothary et al. 1988; Logan et al. 1998; a generous gift from A. Joyner (New York University Medical Center) and J. Rossaint (Samuel Lunenfeld Research Institute, Mount Sinai Hospital)]. For all the constructs, lacZ fragments free of vector sequences were isolated by gel electrophoresis and used for microinjection.

Transient transgenic assay

Transgenic mice were generated by pronuclear injection of lacZ fragments (4 ng/µl) into fertilized eggs obtained from intercrosses between C57BL/6× C3H F1 mice (Hogan et al. 1994; Sasaki and Hogan 1994). The injected embryos were transferred into pseudopregnant recipients, and allowed to develop in utero until E8.2. Because of the narrow window of lefty expression, it was necessary to recover the embryos at a stage between three and eight somite pairs, for which the recipient mice were anesthetized with Nembutal and the stage of embryos was estimated by a size of the decidua. The E8.2 embryos were examined for the presence of the transgene (by PCR) and for lacZ expression

Regulation of lefty-1 and lefty-2 by signals downstream of iv and inv

Given that the expression of lefty-1, lefty-2, and nodal is affected in iv/iv and inv/inv mutant embryos (Collignon et al. 1996; Lowe et al. 1996; Meno et al. 1996, 1997), these three genes all likely act downstream of the iv and inv genes. Thus, signals downstream of the iv and inv genes must reach the transcriptional regulatory regions of lefty-1 and lefty-2. The 5.5-kb upstream region of lefty-2 was able to respond to the iv and inv mutations. Furthermore, ASE–hsp lacZ also responded to these mutations. Therefore, signals downstream from the iv and inv genes likely target the 380-bp region of ASE. The 9.5-kb upstream region of lefty-1 was also able to respond to the iv and inv mutations.

Regulation of lefty genes by the iv and inv genes must be indirect because these latter two genes appear to act at an earlier stage of L–R determination. Furthermore, the iv gene encodes a protein related to axonemal dynein, not a transcription factor (Supp et al. 1997). Clarification of the mechanism by which signals downstream of iv and inv regulate lefty genes will require identification of transcription factors that interact with ASE of lefty-2 and those that bind to RSS of lefty-1. The 380-bp region of ASE and the 0.7-kb region of RSS each possess a number of potential binding sequences for known transcription factors. To identify transcription factors that bind to ASE and RSS, detailed mutational analysis of ASE and RSS as well as the sequence information on other asymmetric enhancers (such as the ones found in nodal gene and human lefty-2) are needed.

Figure 8. Model for transcriptional regulation of lefty-1 and lefty-2. The asymmetric expression of lefty-1 (top) and lefty-2 (bottom) is determined by RSS and ASE, respectively.
(by X-gal staining). The activity of β-galactosidase in dissected embryos was detected by a standard protocol (Hogan et al. 1994). For each construct, >120 embryos were microinjected and transferred, and >10 transgene-positive embryos were examined by X-gal staining. The amount of β-galactosidase activity was estimated from the extent of X-gal staining after incubating in the staining buffer for 2, 8, and 24 hr. Primers used to amplify the lacZ sequence were 5′-TTCGCGTCTGAAATTTGACCTG-3′ and 5′-TCTGCTTCAATCAGCGTGCC-3′ (Sasaki and Hogan 1996).

Permanent transgenic lines

For some lacZ fragments, the transgenic embryos were allowed to develop to term and permanent transgenic lines were established. The presence of the transgene was examined by Southern blot analysis with the lacZ fragment as probe. The lacZ transgenes were introduced into the background of iv/iv or inv/+, by first mating transgenic mice with iv/iv and inv/+ mice, respectively. The genotype of the resulting offspring was determined by PCR or Southern blotting analysis. iv/iv, lacZ or inv/+; lacZ mice thus obtained were mated, and the expression of the transgenes at E 8.2 was examined. The iv alleles were genotyped by PCR with the primers, 5′-GCGAGAATGAC-GAGGTCCAT-3′ and 5′-AGCTCTGAAAACC-GTGGCTGGTGTGGCTGT-3′. The primers used to detect the wild-type allele are 5′-AGCTCTGAAAACC-GTGGCTGGTGTGGCTGT-3′ and 5′-TCTGCTTCAATCAGCGTGCC-3′ (Sasaki and Hogan 1996).

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