Conserved regulation and role of Pitx2 in situs-specific morphogenesis of visceral organs

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Pitx2 is expressed in developing visceral organs on the left side and is implicated in left-right (LR) asymmetric organogenesis. The asymmetric expression of Pitx2 is controlled by an intronic enhancer (ASE) that contains multiple Foxh1-binding sites and an Nkx2-binding site. These binding sites are essential and sufficient for asymmetric enhancer activity and are evolutionarily conserved among vertebrates. We now show that mice that lack the ASE of Pitx2 (Pitx2^{ASE/ASE} mice) fail to manifest left-sided Pitx2 expression and exhibit laterality defects in most visceral organs, although the position of the stomach and heart looping remain unaffected. Asymmetric Pitx2 expression in some domains, such as the common cardinal vein, was found to be induced by Nodal signaling but to be independent of the ASE of Pitx2. Expression of Pitx2 appears to be repressed in a large portion of the heart ventricle and atriовentricular canal of wild-type mice by a negative feedback mechanism at a time when the gene is still expressed in its other domains. Rescue of the early phase of asymmetric Pitx2 expression in the left lateral plate of Pitx2^{ASE/ASE} embryos was not sufficient to restore normal organogenesis, suggesting that continuous expression of Pitx2 in the lineage of the left lateral plate is required for situs-specific organogenesis.

KEY WORDS: Left-right asymmetry, Organogenesis, Pitx2, Mouse

INTRODUCTION

Although recent studies have provided insight into how left-right (LR) asymmetry is generated during vertebrate development (Hamada et al., 2002; Levin, 2005; Tabin, 2005), knowledge of this process remains limited. One of many important questions still unanswered concerns the mechanism by which situs-specific organogenesis is executed.

Situs-specific morphogenesis in the mouse begins soon after the loss of asymmetric expression of Nodal on the left side of the lateral plate mesoderm (LPM). The looping of the heart tube toward the right side is followed by asymmetric lobe formation in the lungs, rotation of the digestive tract and remodeling of the vascular system. This asymmetric morphogenesis occurs in response to Nodal, which functions as a left-side determinant. Organ primordia that have received the Nodal signal thus adopt a left-side morphology, whereas they adopt a right-side morphology in the absence of this signal. At the cellular level, the left and right sides of organ primordial may differ with regard to rate of cell proliferation, apoptosis or migration. However, the cellular basis of the generation of morphological asymmetry is poorly understood.

The bicoid-type homeobox transcription factor Pitx2 is thought to play a major role in asymmetric morphogenesis (Campione et al., 1999; Logan et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998). The Pitx2 gene encodes three isoforms: Pitx2a, Pitx2b and Pitx2c. Pitx2a and Pitx2b mRNAs are generated by alternative splicing and are expressed bilaterally, while Pitx2c mRNA, a transcript from an alternative promoter, is expressed asymmetrically (Kitamura et al., 1999; Liu et al., 2002; Schweickert et al., 2000). Pitx2 knockout mice manifest LR defects in many organs, including the typical right isomerism of the lungs (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999). However, the exact role of Pitx2 in situs-specific organogenesis is unknown.

Asymmetric expression of Pitx2 begins in the left LPM concomitantly with that of Nodal, but it persists after Nodal expression disappears. Pitx2 expression is thus still apparent in the LPM-derived mesenchyme of various visceral organs at the late somite stage. Asymmetric expression of mouse Pitx2 is conferred by an enhancer (ASE) that contains three binding sites for the transcription factor Foxh1, a target of Nodal signaling, as well as a binding site for the homeobox transcription factor Nkx2 (Shiratori et al., 2001). The Foxh1 binding sites are essential for the initiation of asymmetric Pitx2 expression, whereas the Nkx2-binding site is dispensable for such initiation but necessary for maintenance of late-stage expression. The left-sided expression of Pitx2 is thus initiated by Nodal signaling and maintained by Nkx2. Consistently, asymmetric Pitx2 expression is lost in mutant mice lacking any of the Nodal signaling components, such as Foxh1 (Yamamoto et al., 2003) and cryptic [Cfc1 – Mouse Genome Informatics; a co-receptor for Nodal (Shen and Schier, 2000; Yan et al., 1999)]. The LR asymmetric expression pattern of Pitx2 is conserved among all vertebrates examined.

We have now studied the regulation and role of Pitx2 in asymmetric organogenesis by examining the asymmetric enhancer ASE of this gene. Our data suggest that asymmetric Pitx2 expression in vertebrates other than the mouse is also regulated by this highly conserved enhancer. Generation of mice that lack the Pitx2 ASE confirmed its essential role in regulation of Pitx2 and revealed the precise role of Pitx2 in situs-specific morphogenesis. We also tested the importance of two-step regulation of Pitx2 expression by rescue experiments with a Pitx2 transgene.

MATERIALS AND METHODS

Transgenic mouse assay

Genomic DNA libraries for rat, human and zebrafish (Stratagene) or for chicken (Clontech) were screened by hybridization with mouse Pitx2 cDNA as a probe. DNA fragments containing the last intron of Pitx2 were obtained

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from the isolated clones by PCR with a pair of primers corresponding to exons 4 and 5. DNA fragments containing the whole intron 5 of vertebrate Pitx2 genes were ligated to the 5’ end of an hsp68-lacZ reporter construct (Kothary et al., 1988). Seven tandem repeats of the Nkx2-binding sequence of mouse Pitx2 were ligated to the 5’ end of a 380 bp DNA fragment containing the ASE of mouse Left2 (Sajioh et al., 1999) and to the hsp68-lacZ construct. A Foxh1-lacZ transgene was constructed from a 234 kb bacterial artificial chromosome (BAC) clone (RP23-156P23) containing mouse Foxh1; an internal ribosomal entry site (IRES)-lacZ cassette was then inserted into the 3’ untranslated region of Foxh1 upstream of the poly(A) addition sequence in the BAC clone. Transgenic mice were generated by injection of these various lacZ constructs into the pronucleus of fertilized eggs as described previously (Sajioh et al., 1999). They were then collected at embryonic day (E) 8.2 or 10.5 and stained with the β-galactosidase substrate X-gal.

**RESULTS**

**Conserved role of the asymmetric enhancer ASE in Pitx2 regulation among vertebrates**

Asymmetric expression of mouse Pitx2 is controlled by an enhancer (ASE) located in the last intron, intron 5 (Shiratori et al., 2001). The ASE contains three Foxh1-binding sequences and an Nkx2-binding sequence (Fig. 1). The former sites are required for initiation of Pitx2 expression in left LPM at E8.2 (Fig. 2A), whereas the latter is responsible for maintenance of asymmetric expression from E9.5 to 10.5 (Fig. 2F,K,P,U) (Shiratori et al., 2001). We therefore examined Pitx2 genes of other vertebrates to determine whether this transcriptional regulatory mechanism for achieving asymmetric expression is conserved.

We isolated Pitx2 genes from human, rat, chicken and zebrafish. For chicken Pitx2, a 16 kb region containing the last intron, which is located between the exons encoding the homeodomain in all vertebrates examined, was tested for ASE activity with a transgenic mouse assay. ASE activity was initially localized to a 9.0 kb region and was subsequently mapped to a 2.0 kb region containing the last intron. An hsp68-lacZ reporter construct driven by the 9.0 kb fragment of chicken Pitx2 thus gave rise to asymmetric X-gal staining not only in left LPM at E8.2 (Fig. 2C) but also in various primordial organs, including the common atrial chamber, lung bud, septum transversum and gut dorsal mesentery at E10.5 (Fig. 2H,M,R,W). Nucleotide sequencing of the 2.0 kb region containing intron 5 revealed the presence of two Foxh1-binding sequences and an Nkx2-binding sequence in the last intron (Fig. 1). For the Pitx2 genes of human, rat and zebrafish, DNA fragments containing the entire last intron were isolated by PCR and sequenced. Each fragment contained two or three Foxh1-binding sequences and an Nkx2-binding sequence (Fig. 1). The fragment of human Pitx2 showed typical ASE activity, with the corresponding hsp68-lacZ transgene giving rise to left-sided X-gal staining in LPM at E8.2 (Fig. 2B) as well as in various organs at E10.5 (Fig. 2G,L,Q,V). The DNA fragment containing the last intron of zebrafish Pitx2 conferred asymmetric expression of the reporter construct in left LPM at E8.2 (Fig. 2E) but not in primordial organs at E10.5 (Fig. 2J,O,T,Y). Moreover, expression of the transgene in left LPM was apparent

![Fig. 1. Conservation of an asymmetric enhancer (ASE) of Pitx2 among vertebrates.](image) Structures of the Pitx2 ASE from various vertebrates (located in the last intron of Pitx2) are shown on the left. Red and blue circles represent Foxh1- and Nkx2-binding sites, respectively, with the arrows indicating their orientation. The enhancer activity of each ASE in transgenic mouse assays at E8.2 and 10.5 is summarized on the right. The ASE of zebrafish Pitx2 possesses activity only in the anterior portion of left LPM (asterisk). ND, not determined.
only in the anterior portion (Fig. 2E). As we showed previously (Shiratori et al., 2001), the ASE of frog Pitx2 was active in left LPM at E8.2 (Fig. 2D) as well as in the common atrial chamber and gut dorsal mesentery but not in the lung bud or septum transversum at E10.5 (Fig. 2I,N,S,X). These results thus showed that an enhancer that regulates asymmetric expression of Pitx2 (ASE) is evolutionarily conserved among vertebrates. In all species examined, the ASE contains multiple Foxh1-binding sites and an Nkx2-binding site, suggesting that asymmetric expression of vertebrate Pitx2 is initiated by Nodal signaling and maintained by Nkx2.

The role of the Nkx2-binding sequence in the Pitx2 ASE was examined further by linking it to the asymmetric enhancer (ASE) of mouse Lefty2. The Lefty2 ASE contains two Foxh1-binding sequences but no Nkx2-binding sequence, and it gives rise to asymmetric expression in left LPM at E8.2 (Fig. 3A) (Saijoh et al., 1999) but not in organ primordia at E10.5 (Fig. 3C,E,G) (Shiratori et al., 2001). However, the addition of seven copies of the Nkx2-binding sequence of mouse Pitx2 to the Lefty2 ASE (N7-Lefty2 ASE) (Fig. 3B) resulted in asymmetric expression of the corresponding hsp68-lacZ reporter construct in various visceral organs at E10.5 (Fig. 3D,F,H). An Nkx2-binding sequence is thus not only essential but also sufficient for maintenance of asymmetric gene expression.

**Loss of LR asymmetric Pitx2 expression and impairment of situs-specific organogenesis of many but not all organs induced by ASE deletion**

To establish the role of the ASE in Pitx2 regulation, we generated a mutant Pitx2 allele that lacks the ASE (Pitx2ΔASE) (Fig. 4). We first examined how asymmetric Pitx2 expression was affected by ASE deletion. Pitx2ΔASE/ΔASE embryos developed normally until the early somite stage. As expected, asymmetric Pitx2 expression in left LPM was abolished at the early somite stage, whereas bilateral Pitx2 expression in the head mesenchyme remained unaffected (Fig. 5A,B). At later stages (E9.0 to 9.5), left-sided Pitx2 expression in various primordial organs also did not develop in Pitx2ΔASE/ΔASE embryos (Fig. 5D,E,G,H,J,K,M,N,P,Q). These results thus showed that asymmetric expression of Pitx2 was lost as a result of specific deletion of the ASE, confirming the role of the ASE in such expression.

Pitx2ΔASE/ΔASE newborn mice manifested various LR defects, including those previously reported for Pitx2 null mice (Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999) and mutant mice specifically lacking Pitx2c (Liu et al., 2002). Thus, Pitx2ΔASE/ΔASE mice showed right isomerism in the lungs (Fig. 6A,B; 18/18 mice examined), reversed positioning of the great arteries (Fig. 6C,D; 18/18), reversed positioning of the heart apex toward the right (Fig.
6C,D; 5/18), bilateral inferior vena cava (Fig. 6C,D; 5/18), right isomerism in the atrium (Fig. 6E,F; 18/18), and an endocardial cushion defect, as well as a common atrioventricular (AV) valve with a ventricular septal defect (VSD) and atrial septal defect (ASD) (Fig. 6G,H; 4/4), and double-outlet right ventricle (DORV).

To know the cellular basis of the endocardial cushion defects, we also examined the AV cushions at E12.5 by scanning electron microscope (Fig. 6I,J) and by sagittal sectioning (Fig. 6K,L). The AV cushions consist of two portions, the superior AV cushion (SAVC) and inferior AV cushion (IAVC). The SAVC and IAVC are fused with each other at E12.5 in the wild-type embryo (Fig. 6I,K), but they remained separated in the Pitx2<sup>-ΔASE/ΔASE</sup> embryo (Fig. 6J,L). In the mutant embryo, SAVC was hypoplastic while IAVC appeared relatively normal in size (Fig. 6J,L). These results suggest that a hypoplastic SAVC is responsible for the common AV valve with VSD and ASD in Pitx2<sup>-ΔASE/ΔASE</sup> mice. This is consistent with the fact that Pitx2 is expressed in the myocardium adjacent to SAVC but is largely absent in the myocardium adjacent to IAVC (Kitamura et al., 1999).

We also noted previously unrecognized LR defects in the Pitx2<sup>-ΔASE/ΔASE</sup> mice, including reversed positioning of the azygos vein (12/18) and aorta (9/18) on the right side of the thorax (Fig.

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**Fig. 3.** Prolongation of the transient enhancer activity of the ASE of Lefty2 by addition of Nkx2-binding sequences. (A,B) Structures of two lacZ transgenes are shown at the top. Red circles and blue ovals indicate Foxh1- and Nkx2-binding sequences, respectively. The partially blue circles and boxes indicate the hsp68 promoter and lacZ, respectively. The construct in A includes the Lefty2 ASE, which contains two Foxh1-binding sequences. The construct in B contains the Lefty2 ASE plus seven tandem repeats of the Nkx2-binding sequence derived from the Pitx2 ASE. X-gal staining patterns of E8.2 mouse embryos harboring each transgene are shown below. Both lacZ constructs gave rise to typical asymmetric staining in left LPM. (C-H) Transverse sections of X-gal-stained transgenic embryos at E10.5. Expression of lacZ is shown for the common atrial chamber (C,D), foregut (E,F), and gut dorsal mesentery (G,H). Black arrows indicate the domains where asymmetric X-gal staining was detected. Encircled + or – signs indicate whether the enhancer was active or inactive, respectively. The Lefty2 ASE construct did not give rise to asymmetric staining (C,E,G), whereas the N<sup>2</sup>-Lefty2 ASE construct did so in the common cardinal vein, lung bud and gut dorsal mesentery (D,F,H). Scale bars: 200 µm.

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**Fig. 4.** Generation of mice that lack the ASE of Pitx2. (A) Schematic representation of the mouse wild-type Pitx2 allele, a targeting vector designed to delete the 0.6 kb region of Pitx2 that contains the ASE (red oval), as well as the targeted allele before and after Cre-mediated recombination. Pitx2 contains six exons (black boxes) and two promoters, with transcription beginning at exon 1 or exon 1c. (B) Southern blot analysis of Nsi-digested DNA from ES cells of the indicated Pitx2 genotypes before Cre-mediated recombination. The probe used for hybridization is shown by the blue bar on the top. The wild-type and mutant alleles give rise to hybridizing fragments of >23 and 11 kb, respectively. Green triangles, loxP sequences; neo, neomycin resistance gene under the control of the phosphoglycerate kinase gene promoter; DT, diphtheria toxin gene.
6M,N), reversed relation of the aorta and vena cava in the abdomen (Fig. 6U,V; 4/16), abnormal location of the portal vein on the ventral side of the duodenum (Fig. 6O,P; 1/11), abnormal rotation of the gut, such as aberrant looping of the duodenum (Fig. 6Q,R; 12/12), and mal-location of the pancreas on the ventral or right side of the duodenum (Fig. 6R; 12/12).

These morphological defects of Pitx2 mice resemble those of cryptic knockout mice (Yan et al., 1999) and of conditional mutant mice that lack Foxh1 expression in the lateral plate (Yamamoto et al., 2003). Unlike the cryptic or Foxh1 mutant animals, however, some laterality-dependent events remained unaffected in Pitx2 mice. In particular, the directions of heart looping and embryonic turning were normal in all (12/12) Pitx2 embryos examined. Furthermore, the stomach was always located on the left side (12/12) and the position of the spleen was normal (12/12), although the latter was reduced in size (12/12), as has been described for Pitx2 knockout mice (Lin et al., 1999; Lu et al., 1999). These events and organ positions are thus probably regulated by a mechanism dependent on Nodal-cryptic-Foxh1 signaling but independent of the Pitx2 ASE.

Expression domains of Pitx2 that are independent of the ASE but dependent on cryptic and Foxh1

In Pitx2 mice, asymmetric expression of Pitx2 was not detected in left LPM at E8.2 (Fig. 5B) and most asymmetric expression domains normally apparent at E9.0 to 9.5 were also lost (Fig. 5E,H,K,N,Q). A reduced but substantial level of asymmetric Pitx2 expression was detected in the common cardinal vein and vitelline vein of Pitx2 embryos at E9.0, with the exception of a reduced level of expression remaining in the common cardinal vein and vitelline vein (green arrow in E). Asymmetric Pitx2 expression was completely absent from cryptic embryos at E9.0. Asymmetric Pitx2 expression remained apparent at a reduced level in the common cardinal vein and vitelline vein of Pitx2 embryos at E9.5 (green arrows in H, N and Q) but was absent from cryptic embryos at this time. Scale bars: 200 μm. cac, common atrial chamber; ccv, common cardinal vein; dm, gut dorsal mesentery; lb, lung bud; st, septum transversum; vv, vitelline vein.
inferior vena cava and portal vein are derived from the common cardinal vein and vitelline vein, respectively, in both of which a reduced level of asymmetric Pitx2 expression remained in Pitx2\(^{2A5E/\Delta A5E}\) embryos. These results suggest that the Pitx2 expression in these veins of Pitx2\(^{2A5E/\Delta A5E}\) mice is also induced by Nodal signaling.

We also examined whether cryptic, Foxh1 and Nodal are expressed in the ASE-independent expression domains of Pitx2. Cryptic expression, as revealed by whole-mount in-situ hybridization, was apparent in LPM at E8.2 (Fig. 7A) but not in the common cardinal vein or vitelline vein at E9.0 or 9.5 (Fig. 7D,G,J,M,P,S). To detect expression of Foxh1 or Nodal, we examined the Foxh1-lacZ BAC transgenic mice (see Materials and methods) and Nodal\(^{lacZ/\Delta lacZ}\) mice (Collignon et al., 1996), respectively. The Foxh1-lacZ BAC transgene was expressed in left LPM at E8.2 (Fig. 7C), consistent with the distribution of Foxh1 mRNA revealed by whole-mount in-situ hybridization (Saijoh et al., 2000). Expression of the Foxh1-lacZ transgene was apparent in the heart at E9.0 and 9.5, where Pitx2 is expressed, but it was absent from the common cardinal vein and vitelline vein (Fig. 7F,I,L,O,R,U).
Similarly, Nodal expression was apparent in left LPM at E8.2 (Fig. 7B), but it was detected at only a low level at E9.0 (Fig. 7E,H,K) and not at all at E9.5 (Fig. 7N,Q,T) in the common cardinal vein and vitelline vein.

These results suggest that asymmetric expression of Pitx2 in the common cardinal vein and vitelline vein is induced by Nodal signal and is subsequently maintained in the absence of Nodal signal, which is consistent with our previous observations that asymmetric expression in various organs is maintained by Nkx2 in the absence of Nodal signal (Shiratori et al., 2001).

**Negative feedback regulation by Pitx2**

To study the fates of Pitx2-expressing cells, we established transgenic mice that express Cre under the control of a 17 kb region of Pitx2 that contains the ASE (Shiratori et al., 2001). These Pitx2-Cre mice were crossed with CAG-CAT-lacZ mice, which harbor a Cre-responsive lacZ transgene (Sakai and Miyazaki, 1997), and the resulting embryos were stained with X-gal. Stained cells were specifically located in the left LPM at E8.2 (C) and were evident in the heart, but not in the common cardinal vein and vitelline vein, at E9.0 (F,I,L) and E9.5 (O,R,U). Transverse sections were prepared after whole-mount in-situ hybridization (G,J,P,S) or X-gal staining (H,I,K,Q,R,U). Black and yellow arrowheads in D-U indicate the region that corresponds to the common cardinal vein and vitelline vein and in which asymmetric Pitx2 expression persists in Pitx2-ASE/ASE embryos. Scale bars: 200 μm.
adjacent to the IAVC of the AV canal was X-gal negative for Pitx2 17-lacZ (Fig. 8A-D) but X-gal positive for Pitx2-Cre (Fig. 8I-L). This differentially stained region thus represents a domain in which Pitx2 expression is induced at E8.2 but is repressed by E9.5, earlier than the remaining expression domains are repressed.

The X-gal staining pattern yielded by Pitx2 17-lacZ in Pitx2^ΔASE/ΔASE embryos at E9.5 (Fig. 8E-H), however, was virtually identical to that conferred by Pitx2-Cre in wild-type embryos (Fig. 8I-L). The large portion of the ventricle and AV canal in which Pitx2 17-lacZ was not expressed in wild-type embryos was thus X-gal positive in Pitx2^ΔASE/ΔASE embryos harboring this transgene. Such expansion of X-gal-positive cells in Pitx2^ΔASE/ΔASE embryos might result from either an abnormal contribution of cells in which the ASE was once active or from the loss of negative feedback by Pitx2 itself. To distinguish between these possibilities, we examined the pattern of X-gal staining conferred by Pitx2-Cre in Pitx2^ΔASE/ΔASE embryos (Fig. 8M-P). The staining pattern was indistinguishable from that of wild-type embryos harboring the same transgene (Fig. 8I-L), which favors the latter possibility. These results are in principle consistent with the fate mapping data in Xenopus (Ramsdell et al., 2005) but suggest a negative feedback loop in a portion of the AV canal. However, our findings require further work because a transgene does not always recapitulate correct expression patterns of a gene [as illustrated in Table 1 and the previous reports; for example, Mortlock et al. (Mortlock et al., 2003)].

**Requirement for continuous expression of Pitx2 in situs-specific morphogenesis**

Whereas asymmetric expression of Nodal is transient, asymmetric Pitx2 expression induced in LPM at E8.2 is maintained in LPM-derived cells of visceral organs for an additional 2 days. We next examined whether the earlier expression of Pitx2 in LPM is sufficient for situs-specific organogenesis of some organs or whether continuous expression is necessary for all organs.

![Fig. 8. Regulation of Pitx2 expression by negative feedback in the ventricle and AV canal of the heart.](Image)
To address this issue, we generated permanent transgenic mouse lines that express Pitx2 either transiently or continuously. These mice thus harbor a Pitx2 transgene driven either by a mutant ASE (NmASE) that lacks the Nkx2-binding site or by the wild-type ASE (Fig. 9A). In the former transgenic mice (line Tg50), the transgene was expressed in LPM at E8.2 (Fig. 9D) but not in visceral organs at E9.5 or 10.5 (Fig. 9G,J,M; data not shown), as expected. In the latter transgenic mice (lines Tg39 and Tg55), asymmetric expression of the transgene was apparent in LPM at E8.2 (Fig. 9B,C) and was maintained at E10.5. However, transgene expression...
was maintained at E10.5 only in subsets of visceral organs; it was highly expressed in the truncus arteriosus, weakly in the gut dorsal mesentery and not in lung bud in Tg39 mice (Fig. 9E,H,K), whereas it was highly expressed only in the gut dorsal mesentery in Tg55 mice (Fig. 9F,II). A high level of Pitx2 expression in some domains may be nonpermissive because we were unable to establish transgenic mouse lines that express the transgene in many visceral organs.

The transgenes in the Tg50, Tg39 and Tg55 lines were then examined for their ability to rescue the LR defects of Pitx2<sup>ΔASE/ΔASE</sup> mice. The transgene of line Tg39, which was highly expressed in the truncus arteriosus (Fig. 9E), was able to rescue the morphological defect of the great vessels (11/13 mice, 85%) (Fig. 10A) but not the internal defects of the heart (including ASD, VSD and DORV) or other defects (Fig. 10D,G; data not shown) of Pitx2<sup>ΔASE/ΔASE</sup> mice. The transgene of line Tg55, which was highly expressed in the gut dorsal mesentery (Fig. 9L), was able to rescue the abnormal rotation of the gut (7/12, 58%) and aberrant positioning of the pancreas (7/12, 58%) (Fig. 10H, data not shown) but not other defects (Fig. 10B,E) of Pitx2<sup>ΔASE/ΔASE</sup> mice. Thus, defects were rescued for the organs in which the transgene was highly expressed. Finally, the transgene of line Tg50 was unable to rescue any of the morphological defects of Pitx2<sup>ΔASE/ΔASE</sup> mice (Fig. 10C,F,I). These results demonstrate that situs-specific morphogenesis requires continuous expression of Pitx2 on the left side of primordial organs.

**DISCUSSION**

A conserved mechanism for regulation of asymmetric Pitx2 expression

Pitx2 is expressed asymmetrically for a long period during organogenesis and executes LR asymmetric morphogenesis in vertebrates (Campione et al., 1999; Logan et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998). Mouse Pitx2 is regulated by an ASE that contains three Foxh1-binding sequences and an Nkx2-binding sequence, both of which are essential for asymmetric expression (Shiratori et al., 2001). The phenotype of Pitx2<sup>ΔASE/ΔASE</sup> mice described in the present study now establishes an essential role for the ASE in Pitx2 regulation. Addition of multiple copies of the Nkx2 binding sequence derived from the ASE of Pitx2 to the ASE of Lefty2 transformed the transient action of the latter into a longer lasting one, confirming the essential role of the Nkx2-binding sequence in the ASE of Pitx2. We have also now shown that Pitx2 of all vertebrates (mouse, rat, human, chicken, frog, zebrafish) examined possesses an ASE in the last intron with a similar organization, namely two or three Foxh1-binding sequences and one Nkx2-binding sequence. Asymmetric Pitx2 expression in vertebrates is thus regulated by a highly conserved enhancer, ASE.

The ASEs of Pitx2 from each of the various vertebrates examined showed largely similar activities in mouse embryos. The ASE of mouse, human, or chicken Pitx2 showed activity in the left LPM at E8.2 as well as in many organs, including the common atrial chamber, lung bud, septum transversum and gut dorsal mesentery, at E10.5. By contrast to the ASE of Pitx2 from other vertebrates, however, that of zebrafish Pitx2 was active only in the anterior portion of left LPM at E8.2 and was inactive in all organs examined at E10.5. It is thus possible that the Nkx2-binding sequence in the ASE of zebrafish Pitx2 is recognized by zebrafish Nkx2 but not by mouse Nkx2. Alternatively, the Nkx2-binding sequence of the ASE of zebrafish Pitx2 alone may not be sufficient for maintenance of Pitx2 expression.

**Pitx2-dependent and -independent LR organogenesis**

The Pitx2<sup>ΔASE/ΔASE</sup> mice manifested LR defects in many organs. LR asymmetry in some organs, however, remained normal in the Pitx2<sup>ΔASE/ΔASE</sup> mice. This latter finding is not due to residual Pitx2 expression, which was detected only in the common cardinal vein and vitelline vein. In mutant mice that lack expression of cryptic Pitx2 (Yan et al., 1999) or Foxh1 (Yamamoto et al., 2003), however, those organs that remain normal in Pitx2<sup>ΔASE/ΔASE</sup> mice are abnormal. The asymmetric morphogenesis of such organs thus appears to be regulated in a manner independent of Pitx2 but dependent on Nodal signaling.

LR asymmetric morphogenesis is achieved by three mechanisms: (1) directional looping of a tube; (2) differential lobation, as in the lungs or liver; and (3) one-sided regression, as in blood vessels (Hamada et al., 2002). LR asymmetric events regulated in a Pitx2-independent manner include heart looping, embryonic turning and looping of the duodenum-stomach, all of which correspond to the first pattern of morphogenesis. The corresponding organs form initially as a straight tube at the midline that subsequently undergoes looping or turning. Directional looping of the developing heart, for example, may be achieved by physical forces intrinsic to the heart, such as those generated by changes in the arrangement of intracellular actin bundles (Itasaki et al., 1991; Itasaki et al., 1989), changes in myocardial cell shape (Manasek et
al., 1972) or differential rates of cell proliferation. Embryonic turning may also involve LR asymmetric rates of cell proliferation in LPM (Miller and White, 1998). Alternatively, the force for heart looping may be provided externally, such as by the adjacent splanchnopleure (Voronov et al., 2004). Although most situs-specific organogenesis depends on Pitx2, the mechanism of Pitx2 action remains unknown. Pitx2-dependent events include the development of asymmetries in lung lobation, blood vessel remodeling and atrial shape, and it remains to be determined how Pitx2 regulates such seemingly different cellular processes.

**Significance of two-step regulation of asymmetric Pitx2 expression**

Pitx2 is expressed asymmetrically for a long period and is regulated in two steps: initiation by Nodal signaling and maintenance by Nkx2. In the present study, we examined whether the early phase of Pitx2 expression is sufficient or whether continued expression is necessary for situs-specific organogenesis. Our data obtained from transgenic rescue experiments demonstrate that the persistent expression of Pitx2 is required. It is possible that Pitx2 regulates various cellular events in organs undergoing LR asymmetric morphogenesis. If so, what is the role of the early phase of Pitx2 expression in left LPM? The early-phase expression may play a positive role in LR morphogenesis by activating target genes that are essential for morphogenesis in the late phase. Alternatively, the early-phase expression may simply be necessary for the late-phase expression; although Pitx2 might play a role only during the late phase, its expression may need to be initiated at the early phase in response to Nodal signaling. Distinguishing between these possibilities will require the generation of transgenic mice that asymmetrically express Pitx2 only at the late phase.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/15/3015/DC1

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