Asymmetric and node-specific *nodal* expression patterns are controlled by two distinct *cis*-acting regulatory elements

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The TGF β -related molecule Nodal is required for establishment of the anterior–posterior (A–P) and left–right (L–R) body axes of the vertebrate embryo. In mouse, several discrete sites of *nodal* activity closely correlate with its highly dynamic expression domains. *nodal* function in the posterior epiblast promotes primitive streak formation, whereas transient *nodal* expression in the extraembryonic visceral endoderm is essential for patterning the rostral central nervous system. Asymmetric *nodal* expression in the developing node and at later stages in left lateral plate mesoderm has been implicated as a key regulator of L–R axis determination. We have analyzed the *cis*-regulatory elements controlling *nodal* expression domains during early development. We show that the regulatory sequences conferring node-specific expression are contained in an upstream region of the locus, whereas early expression in the endoderm and epiblast and asymmetric expression at later stages on the left side of the body axis are controlled by a 600-bp intronic enhancer. Targeted deletion of a 100-bp subregion of this intronic enhancer eliminates *nodal* expression in the early epiblast and visceral endoderm and disrupts asymmetric expression in the node and lateral plate mesoderm. Thus, developmentally regulated *nodal* expression at distinct tissue sites during A–P and L–R axis formation is potentially controlled by common transcriptional activators.

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The activities of secreted growth factors belonging to the TGF β superfamily are critical for establishing the body axes of the vertebrate embryo. In particular, *nodal* homologs have been shown to contribute at distinct stages both during the initial specification and subsequent elaboration of the anterior–posterior (A–P) axis. In mouse, *nodal* signals are required for the formation of the primitive streak and initiation of gastrulation (Zhou et al. 1993; Conlon et al. 1994). Similarly, *Xnr-1* and *Xnr-2* function in *Xenopus* to promote mesoderm induction and patterning (Jones et al. 1995; Smith et al. 1995). Recent studies in both mouse (Varlet et al. 1997) and zebrafish (Feldman et al. 1998; Rebagliati et al. 1998a; Sampath et al. 1998) demonstrate *nodal* activities are necessary for development of the anterior central nervous system (CNS). *nodal* genes also act in a conserved molecular pathway to determine the left–right (L–R) axis, the process by which distinct and invariant handedness is conferred upon the embryonic body plan. Thus in all vertebrates examined, expression of a *nodal* family member exclusively in left lateral plate mesoderm (LPM) precedes the development of overt differences between the left and right sides of the embryo (Levin et al. 1995;

Collignon et al. 1996; Lowe et al. 1996; Rebagliati et al. 1998b). In chick and mouse only a single *nodal* gene has been identified to date, whereas multiple *nodal*-related genes have been cloned from zebrafish (Rebagliati et al. 1998b) and *Xenopus* (Jones et al. 1995; Smith et al. 1995; Lustig et al. 1996; Joseph and Melton 1997). Biochemical and genetic studies show these related ligands have both unique and partially overlapping functions. Thus, *Xnr-1* and *Xnr-2* are both potent inducers of dorsal mesoderm (Jones et al. 1995), but ectopic expression of *Xnr-1* selectively leads to reversal of body situs (Sampath et al. 1997). By contrast, *Xnr-3* acts as a neural inducer (Hansen et al. 1997). In zebrafish, the *cyclops* and *squint* mutants represent loss-of-function alleles at two independent *nodal* loci (Feldman et al. 1998; Rebagliati et al. 1998a; Sampath et al. 1998). Interestingly, *squint* has been shown to act as a recessive enhancer of the *cyclops* phenotype, suggesting these ligands have partially redundant functions during anterior patterning (Feldman et al. 1998).

In the postimplantation mouse embryo, sites of *nodal* functional activity closely correlate with highly dynamic expression domains. Thus, our previous studies have shown that *nodal* expression in the posterior epiblast is required for primitive streak formation, whereas transient *nodal* expression in the extraembryonic vis-

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ceral endoderm is essential for patterning the most anterior regions of the CNS (Varlet et al. 1997). These experiments suggest that *nodal* plays a key role during establishment of the anterior organizing center or AVE in the pregastrulation stage embryo (for review, see Beddington and Robertson 1998). Based on its node-specific expression pattern at later stages (Zhou et al. 1993; Conlon et al. 1994; Collignon et al. 1996), *nodal* has also been implicated in the formation and/or function of the mouse node, the organizing center responsible for patterning the midline and posterior regions of the embryo (Beddington and Robertson 1999). By early somite stages, asymmetric *nodal* expression domains are detected both in the node as well as in the left LPM (Collignon et al. 1996; Lowe et al. 1996). A second TGF β family member *lefty-2* is also asymmetrically expressed in the left LPM (Meno et al. 1997), suggesting that *nodal* and *lefty-2* may share transcriptional regulatory elements. Consistent with this idea, asymmetric expression of both genes is similarly perturbed in *inversion of embryonic turning* (*inv*) and situs inversus viscerum (*iv*) mutant embryos that develop abnormal body situs (Collignon et al. 1996; Lowe et al. 1996; Meno et al. 1996, 1997). Functional studies in chick have also demonstrated that *nodal* misexpression affects heart looping and polarity of visceral organs (for review, see Harvey 1998). Recent studies demonstrate that *pitx2*, a homeodomain transcription factor of the bicoid class, is an important downstream target of the *nodal* pathway (for review, see Harvey 1998). Thus, ectopic *nodal* expression in chick embryos induces *pitx2* (Logan et al. 1998; Piedra et al. 1998; Ryan et al. 1998), and right-sided *pitx2* expression alone is sufficient to interfere with establishment of situs (Logan et al. 1998; Ryan et al. 1998). In chick, *nodal* may also act to repress left-sided expression of molecules such as the transcription factor *Snr-1* (Issac et al. 1997). Collectively these data suggest that asymmetric *nodal* and *lefty-2* expression domains play a key role during specification of the L-R body axis (Beddington and Robertson 1999).

As for other TGF β family members, Nodal is thought to act by binding to cell surface serine–threonine receptor kinases, classified as type I or type II according to structural and functional criteria (for review, see Massagué 1996). The diverse ligand binding and signaling properties of TGF β receptor kinases have been analyzed extensively (for review, see Massagué 1998). The formation of a heteromeric complex between type I and type II receptor kinases results in the activation of downstream intracellular targets (Wrana et al. 1994). Functionally distinct type I receptors selectively phosphorylate different Smad effector proteins that function as transcriptional activators (for review, see Massagué 1998; Whitman 1998). In *Xenopus* overexpression of *Xnr-1* and *Xnr-2* leads to formation of dorsal mesoderm (Jones et al. 1995), an effect that can be blocked by coinjection of a dominant-negative form of the *Xenopus* type I activin receptor ALK4 (Piccolo et al. 1999). Thus, these *nodal* homologs may normally activate the TGF β /activin–Smad2/Smad3 pathway to influence cell fate. In mouse, loss-of-function mutations in ALK3 (Mishina et al. 1995)

and ALK4 (Gu et al. 1998), corresponding to BMP and activin type I receptors, respectively, arrest embryonic development prior to gastrulation, suggesting these receptors may be involved in transducing *nodal* signals at early stages. Consistent with this idea, both ALK4 and Smad2, an effector of the TGF β /activin pathway, are essential for the contribution made by the extraembryonic lineages in establishing anterior pattern in the early embryo (Gu et al. 1998; Waldrip et al. 1998), as expected for molecules acting in the *nodal* pathway. However, the various components of Nodal-specific receptor complexes and cell-type specific downstream targets responsible for *nodal* signaling at diverse tissue sites have yet to be elucidated.

We now have a rudimentary understanding of the tissue interactions and some of the molecular components essential for establishment of the key organizing centers of the early mouse embryo, namely the AVE and the node (for review, see Beddington and Robertson 1998, 1999). For example, HNF3 β , a member of the Fork head family of transcription factors, is necessary for the formation of the node (Ang and Rossant 1994; Weinstein et al. 1994). A complex pattern of HNF3 β expression is generated via the combined activities of multiple enhancer and repressor elements (Sasaki and Hogan 1996). Considerable data suggest that HNF3 β acts to control the expression domains of important downstream signaling molecules, such as Sonic hedgehog (Shh), that pattern the later-stage embryo (Epstein et al. 1999). Transcription factors acting at restricted sites may similarly be responsible for establishment of *nodal* and *lefty* asymmetries. Given the central role played by *nodal* during anterior patterning, formation of the primitive streak, and L-R axis determination, it is of considerable interest to understand *nodal* transcriptional control mechanisms at these different stages of embryonic development.

Here we used a conventional transgenic approach to map *cis*-acting regulatory elements controlling *nodal* expression domains. The sequences that confer node-specific expression are contained in a 2.7-kb upstream flanking region. By contrast, early expression in the visceral endoderm and epiblast and asymmetric expression at later stages on the left side of the body axis are controlled by a 600-bp intronic enhancer. Moreover, expression of transgenes carrying this asymmetric enhancer element is perturbed in both the *inv* and *iv* mutant backgrounds. Targeted deletion of a 100-bp region contained within the asymmetric enhancer of the endogenous *nodal* locus selectively eliminates *nodal* expression in the early epiblast and visceral endoderm lineages, and also renders the locus insensitive to the signals governing asymmetric expression in the node and LPM. The present experiments thus identify two distinct transcriptional enhancer regions governing highly dynamic patterns of *nodal* gene expression.

Results

Identification of the minimal promoter

The *nodal* locus is comprised of three coding exons span-

ning ~8 kb (Zhou et al. 1993; Fig. 1a). The transcriptional start site has not been previously mapped, and the first 327 bp of the published cDNA sequence in exon 1 originate from genomic sequence 5' to the presumed ATG translational start site. This 327-bp region has all the hallmarks of a classical Pol II promoter and transient transfection assays established that the 5' region has promoter activity (D.P. Norris and E.J. Robertson, unpubl.). Thus, the transcription start site is 12 bp upstream of the ATG. RNase protection assays have confirmed that *nodal* transcripts contain an unusually short 5' UTR (D. Constam and E.J. Robertson, unpubl.). Northern blots similarly show a single 1.8–2.0-kb *nodal* transcript lacking an appreciable 5' UTR (Conlon et al. 1994). DNase I hypersensitivity assays comparing chromatin prepared from embryonic stem (ES) cells, known to express *nodal*, with that from liver cells as a negative control identified two strong hypersensitive sites (HSs) specific to the expressed allele (denoted by arrows in Fig. 1a), the first mapping within the minimal promoter region and the second located ~1 kb downstream of the first exon.

Two distinct enhancer regions control *nodal* expression at early somite stages

To identify enhancers responsible for *nodal* transcriptional regulation, we constructed transgenes containing *nodal* genomic subregions, a LacZ reporter cassette, and either the endogenous *nodal* or the hsp68 minimal promoter (Sasaki and Hogan 1996). We initially tested four nonoverlapping fragments encompassing 25 kb of genomic sequence (Fig. 1b). Constructs C1, C2, and C4 included the hsp68 minimal promoter whereas the C3 con-

struct used the endogenous *nodal* minimal promoter. Initial screening was by transient analysis of embryos recovered at ~8.5 days of development, a stage at which *nodal* expression in both the node and the left LPM is readily detected (Collignon et al. 1996; Lowe et al. 1996; Fig. 2e). Age-matched embryos carrying the *nodal.lacZ* reporter allele were analyzed in parallel. The C1- and C3-containing transgenes resulted in variable ectopic *lacZ* expression patterns indicating that these are position dependent and are usually repressed in the context of the endogenous locus (data not shown). We found a reproducible pattern of node specific β -gal expression in embryos carrying the C4 transgene (–5 to –12 kb; Fig. 1b). Expression was strictly confined to the periphery of the notochordal plate in a manner faithful to the endogenous pattern of *nodal* expression (Fig. 2). Moreover, in agreement with the pattern seen using the *nodal.lacZ* allele, staining is also observed within the posterior notochord. After prolonged staining both the stable C4 line and the F₀ transgenic embryos show punctate expression in a minor population of cells surrounding the node.

In striking contrast, the C2 transgene (+0.5 to +8 kb) gave asymmetric β -gal expression confined to the left LPM. This expression domain extends along the entire length of the left LPM from the base of the allantois posteriorly to the level of the heart anteriorly (Fig. 3e). Expression is conspicuously absent from both the node and notochord. Analysis of tissue sections confirmed that *lacZ* expression is confined to the mesoderm of the splanchnopleure and somatopleure. We also consistently observe LacZ staining in floor-plate cells of the neural tube. This cell population normally lacks *nodal* expression as assessed by whole-mount mRNA in situ or via

Figure 1. The *nodal* locus contains two physically distinct *cis*-acting regulatory elements. (a) Genomic organization and restriction sites used to produce the reporter constructs; the extreme 5' end corresponds to the 5' extent of the λ clone. Exons are shown as solid boxes. The positions of two DNase I hypersensitive sites specific to the expressed locus are signified by vertical arrows. Probes used for the hypersensitivity analysis are indicated in red. (E) *EcoRI*; (H) *HindIII*; (N) *NotI*; (S) *SalI*; (Sm) *SmaI*; (Sp) *SpeI*. (b) Genomic subregions used for production of reporter constructs. The fragments shown in blue gave LacZ staining patterns corresponding to *nodal* expression domains whereas those in black did not.

Figure 2. Fine mapping of the upstream region controlling node-specific expression. (a) Genomic fragments analyzed in the delineation of the node specific element. Constructs giving expression within the node at 8.5 dpc are shown in blue; nonexpressing ones are in black. The total number of positive embryos isolated is indicated over the total number of embryos analyzed in the transient analysis. The number of stable transgenic lines expressing within the node is indicated, together with the total number of transgene positive lines identified (in parentheses). (*) Three out of four C4a integrations expressed *lacZ*, but the staining patterns did not correspond to *nodal* expression domains, nor were results consistent between lines. (b–e) Control *nodal.lacZ*/+ embryos were stained using Salmon-gal as the substrate. For 6.5- and 7.5-dpc embryos anterior (A) is at left and posterior (P) at right. For 8.5-dpc embryos the left (L) and right (R) sides of the axis are indicated. Expression patterns in embryos carrying a stable integration of the C4 transgene (f–i) or a stable integration of the C4d transgene (j–l) are shown. In C4d embryos, expression is not visible prior to node formation. (m) Transverse section of 6.5-dpc C4 embryo. Expression is detected within the epiblast (e) and mesoderm (m) but not the visceral endoderm (ve). (n) Frontal section through an 8.5-dpc C4 transgenic embryo. Expression is detectable within the periphery of the ventral layer of the node. Notably, transgene expression in the node lacks the marked asymmetry seen for the endogenous *nodal* locus. (o) Section through an 8.0-dpc C4d transgenic embryo. LacZ protein is localized symmetrically in the lateral edges of notochordal plate. (ex) Extra-embryonic region; (em) embryonic region; (nd) node; (ps) primitive streak; (lpm) lateral plate mesoderm.

Figure 3. Fine mapping of the intronic enhancer responsible for asymmetric *nodal* expression. (a) Genomic fragments analyzed in the characterization of a minimal asymmetric enhancer. Positive embryos showed *lacZ* expression in the left LPM at 8.5 dpc. Expression patterns in embryos carrying a stable integration of the C2 transgene (b–e) or a stable integration of the C2g transgene (f–i) are shown. (j,k) Transverse sections of C2 transgenic embryos at day 5.5 and 6.5 dpc, respectively, showing expression within the epiblast and visceral endoderm. (l) Transverse section of C2g embryo at 6.5 dpc showing expression in the epiblast and visceral endoderm. (m) Frontal section through an 8.5-dpc C2 transgenic embryo. *lacZ* expression is detectable in the splanchnopleure and somatopleure of the lateral plate mesoderm, as well as in the left side of the floor-plate (fp), but is absent from the somites (s). Orientation of embryos and labeling is as in Fig. 2.

analysis of the more sensitive *nodal.lacZ* allele. As shown in Figure 3m, floor-plate expression is distinctly asymmetric, with β -gal activity confined to cells on the left side of the midline (see below). Moreover three founder lines carrying C2 gave similar patterns of β -gal expression. Thus, at 8.5 days of development the C2 and C4 transgenic constructs gave nonoverlapping expression patterns strongly suggesting that *nodal* expression is controlled by two distinct enhancer regions.

A 2.7-kb upstream region contains the *node* enhancer

The onset of *nodal* expression occurs at 5.0 days post coitum (dpc) in the visceral endoderm and throughout the epiblast (Varlet et al. 1997). *nodal* transcripts are initially detected throughout the visceral endoderm overlying the epiblast, but this expression is rapidly lost coincident with the initiation of gastrulation (Varlet et al. 1997). *nodal* expression is gradually down-regulated in the distal tip of the epiblast and becomes confined to cells within the prospective posterior proximal quadrant precisely marking the site of primitive streak formation (Collignon et al. 1996; Varlet et al. 1997; Fig. 2b,c). Shortly after the onset of gastrulation and elongation of the primitive streak, *nodal* expression becomes restricted to cells of the developing node (Collignon et al. 1996). To further describe *cis*-acting elements control-

ling these complex expression domains we analyzed stable lines containing C2 and C4 transgenes. Embryos carrying a stable integration of the C4 transgene show very low levels of *lacZ* expression detectable throughout the epiblast at 5.0 dpc (data not shown). Approximately 12 hr later, *lacZ* expression is confined to the proximal regions of the epiblast and subsequently becomes localized to the posterior side (Fig. 2f,g). In contrast there was no detectable expression in visceral endoderm cells (Fig. 2m, data not shown). Sectioning of early gastrulation stage embryos shows LacZ staining restricted to nascent mesoderm (Fig. 2m), as previously described for endogenous *nodal* transcripts (Varlet et al. 1997). At 7.5 dpc, expression was observed along the length of the primitive streak and in a patch of cells at the distal tip of the fully extended streak (Fig. 2h). As mentioned above, the C4 genomic subregion drives expression within the periphery of the node (Fig. 2i) specifically within the ventral layer of the notochordal plate (Fig. 2n).

To further dissect *cis*-acting regulatory elements, the 7.0-kb C4 genomic region was subdivided (Fig. 2a). The shorter 2.0-kb C4a transgene failed to give detectable expression in F₀ embryos recovered at 8.5 dpc, and four independent founder lines similarly gave negative results at all embryonic stages examined. In contrast, F₀ embryos carrying the C4b transgene clearly showed *lacZ* expression confined to the node (data not shown). This

4.6-kb region was then further subdivided. At 8.5 dpc, the 2.0-kb C4c transgene failed to give expression in the node. The possible activity of the C4b and C4c constructs at earlier stages of embryogenesis has not yet been analyzed. Five transgenic lines carrying stable integrations of C4d were generated, and four of these were found to express *lacZ* specifically within the node (Fig. 2l). Thus, the node enhancer maps to the 2.7-kb C4d fragment (−7.5 kb to −10.2 kb). However, in marked contrast to the C4 construct, we fail to detect early *lacZ* expression conferred by the C4d transgene prior to node formation (Fig. 2j,k). Thus, further work is needed to identify C4 enhancer(s) that contribute to *nodal* transcription in the epiblast.

At the three- to four-somite stage normal embryos show asymmetric *nodal* expression within the node, reflecting approximately twofold higher numbers of *nodal*-positive cells on the left side (Collignon et al. 1996). In contrast, embryos carrying either the C4 or C4d transgenes show equivalent node-specific staining on the left and right sides (Fig. 2n,o), implying that the asymmetric node element(s) maps outside the 7.0-kb C4 region.

The minimal asymmetric enhancer overlaps with the HS site in intron 1

As shown above, the C2 transgene is sufficient to direct *lacZ* expression exclusively on the left side of the body axis (Fig. 3e). To examine enhancer activities at earlier stages of development, we analyzed three independent transgenic lines. *LacZ* staining was consistently observed at 5.5 dpc at high levels throughout the epiblast (Fig. 3b) and in the overlying visceral endoderm (Fig. 3j). Shortly before the initiation of gastrulation at 6.5 dpc, highest levels of expression are seen in the most proximal posterior regions of the epiblast (Fig. 3c). Transverse sections confirm β -gal staining is restricted to visceral endoderm and posterior epiblast cells (Fig. 3k). At 6.75 dpc, C2 expression is initially confined to the posterior epiblast (Fig. 3d) and by 7.5 dpc largely disappears (data not shown). From the three-somite stage on asymmetric expression is observed within left LPM (Fig. 3e). Intriguingly, β -gal expression is also detectable in a small number of cells exclusively on the left side of the floor plate (Fig. 3m). As mentioned above, neither *nodal* mRNA nor *nodal.lacZ* activity is normally present in this cell population (Collignon et al. 1996; Lowe et al. 1996). However this expression pattern closely resembles that reported for the *lefty-1* and *lefty-2* genes (Meno et al. 1996, 1997). It is possible that the C2 genomic subregion lacks a repressor element that normally silences *nodal* expression at this site. Alternatively, the increased sensitivity afforded by high-copy transgenes (all three lines carry 5–8 copies of the transgene) potentially reveals a minor *nodal* expression domain.

The 8-kb C2 genomic subregion extends across the two downstream exons including a DNaseI HS within the first intron (Fig. 3a). To further delineate the asymmetric enhancer, we analyzed a nested series of transgenes. As shown in Figure 3, these fragments include and

extend downstream of the HS. Results obtained in both transient transgenic embryos and founder lines map the left LPM enhancer to a 1.0-kb subregion defined by the C2f transgene (Fig. 3a). The 596-bp fragment (C2g) containing the DNase I HS in intron 1 was subsequently amplified by PCR and analyzed by F₀ transgenesis. The C2g subregion gave expression within the left LPM at 8.5 dpc. Two stable lines carrying the C2g transgene show expression throughout the epiblast at 5.5 dpc (Fig. 3f), and this domain gradually resolves to the posterior side over the next day of development (Fig. 3g). Transverse sections through the embryonic region show expression within the visceral endoderm (Fig. 3l). Following extension of the streak to its distal limit, expression is greatly reduced but remains detectable on the posterior side of the epiblast (Fig. 3h). From the three-somite stage onwards, the C2g transgene faithfully recapitulates the asymmetric expression of the endogenous gene in the lateral plate (Fig. 3i). *lacZ* expression is detected synchronously along the full length of the left LPM and is down-regulated shortly after the initiation of embryonic turning (data not shown). Interestingly, the C2g transgene is not expressed in the midline of the floor plate, possibly because of the loss of a C2 transgene floor plate enhancer. Overall, these results demonstrate that the 596-bp minimal enhancer region spanning the intron 1 HS site includes *cis*-acting elements necessary for expression within the primitive endoderm, epiblast, and LPM.

*Asymmetric enhancer activity is perturbed in *iv* and *inv* mutant embryos*

The mutations *iv* and *inv* disrupt establishment of the L-R axis during early mouse development. *iv*, a recessive mutation resulting from an amino acid substitution in the L-R dynein (Lrd) protein (Supp et al. 1998), causes randomization of body situs (Hummel and Chapman 1959). The *inv* mutation in contrast causes a reversal of situs (Yokoyama et al. 1993). The transgene insertion at the *inv* locus largely deletes the coding region of a novel ankyrin repeat-containing protein (Mochizuki et al. 1998; Morgan et al. 1998). Both of these mutations perturb the normally asymmetric *nodal* and *lefty* expression patterns. In *iv/iv* embryos, *nodal* and *lefty-2* expression domains are absent, bilateral, or random (Lowe et al. 1996; Meno et al. 1997), whereas *nodal* and *lefty* expression are for the most part reversed in *inv/inv* embryos (Collignon et al. 1996; Lowe et al. 1996; Meno et al. 1996).

To test whether the asymmetric enhancer element responds appropriately to regulatory cues, independent C2 transgenic lines were crossed into these mutant backgrounds. As shown in Figure 4, in the context of the *inv* mutation the majority of C2 transgenic embryos showed bilateral *lacZ* expression (Table 1). For the most part, roughly equivalent staining was observed on both sides of the axis, but occasionally embryos showed enhanced expression on the right (Fig. 4a,e). Thus, C2 transgene expression differs significantly from that observed for en-

ogenous *nodal* transcripts found only on the right side of the axis in *inv/inv* embryos (Collignon et al. 1996; Lowe et al. 1996). On the other hand, C2 transgene expression more closely resembles that previously documented for endogenous *nodal* in the *iv/iv* background (Lowe et al. 1996). Thus, we observed unilateral expression on the left or right side, or bilateral expression (Fig. 4f,g), although, as for the *inv* mutants, an unexpectedly high proportion of embryos displayed bilateral expression (Table 1). Moreover, asymmetric expression in the floor plate mirrored that seen in the lateral plate (Fig. 4g,h). In striking contrast, as shown above, the C2 transgene was exclusively expressed on the left side in wild-type embryos.

Targeted deletion of the asymmetric enhancer

The 596-bp C2g genomic fragment (Fig. 5) contains several putative transcription factor binding sites, including those for GATA, HoxD8-10, Myf-3, MyoD, Sp1, and NF- κ B. Except for the GATA family, none of these transcription factors is thought to be essential for early postimplantation mouse development. A retinoic acid response element (RARE) is present at position 360. This minimal asymmetric enhancer lacks consensus binding sites for the Gli proteins, effectors of the hedgehog signaling pathway. A novel 10-bp motif (CAATCCACAT) appears twice within a 100-bp region. Database searches (Heinemeier et al. 1998) reveal no homology with any previ-

Table 1. Expression of the nodal asymmetric enhancer in *iv/iv* and *inv/inv* mutant embryos

	C2/+, <i>inv</i> /+ × +/+, <i>inv</i> /+	C2/+, <i>inv</i> /+ × C2/+, <i>inv</i> /+	C2/+, <i>iv</i> /+ × +/+, <i>iv</i> /+
Total	24	34	33
No expression	10 (12)	11 (8.5)	17 (17.5)
Left	11 (9)	17 (19)	8 (13.5)
Right	0 (3)	1 (6.5)	2 (1)
Bilateral	3 (0)	5 (0)	6 (1)

Crosses between mice doubly heterozygous for the C2 transgene and either *iv* or *inv*, result in a greater frequency of embryos expressing LacZ bilaterally than expected from previous published results. The number of embryos showing expression in either the left lateral plate (left), the right lateral plate (right), in both lateral plates (bilateral), or not at all (no expression) is indicated. The numbers shown in parentheses represent the expected number of embryos in each class (Collignon et al. 1996; Lowe et al. 1996). The total number of embryos collected for each cross is indicated at the top of each column.

ously characterized transcription factor binding sites but, interestingly, the minimal 380-bp asymmetric enhancer region recently identified for the *lefty-2* locus (Saijoh et al. 1999) also contains two copies of this 10-bp sequence.

To further test the minimal 596-bp enhancer for its activity in conferring asymmetric expression, we designed targeting vectors to eliminate these sequences

Figure 4. Perturbed C2 transgene expression in *inv* and *iv* mutant backgrounds. (a) Bilateral C2 expression in a presumed *inv/inv* embryo at 8.5 dpc. Notably staining is stronger on the right (R) vs. left (L) side of the embryo and expression in the floorplate is broader. (b) Anterior view of presumed *inv/inv* embryo. In addition to bilateral staining in the LPM, the heart tube (ht) is looping to the left rather than the right side, highlighting the reversal of situs. (c) Posterior view of a presumed *inv/inv* embryo showing increased *lacZ* expression on the left side. (d) Section through C2 *inv/inv* embryo. Staining is visible in the LPM on both sides of the axis. *lacZ* is expressed equally on both sides of the floorplate. (e) Posterior view of presumed *inv/inv* embryo in which *lacZ* expression is confined to the LPM on the right side of the axis. (f) Posterior view of a presumed *iv/iv* C2 transgenic embryo showing *lacZ* expression is detectable in the right and left LPM. (g) Anterior view of a presumed *iv/iv* C2 transgenic embryo. Expression is bilateral and truncated on the right as compared to the left side. (h) Section through a C2 *iv/iv* embryo showing LacZ staining in both the right and left lateral plate.

941 GTAGTTTAGA AATCGTAAGA CTTTGTGTAT AGACTTATAG AAGGGACTAG
 951 GTGACACCGC CCGATCTCCG CCAGAAAGCG TCTGCGCAGA ACCGGGGCTA
 101 AATTAATACA AGTCGTTACT GCTTTATAC CCGTTGCAGC AAGTGAACAC
 151 AGGAAGTAGG GCTCAAGAGT CCGAATATTAG AGATCGGGTG TAGAAAGTCC
 201 AGAAGTCAGG CAATCTCTCT GGTGCGGGCG TCGAGGTGCC TTGGTGAACA
 251 ACAGGCTCGG AACCTACTTG GATCGAGGTG TCGGTGTGTG TGTGTGTGTG
 301 TGTGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTGTGTG
 351 ACCGAGCGAC CTGATGTGAT CTCACACAGG TAGGCGGACA GTGGGTAGCC
 401 CAGGAGAGGG AGTTCCGACT CAGGACTACC TTCTCGGGCG CAATCCGACT
 451 CTGCGCTCTT GGGCAGCTTG GACAGCGCCG TCTCTGTCTC TCTGGGGACA
 501 GAGGGGCTTC CGGGGGAACC GGACCCTATC CACGCTTCTC AATCGGCTCT
 551 GAGCGGCGCG CCGCGGCGCC TCACGACCTG GAGGCGGAGT CCTTAG
 Δ100
 Δ600

Figure 5. Nucleotide sequence of the minimal asymmetric enhancer element. The positions of putative transcription factor binding sites are indicated by the colored boxes. (Green) GATA; (yellow) HoxD; (pink) Mef1, MyoD, or Myf3; (blue) RXRα. (red) A conserved 10-bp motif also present in the *lefty-2* minimal asymmetric enhancer (Saijoh et al. 1999). Seven Sp1 and two NF-κB sites and a 65-bp CA repeat (between bases 284 and 350) are also present in this region. The arrows indicate the exact positions of the Δ100 and Δ600 deletions generated by homologous recombination.

within the endogenous *nodal* locus via homologous recombination in ES cells. We engineered a large deletion (Δ600) to eliminate the entire 596-bp subregion and also a second smaller 100-bp deletion (Δ100) that selectively removes the two potential 10-bp transcription factor binding sites (Fig. 5). The targeting vectors were designed to insert a LoxP-flanked hygromycin resistance cassette in place of the corresponding genomic region that, following drug selection, was removed by transient expression of Cre recombinase. We used ES cells heterozygous for the IRES-*lacZ* reporter allele (Collignon et al. 1996) to facilitate analysis of *nodal* expression patterns. Figure 6 shows the targeting strategy and Southern blot analysis used to identify recombinant ES cell clones harboring deletions in the context of the IRES-*lacZ* allele.

We initially characterized LacZ staining patterns in chimeric embryos. As ES cells show a marked developmental bias when injected into recipient blastocysts (Beddington and Robertson 1989), this strategy only allowed us to examine potentially altered *nodal* expression patterns in derivatives of the epiblast. At ~6 days of development (the earliest stage recovered), chimeric em-

Figure 6. Targeted deletion of the minimal asymmetric enhancer. (a) The targeting vectors contained a lox-flanked PGK-hygromycin cassette in place of intron 1 sequences (green circle). Homologous recombination in ES cells heterozygous for the *nodal.lacZ* allele (Collignon et al. 1996), specifically at the *nodal.lacZ* locus results in the appearance of a 12-kb *SpeI* (Sp) fragment. Following Cre-mediated recombination, clones in which the hygromycin cassette has been correctly excised were identified by digestion with *HindIII* (H) and probing with SS1/1 (Conlon et al. 1991). In the case of the Δ600 deletion construct, Cre-mediated excision converts the 6-kb fragment to 3.5 kb. In the case of the Δ100 deletion construct, a 4.0-kb *HindIII* fragment is generated. (b) Southern blot analysis of *SpeI*-digested DNA from drug resistant clones. (c) Southern blots of *HindIII*-digested DNA isolated following transient transfection with Cre-recombinase. The 6-kb band corresponds to the correctly targeted *nodal.lacZ* allele (T) whereas the wild-type allele gives rise to a 4-kb fragment. In the case of the Δ600 construct, correct excision of the *hygro* cassette yields a 3.5-kb fragment. In the case of the Δ100 construct, correct excision results in a 4.0-kb fragment that comigrates with the wild-type product.

bryos carrying the $\Delta 600$ allele showed most intense expression in a ring of epiblast cells adjacent to the embryonic/extraembryonic boundary, and LacZ staining is largely absent from the prospective anterior of the embryo (Fig. 7a). A few hours later, this expression domain has resolved to the posterior side where the streak is developing (Fig. 7a). By four- to five-somite stages, expression is exclusively detected in the node (Fig. 7b). The $\Delta 100$ allele gave similar results in this transient analysis (Fig. 7c,d).

Based on these observations, ES cell clones carrying the $\Delta 100$ allele were used to generate germ-line chimeras, and embryos carrying this deletion were analyzed in detail. The onset of β -gal staining differs markedly from that normally observed for the endogenous locus, as the $\Delta 100.lacZ$ allele fails to show any appreciable expression at 5.5 dpc (data not shown). At ~ 6.0 dpc, expression is detectable in a circumferential ring of proximal epiblast cells (Fig. 8a). As for wild-type *nodal*, expression becomes restricted proximally and then posteriorly within the epiblast immediately prior to streak formation (Fig. 8b,d). Notably, *lacZ* activity is absent from the visceral endoderm (Fig. 8c). Concomitant with the onset of gastrulation, expression is confined to the posterior epiblast and persists in nascent mesoderm emerging from the proximal streak (Fig. 8e). We failed to detect *lacZ* expression at three- to four-somite stages in LPM on either side

of the axis (Fig. 8g). Moreover, asymmetric expression within the notochordal plate is also eliminated (Fig. 8j). Interestingly, we observed very weak expression of β -gal activity in the left LPM in seven- to eight-somite stage embryos (Fig. 8h). This highly transient expression domain is no longer detectable by the 10-somite stage (Fig. 8i). Overall the early pre- and postgastrulation staining patterns associated with the $\Delta 100.lacZ$ allele are quite similar to those seen above with the C4 transgene. The results above suggest that distinct regulatory elements controlling *nodal* transcription in the proximal epiblast likely map outside of the $\Delta 100$ -bp deletion.

Discussion

Highly dynamic and transient *nodal* expression patterns control functional activities essential for anterior patterning, primitive streak formation, and L-R axis determination. Our results demonstrate that node-specific and asymmetric *nodal* gene expression is governed by two distinct *cis*-acting regulatory elements. Thus, a 5'-flanking region mapping between -7.5 and -10.2 kb upstream of the *nodal* promoter was found to direct node-specific expression, whereas a 596-bp intronic enhancer confers early expression in the epiblast and the visceral endoderm and also acts at later stages to direct asymmetric expression in left LPM. The essential contribution made by a 100-bp genomic subregion in regulating early expression in the visceral endoderm and the epiblast, as well as asymmetric expression at later stages in the lateral plate, has been established via targeted deletion.

Classical tissue grafting experiments clearly demonstrate that the signals responsible for organizing the posterior body plan originate in the node, a distinct embryonic structure originally identified in amphibian embryos by its ability to induce an ectopic second axis. Similarly, the mouse node isolated from late-streak stage embryos can induce the formation of a second axis containing host-derived neural and somitic tissues (Beddington 1994). However, the molecular pathways responsible for formation and maintenance of the node remain ill defined (for review, see Beddington and Robertson 1999). The mouse node is comprised of two distinct cell layers. The proximal layer is thought to give rise to the floor plate of the neural tube, whereas the ventral surface, the notochordal plate, gives rise to the notochord and perhaps the definitive endoderm of the gut (Sulik et al. 1994; Tam and Behringer 1997). *nodal* expression is strictly confined to the lateral edges of the notochordal plate during late gastrulation stages.

The present experiments identify a region upstream of the *nodal* promoter that specifically directs expression in the developing node. As for endogenous *nodal*, transgene expression is initially observed at the base of the primitive streak in a population of presumed node precursors and, following morphological appearance of the overt node, becomes highly restricted to the lateral edges of the ventral layer of cells constituting the notochordal plate (Collignon et al. 1996). A number of potential tran-

Figure 7. Transient analysis of *nodal* expression in chimeric embryos. (a) Chimeric embryos recovered at 6.5 dpc following injection of ES cells carrying the $\Delta 600$ deletion. *nodal.lacZ* expression is observed primarily in cells occupying the most proximal posterior regions of the embryo and absent from the distal and prospective anterior epiblast. (b) In chimeric embryos recovered at 8.5 dpc (3–5 somites), *nodal.lacZ* expression is confined to cells in the periphery of the mature node. No *lacZ*-positive cells are detectable in left lateral plate mesoderm. (c) Chimeric embryos recovered at 6.5 dpc following injection of ES cells carrying the $\Delta 100$ deletion. *nodal.lacZ* expression is confined to the proximal posterior region of the epiblast marking the site of the primitive streak formation. (d) At 8.5 dpc (3–5 somites) strong *nodal.lacZ* expression can be seen in the ventral lateral cells of the node, but no staining is visible in cells of the lateral plate.

Figure 8. Node-specific *nodal* expression domains in embryos carrying a germ-line deletion of the 100-bp minimal asymmetric enhancer. (a) $\Delta 100$ *nodal.lacZ* expression is initially detected at 5.5 dpc and is strictly confined to proximal epiblast cells. (b,c) At pregastrulation stages (6.0 dpc) $\Delta 100$ *nodal.lacZ* expression has resolved to the proximal epiblast population on the prospective posterior side of the embryo. Expression is noticeably absent from the visceral endoderm. (d–f) At later stages subsequent to formation and elongation of the primitive streak, $\Delta 100$ *nodal.lacZ* expression closely resembles that seen from the endogenous locus (see Fig. 2). (g) At the four-somite stage $\Delta 100$ *nodal.lacZ* expression is confined to the node. The modified allele is not active in the lateral plate mesoderm. (h) At the eight-somite stage weak LacZ staining is detected in the left lateral plate mesoderm along the length of the axis. (i) By the ten-somite stage $\Delta 100$ *nodal.lacZ* expression is strictly confined to the developing tail bud and posterior notochord. (j) Frontal section through the posterior region of a five-somite stage $\Delta 100$ *nodal.lacZ*/+ embryo, showing symmetric expression at the lateral edges of the notochordal plate. The modified $\Delta 100$ *nodal* allele is not expressed in LPM. (psm) Presomitic mesoderm.

scription factor binding motifs including HNF3-like and LEF-1/TCF sites appear scattered across the 2.7-kb C4d genomic fragment (J. Brennan, D.P. Norris, and E.J. Robertson, unpubl.). The winged helix factor HNF3 β is strongly expressed in the anterior streak and then in the node, notochord, and floor plate (Ang et al. 1993; Monaghan et al. 1993; Sasaki and Hogan 1993), where it is required for the formation of these midline organizing structures (Ang and Rossant 1994; Weinstein et al. 1994). *Wnt8* and *Wnt11* are abundantly expressed in the node (Bouillet et al. 1996; Kispert et al. 1996) and are also potential candidates for regulating *nodal* transcription. Moreover LEF-1/TCF-binding sites in the *Xnr-3* promoter have been implicated in regulating expression, suggesting *Xnr-3* is a *wnt* signaling target (McKendry et al. 1997). The possible contributions made by HNF3 β and LEF-1/TCF transcription factors in controlling this node-specific expression domain are currently being investigated.

A strong argument has been made in chick that *nodal* asymmetry in Hensen's node initiates downstream signaling events essential for asymmetric *nodal* expression in the lateral plate (Levin et al. 1995, 1997). In mouse, mutations disrupting node morphogenesis also lead to defects in the establishment of L-R asymmetry (Dufort et al. 1998; Nonaka et al. 1998; King et al. 1999). The present data strongly suggest that the transient but pronounced asymmetry of *nodal* expression in the mouse node requires cooperative actions of two distinct enhancer regions. Thus, we observe that the node enhancer

alone drives equivalent expression on both sides of the node, and targeted deletion of the asymmetric enhancer results in a similar pattern of node-specific expression. The upstream enhancer region is thus sufficient for activating expression in the notochordal plate, but these sequences alone fail to confer asymmetry in the node. We suggest the additional regulatory sequences responsible for node asymmetry may be contributed by the intron 1 enhancer. It will be of considerable interest to learn whether *nodal* expression in the node is a prerequisite for establishing asymmetric *nodal* and *lefty-2* expression domains in the lateral plate, and whether asymmetric *nodal* signals are causally involved in inducing asymmetric *lefty-1* expression in the floor plate. In normal embryos, *nodal* and *lefty-2* transcripts are broadly expressed along most of the left side of the axis. Asymmetric expression is highly transient, and both transcripts are largely undetectable following the onset of embryonic turning. Assessment of the minimal asymmetric *nodal* and *lefty-2* enhancers identified in transgenic experiments (Saijoh et al. 1999; Adachi et al. 1999; Fig. 3), together with the analysis of the 100-bp deletion allele, strongly implicate the highly conserved 10-bp motif (CAATCCAACAT) as a key regulatory element responsible for controlling asymmetric gene expression. Identification of transcription factor(s) binding to this minimal enhancer element promises to provide important insights into upstream components of the L-R pathway in mammals.

Cell-specific nuclear factors that potentiate left-sided

expression or repress transcription on the right side of the embryo may cooperatively generate *nodal* and *lefty* asymmetry. Recent experiments suggest that both regulatory mechanisms govern asymmetric *nodal* expression in chick and *Xenopus*. Repression of *shh* transcription on the right side of the node in chick embryos, mediated by the activin signaling pathway, leads to selective *nodal* expression on the left side of Hensen's node and in the lateral plate (Levin et al. 1995, 1997). Moreover, expression of *shh* on the right side of the body axis causes ectopic *nodal* expression and situs defects (Levin et al. 1995). Misexpression of *Xshh* or *Xbhh* also induces ectopic *Xnr-1* on the right hand side of *Xenopus* embryos (Sampath et al. 1997). In contrast, in mouse the absence of Gli binding sites in both the *nodal* and *lefty-2* asymmetric enhancers (Fig. 5; Saijoh et al. 1999), together with the observation that *shh* mutants establish normal situs (Chiang et al. 1996), implies that Shh signaling is not involved in the establishment of molecular asymmetry. In *Xenopus*, LPM isolated from either side of the body axis expresses *Xnr-1* when cultured in isolation (Lohr et al. 1997) suggesting that negative regulation normally prevents right-sided *Xnr-1* expression. In mouse, *lefty-1* asymmetric expression in the floor plate also appears to be mediated in part by a right-sided repressor contained within the *lefty-1* locus (Saijoh et al. 1999). To date we have failed to identify similar repressor elements mapping to the *nodal* locus. On the other hand, C2 transgenes show asymmetric expression in the floor plate, implicating *cis*-acting regulatory sequences possibly shared with the *lefty-1* and *lefty-2* genes (Meno et al. 1997). The activity of the C2 asymmetric enhancer is disturbed in both the *inv* and *iv* mutant backgrounds. Notably, a high proportion of mutant embryos show bilateral expression of the C2 transgene, whereas, particularly in the case of the *inv* mutation, expression of endogenous *nodal* transcripts is predominantly reversed (Table 1; Collignon et al. 1996; Lowe et al. 1996). This discrepancy is perplexing considering C2 transgene expression is invariantly left-sided in wild-type embryos. Perhaps a repressor selectively acts to eliminate right-sided expression in wild-type embryos, but limiting amounts of transcriptional regulators may fail to bind all possible target sites on multicopy transgenes in the *inv* and *iv* mutant backgrounds.

Activin, a *lefty* homolog recently described in zebrafish, has been shown to antagonize activin signaling, likely by binding to the activin type II receptor (Thisse and Thisse 1999). Thus, coordinately regulated expression of *nodal* and *lefty-2*, a putative receptor antagonist, potentially modulates *nodal* signaling in the lateral plate. Mutation of ActRIIB leads to right isomerisms in mice (Oh and Li 1997), and in *Xenopus* overexpression of dominant-negative forms of this receptor block expression of the *nodal* target *pitx2* (Ryan et al. 1998). Competitive binding of Lefty-2 to activin type II receptors may therefore attenuate *nodal* signaling on the left side of the axis. On the other hand, expression of mouse Lefty proteins leads to ectopic *nodal* expression in chick (Yoshioka et al. 1998), implicating *lefty* as a positive

regulator of *nodal* activity. Much additional work is needed to decipher the unique and shared roles of *nodal* and *lefty* signals during establishment of the mammalian L-R body axis.

Initially, widespread and uniform *nodal* expression in the early epiblast is gradually restricted to the prospective posterior side of the pregastrulation stage embryo (Varlet et al. 1997). Down-regulation first in the distal tip cells and shortly thereafter in the prospective anterior epiblast likely occurs in response to signals from both the overlying AVE as it rotates from the distal tip to the anterior side of the embryo, as well the proximal extra-embryonic ectoderm (for review, see Beddington and Robertson 1999). Our results demonstrate epiblast expression is influenced by two distinct regions of the locus. Thus, a genomic region upstream of the locus (C4; -5 to -12 kb) confers expression in the proximal posterior region of the epiblast (Fig. 2), whereas the intronic enhancer directs the onset of widespread epiblast expression at 5.5 dpc (Fig. 3). Interestingly, expression from the modified $\Delta 100$ allele closely resembles that described for T transcripts (Thomas and Beddington 1996). Thus, expression is first seen in a circumferential pattern in cells throughout the proximal rim of the epiblast and rapidly resolves to the prospective posterior region to mark the position of the streak (Fig. 8). In *Smad2* mutant embryos, both T and *nodal* are uniformly expressed throughout the epiblast (Waldrip et al. 1998) suggestive of coordinately regulated transcription in the proximal epiblast. The factors responsible for highly specific gene expression patterns during the complex process of mesoderm induction have yet to be elucidated.

nodal activities in the primitive endoderm are essential for the formation of the AVE and patterning of the rostral CNS (Varlet et al. 1997). Thus, *Smad2* mutant embryos lacking *nodal* expression in the visceral endoderm fail to activate AVE-specific markers including *cer-1*, *lim-1*, and *hesx-1* and consequently fail to establish A-P polarity in the underlying epiblast (Waldrip et al. 1998). Surprisingly, the minimal asymmetric enhancer also controls *nodal* expression in the visceral endoderm. The minimal 596-bp C2g transgene is strongly expressed in the endoderm, and targeted deletion of a 100-bp fragment abolishes expression in this tissue (Fig. 8). Thus, this discrete genomic subregion not only controls asymmetric expression but also, remarkably, directs early *nodal* expression domains in the epiblast and primitive endoderm. Thus, the same signaling cues that act to induce *nodal* activity during early development to pattern the A-P axis may also govern asymmetric *nodal* expression at later stages to establish the L-R body axis.

Materials and methods

DNase I analysis

Chromatin was produced and DNaseI digestion was carried out according to the methods of Reik et al. (1991). DNA (10 mg) from each DNase I digestion was subsequently digested with *EcoRI* or *SpeI*, fractionated through a 0.8% agarose gel, then

transferred. Southern blots were probed with the SS1/1 (Conlon et al. 1991) and the Nodal 5' probe (Collignon et al. 1996). Restriction sites and probes are indicated in Figure 1, with the exception of the 5' *SpeI* site ~0.5 kb upstream of the C4 fragment.

Cloning and production of reporter constructs

Genomic subclones spanning the entire *nodal* locus were isolated from 129Sv libraries. Fragments downstream from the first exon were recovered from the cosmid clone described previously (Collignon et al. 1996). Lambda clones containing upstream sequence extending ~12 kb 5' from the end of exon 1 were obtained from a Stratagene mouse genomic library (no. 946313) using the *nodal* 5' genomic probe (Collignon et al. 1996). For constructs C1, C2, and C4, the genomic subclones were cloned into the hsp68lacZpA reporter construct (Sasaki and Hogan 1996). However, for C2, the *lacZ* locus was placed immediately downstream of the ATG of *nodal*. The transcriptional start site of *nodal* in the cDNA clone was mutated from ATGAGT to an *NcoI* site (ATGCAT) to allow ligation to the *NcoI* site at the start of the *lacZ*.poly(A) cassette recovered from a Wnt1 minimal promoter.*lacZ* construct (Echelard et al. 1994), with no loss of sequence.

Production of transgenic embryos

DNA for injection was purified using Qiagen P100 columns. Following digestion with appropriate enzymes, fragments were gel fractionated and electroeluted into TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA at pH 8), concentrated using Wizard Prep kit (Promega), and recovered in injection buffer (10 mM Tris at pH 7.5, 0.1 mM EDTA). Fertilized eggs from ICR mice (Taconic) were collected for pronuclear injection according to standard procedures (Hogan et al. 1994). Injected eggs were transferred to pseudopregnant (C57BL/6J × CBA/J) F₁ females. For F₀ analysis, foster mothers were sacrificed and embryos retrieved for further analysis. Live-born offspring were genotyped by Southern blot analysis of tail DNA using LacZ probes to identify transgenic animals.

LacZ staining and histology

Embryos were collected and LacZ stained according to standard protocols (Hogan et al. 1994). Noon on the day of finding vaginal plugs was designated as 0.5 day of development. For sectioning, embryos were washed in PBS and postfixed overnight in 4% paraformaldehyde. Embryos were washed in PBS, dehydrated through an ethanol series, cleared in xylenes, and embedded in paraffin wax. Sections (8 μm) were taken, dewaxed in xylenes, and mounted under coverslips with Cytoseal (Stephens Scientific).

Generation and analysis of targeted mutations

To produce the Δ600 deletion construct, a PCR fragment flanking the 5' extent of the deletion, stretching ~400 bp 3' from the *NotI* site in exon 1 (primers: 5'-GGGGTACCGTCGACGCGGCCGCTTTGCCTCTGTGGA; 3'-GCGGATCCATGCATCTCGAGTCTAGATGGGCAAACTGAGACTCGCAGG), was cloned into pBSII (Stratagene). A LoxP-flanked PGKHygro cassette engineered such that Cre-mediated excision results in loss of both the PGK promoter and hygromycin-resistance gene was ligated 3' to the Δ600 PCR fragment. The 3' end of the Δ600 deletion is a *PstI* site 1 kb 3' to the *NotI* site in exon 1. 3' Homology was therefore provided by the 4-kb *PstI*-*BamHI* fragment, stretching from this *PstI* site to a *BamHI* site within exon

2. 5' Homology was provided by a 6-kb *NotI*-*SalI* genomic fragment stretching 5' from the *NotI* site within exon 1 to the upstream *SalI* site shown in Figure 1. To produce the Δ100 deletion, PCR fragments abutting both sides of the deletion were produced; an 800-bp Δ100 5' PCR fragment stretches from the 5' extent of the deletion to the *NotI* site in exon 1, whereas an 80-bp Δ100 3' PCR fragment stretches from the 3' extent of the deletion to the *PstI* site used in the production of Δ600. Δ100 5' fragment primers: GGGGTACCGTCGACGCGGCCGCTTTGCCTCTGTGGA; TCAGAATTCAGATCTCCAGGAAGGTAGTCCTGAG. Δ100 3' fragment primers: TCAGAATTCGAGACTAGTTCTAATCCGGTCTGACCCGC; TCAGGTACCGGATCCATGCATGCTGCAGGCAGGGGACTAGG. The 5' and 3' fragments were cloned either side of the LoxP-flanked PGKHygro and sequence confirmed. The remaining regions of 3' and 5' homology were provided as for the Δ600 vector. The vectors were linearized with *SalI* and electroporated into *nodal.lacZ*/+ ES cells (Collignon et al. 1996), using standard procedures (Ramirez-Solis et al. 1993). Hygromycin-resistant colonies were picked into 96-well plates and screened by Southern blot analysis (Ramirez-Solis et al. 1993). DNA was digested with *SpeI* and separated on 0.6% agarose gels, blotted onto Hybond N membrane (Amersham), and probed using the 3' cDNA probe. Approximately 1 in 15 drug-resistant colonies had undergone the expected recombination event. Fidelity of the targeting event was confirmed with a *nodal* 5' genomic probe (Collignon et al. 1996; Fig. 6) and hygromycin probe (Fig. 6). Correctly targeted ES cells were expanded and subjected to transient transfection with the MC1Cre expression vector. Cells grown for two days to allow recombination to occur were then low density plated. Colonies were isolated, DNA digested with *HindIII*, and separated through 0.8% agarose gels. Southern blots were analyzed with the SS1/1 probe (Conlon et al. 1991; Fig. 6) and clones that had excised the selection cassette were isolated and expanded. The targeting and excision events were confirmed by Southern blot analysis using the *nodal* 5' genomic probe and the 3' cDNA probe. Recombinant ES cells carrying the deletions at the *nodal.lacZ* allele were injected into ICR blastocysts to generate chimeric embryos using standard procedures (Bradley 1987). Embryos were harvested at 6.5 and 8.5 days of development and LacZ stained. ES cells carrying the Δ100 deletion were injected into C57BL/6J blastocysts to generate live born chimeras. Highly chimeric males were mated to MF1 females and agouti offspring genotyped by Southern blot analysis using the 3' cDNA probe. Chimeric males transmitting the correctly targeted Δ100 allele and their offspring were used to produce embryos for LacZ staining and histological analysis.

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