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Differential expression and functional analysis of *Pitx2* isoforms in regulation of heart looping in the chick

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SUMMARY

***Pitx2*, a *bicoid*-related homeobox gene, plays a crucial role in the left-right axis determination and dextral looping of the vertebrate developing heart. We have examined the differential expression and function of two *Pitx2* isoforms (*Pitx2a* and *Pitx2c*) that differ in the region 5′ to the homeodomain, in early chick embryogenesis. Northern blot and RT-PCR analyses indicated the existence of *Pitx2a* and *Pitx2c* but not *Pitx2b* in the developing chick embryos. In situ hybridization demonstrated a restricted expression of *Pitx2c* in the left lateral plate mesoderm (LPM), left half of heart tube and head mesoderm, but its absence in the extra-embryonic tissues where vasculogenesis occurs. RT-PCR experiments revealed that *Pitx2a* is absent in the left LPM, but is present in the head and extra-embryonic mesoderm. However, ectopic expression of either *Pitx2c* or *Pitx2a* via retroviral infection to the right LMP equally randomized heart looping direction. Mapping of the transcriptional activation function to the C terminus that is identical in both isoforms explained the similar results obtained by the gain-of-function approach. In contrast, elimination of *Pitx2c* expression from the left LMP by antisense oligonucleotide resulted in a randomization of heart looping, while treatment of embryos with antisense oligonucleotide specific to *Pitx2a* failed to generate similar effect. We further constructed RCAS retroviral vectors expressing dominant negative *Pitx2* isoforms in which the C-terminal transcriptional activation domain was replaced by the repressor domain of the *Drosophila* Engrailed protein (En^r). Ectopic expression of *Pitx2c-En^r*, but not *Pitx2a-En^r*, to the left LPM randomized the heart looping. The results thus demonstrate that *Pitx2c* plays a crucial role in the left-right axis determination and rightward heart looping during chick embryogenesis.**

Key words: Pitx2, Isoforms, Chick, Left-right asymmetry, Heart looping

INTRODUCTION

In vertebrates, the overall bilateral symmetry of the adult body is broken by the consistently asymmetric placement of various internal organs such as the heart, liver, spleen and gut, or asymmetric development of paired organs such as brain and lungs. The first morphological indication of the left-right asymmetry is manifested by the rightward looping of the developing heart. A great deal of research has been undertaken into the study of factors involved in left-right axial determination and dextral looping of the heart. Recent advances in this area have led to sophisticated and complex pathways of induction and regulation between numerous signaling molecules and transcription factors (Harvey, 1998; Ramsdell and Yost, 1998). The first demonstration of left-right asymmetric gene expression, preceding organogenesis, was seen in the chick embryo and includes a cascade of signals involving activin receptor IIa (*cAct-IIa*), sonic hedgehog (*Shh*) and Nodal (Levin et al., 1995). During early gastrulation, the asymmetric expression of *cAct-IIa* on the right side of the node restricts *Shh* expression to the left side of the node of a chick embryo. The asymmetric *Shh* expression is then responsible for the asymmetric expression of *Nodal* to the left lateral plate mesoderm (LPM). It was recently found that caronte, a member of the Cerberus/Dan gene family, mediates Shh induction of *Nodal* by antagonizing a repressive activity of BMPs (Rodriguez-Esteban et al., 1999; Yokouchi et al., 1999; Zhu et al., 1999). Studies in other vertebrate species. including mouse and *Xenopus*, indicate that the asymmetrical expression of *Nodal* in the left LPM is conserved across species and is crucial for establishing initial left-right asymmetry (Collignon et al., 1996; Hyatt et al., 1996; Lowe et al., 1996; Sampath et al., 1997). Downstream from this left side signaling cascade reside *lefty1* and *lefty2* (Meno et al., 1996; Meno et al., 1997;

Meno et al., 1998; Rodriguez-Esteban et al., 1999; Yokouchi et al., 1999), and *Pitx2* (Campione et al., 1999; Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; St Amand et al., 1998; Yoshioka et al., 1998). Retinoic acid, which is involved in the left-right patterning (Chen and Solursh, 1992; Smith et al., 1997), has also been shown to regulate the expression of left side-specific genes in a pathway downstream or parallel to *Shh* across different species (Chazaud et al., 1999; Tsukui et al., 1999; Wasiak and Lohnes, 1999; Zile et al., 2000). Several other genes also exhibit asymmetrical expression and participate in the regulation of left-right axis formation, as summarized by King and Brown (King and Brown, 1999), including *Fgf8*, *Snail*-related gene and *Nkx*3.2 (Issac et al., 1997; Sefton et al., 1998; Boettger et al., 1999; Meyers and Martin, 1999; Schneider et al., 1999; Rodriguez-Esteban et al., 1999; Capdevila et al., 2000).

Pitx2 is a member of the Pitx/RIEG family of homeobox containing genes which are closely related to the *Drosophila* gene *bicoid* (Semina et al., 1996, Mucchielli et al., 1996). Two additional family members *Pitx1* and *Pitx3* have also been identified (Lamonerie et al., 1996; Szeto et al., 1996; Lanctôt et al., 1997; Semina et al., 1997). *PITX2* (also known as *RIEG*) was first identified in humans, and mutations in this gene have been shown to be responsible for the autosomal-dominant disorder, Rieger syndrome, which exhibits defects in the tooth and eye (Semina et al., 1996). *PITX2* has also been identified as a downstream target of the human acute leukemia *ALL1* gene (*MLL1* – Human Gene Nomenclature Database), suggesting a role in tumorigenesis (Arakawa et al., 1998). A role for *Pitx2* in vertebrate organogenesis has been suggested by its expression in developing Rathke's pouch, eye, limb bud, heart, brain and tooth germ during embryogenesis (Semina et al., 1996; Mucchielli et al., 1996; Mucchielli et al., 1997; T. R. St Amand, PhD Thesis, Tulane University, 1999; St Amand et al., 2000), and has been further demonstrated by gene knockout experiments (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999).

A prominent function of *Pitx2* in vertebrate organogenesis is its involvement in the regulation of left-right development. *Pitx2* is expressed in the left side LPM and the left side of the cardiac tube and gut, and functions downstream of Shh/Nodal pathway in all vertebrate species examined (Meno et al., 1998; Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; St Amand et al., 1998; Yoshioka et al., 1998; Bisgrove et al., 1999; Campione et al., 1999). Functional analysis indicated that *Pitx2* may interpret and subsequently execute the left-right developmental program (reviewed in Yost, 1999). Ectopic expression of *Pitx2* on the right LPM alters left-right developing of the heart and other internal organs (Logan et al., 1998; Ryan et al., 1998; Campione et al., 1999). On the other hand, loss of *Pitx2* function in mouse by gene targeting also results in defects in the left-right asymmetry of internal organs, including heart (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999). Several *Pitx2* isoforms have been isolated from human, mouse, chick, *Xenopus* and zebrafish (Gage and Camper, 1997; Arakawa et al., 1998; Logan et al., 1998; St Amand et al., 1998; Essner et al., 2000; Schweickert et al., 2000). Thus, the potential role of these *Pitx2* isoforms in the regulation of left-right asymmetry has just begun to be elucidated (Essner et al., 2000; Schweickert et al., 2000).

We have cloned two chick *Pitx2* isoforms (*Pitx2a* and *Pitx2c*), which correspond to the mouse and human isoforms of *Pitx2* and differ only in their N-terminal region. In this study, we have analyzed their differential expression patterns during early chick embryonic development and their involvement in the regulation of heart looping. We revealed the presence of *Pitx2*a and *Pitx2c* but not *Pitx2b* in the developing chick embryos. Whole mount in situ hybridization using *Pitx2c* specific probe, in combination with RT-PCR, indicates that *Pitx2c*, but not *Pitx2a*, is restrictedly expressed in the left LPM. Although ectopic expression of either *Pitx2a* or *Pitx2c* to the right LPM via RCAS retroviral infection randomized heart looping direction, specific repression of *Pitx2* isoforms by antisense oligonucleotide approach, as well as ectopic expression of dominant negative forms of RCAS-*Pitx2* demonstrate that left-right asymmetry in developing chick embryo is regulated by *Pitx2c*.

MATERIALS AND METHODS

Isolation of chick *Pitx2* cDNA isoforms

Chick *Pitx2a* and *Pitx2c* were isolated simultaneously from a day 10 chick embryonic brain cDNA library using a mouse *Pitx2* cDNA probe under low stringency condition, as described previously (St Amand et al., 1998). Briefly, about 1×10^6 phage plaques were plated and hybridized with a mouse *Pitx2* probe in 6× SSPE, 5× Denhardts, 50 mM phosphate, 0.1% SDS, 10 mM $Na_4P_2O_7$, 200 µg/ml salmon sperm DNA at 60°C. Membranes were then washed three times for 30 minutes each with 4× SSPE, 0.2% SDS before exposing to X-ray film.

Probes and whole-mount in situ hybridization

Chick *Pitx2c*-specific probes were generated by PCR amplification of the first 306-bp of the 5′ region, including 76-bp 5′ UTR, from *Pitx2c* cDNA (St Amand et al., 1998). This region exhibits sequence diversity from *Pitx2a.* The PCR amplified fragment was subcloned into pBluescript KS. Pitx2 full-length probes were generated as described previously (St Amand et al., 1998). The *Drosophila engrailed* repressor sequence was released from the *Slax* 13 adaptor plasmid and subcloned into pBluescript KS. All probes were labeled with digoxigenin, as directed by the manufacturer (Boehringer Mannheim). Probe size and yield were determined by comparing with an RNA standard after agarose gel electrophoresis.

Whole-mount in situ hybridization was performed as described previously (St Amand et al., 1998) and signals were visualized using NBT/BCIP substrate (Boehringer Mannheim). For visualization, analysis and photographs, samples were dehydrated with glycerol and mounted onto slides. In the case of samples to be sectioned, the samples were gradually re-hydrated in PBS and dehydrated again in ethanol. Samples were then cleared with xylene followed by embedding in paraffin wax. Embryos were sectioned at 15 µm and mounted with Permount.

Northern blot

Northern blot analysis was performed using NorthernMax kit (Ambion, Austin, TX) according to manufacture's instructions. Poly(A) RNA samples were extracted from whole chick embryos at stages 4, 8, 11, 21, 26 and 31 using the PolyATtract System 1000 (Promega Corp.; Madison, WI). About 2 µg of Poly(A) RNA was denatured in formamide based denaturing buffer and subject to electrophoresis through a 1% agarose-formaldehyde gel. RNA was then transferred to positively charged nylon membrane and immobilized by UV crosslink. A 1-Kb ^{32}P-labeled probe was used that covers both exon 5 and exon 6 which are present in all three *Pitx2* isoforms. Hybridization and washes were carried out

at appropriate temperatures and the blots were exposed to X-ray film.

Retrovirus construction and infection

To make RCAS-*Pitx2* constructs, full-length *Pitx2a* and *Pitx2c* sequences were released from the pBluescript vectors by double digestion with *BamH*I and *EcoR*I, and were cloned into the *Cla* 12 adapter plasmid. The *Cla*I fragments containing *Pitx2a* and *Pitx2c* sequences were cloned into the RCAS retroviral vectors. The orientation was determined by PCR using an upper primer targeting on retroviral sequence and a lower one targeting on *Pitx2* sequence and further by sequencing. To make RCAS-*Pitx2*-En^r constructs, DNA fragments with the open reading frames containing the entire N-terminal region and the homeodomain of *Pitx2a* and *Pitx2c* were generated by high fidelity PCR and cloned into the pBluescript vector. After sequencing, the fragments were released and inserted into a version of *Slax* 13 adaptor plasmid containing the *Drosophila engrailed* repressor domain sequence (En^r; provided by Dr C. Cepko of Harvard Medical School). The *Cla*I fragments harboring *Pitx2* sequence with the C terminus replaced with the En^r domain were further cloned into the *Cla*I site of RCAS retroviral vectors. Generation and concentration of viral supernatant were carried out according to a protocol described previously (Logan and Francis-West, 1999). Retrovirus infection was performed on stage 3+ or stage 4 embryos (Hamburger and Hamilton, 1951) explanted in New culture (New, 1955). RCAS-*Pitx2a* and RCAS-*Pitx2c* viruses were targeted to the right side of the blastoderm by multiple points of injection, while RCAS-*Pitx2a-En^r* and RCAS-*Pitx2c-En^r* were targeted to the left side of the blastoderm. Injected embryos were returned to a 37°C incubator in a humid chamber, and were allowed to develop to stage 11 when heart looping has finished.

Antisense oligonucleotide treatment

Antisense oligonucleotide treatment was carried out on stage 6-7 embryos explanted in New culture as described previously (Srivastava et al., 1995; Issac et al., 1997). The antisense oligonucleotides have sequences (*Pitx2a*, 5′-ACGCAGGCTGAGACAAGT-3′; *Pitx2c*, 5′-GGTGTCAGAGATAGTGTG-3′) that target to the specific N-terminal regions of *Pitx2a* and *Pitx2c*. A random oligonucleotide (5′-AGGCTCGAACTCAGACTT-3′) was used as a control. All the oligonucleotides used were synthesized as phosphorothioate derivatives and HPLC purified (IDT, Corralville, IA). Oligonucleotides were diluted to a concentration of 40 μM in lipofectAMINE (GibcoBRL). Approximate 10 μl of mixed lipofectAMINE was applied directly onto the cultured embryo within the ring. Embryos were allowed to develop to stage 11, and then scored for the heart looping direction.

GAL4/*Pitx2* expression plasmids and CAT assay

The DNA fragments encoding the N-terminal regions (amino acids 2-100 for *Pitx2c*; amino acids 2-38 for *Pitx2a*) and the C terminus (identical in both isoforms; amino acids 161-333 in *Pitx2c* and amino acids 100-271 in *Pitx2a*) of *Pitx2* were amplified from chick *Pitx2a* and *Pitx2c* cDNA plasmids and cloned into pBXG1 which contains the DNA-binding domain of GAL4 (amino acids 1-147) under the control of SV40 enhancer/promoter (Lillie and Green, 1989). The DNA fragments were inserted downstream of and in frame with the GAL4 DNA-binding domain. All constructs, named GAL4/*Pitx2c*N, GAL4/*Pitx2a*N and GAL4/*Pitx2*C, were confirmed by DNA sequencing. Transfection and chloramphenicol acetyltransferase (CAT) assay were carried out using P19 cells. Briefly, The GAL4/*Pitx2* constructs were co-transfected into P19 cells respectively, with the pG_5ECAT reporter plasmid containing five GAL4-binding sites upstream of the adenovirus E1b minimal promoter and the CAT gene (Ma and Ptashne, 1987). As an internal control for transfection efficiency, a CMV-β-gal plasmid was included. Transfection was achieved by addition onto cells plated in a 60 mm culture dishes of a mixture of 1 μg GAL4/*Pitx2*, 1 μg pG_5ECAT, 1 μg CMV-β-gal and 12 μl of lipofectAMINE (GibcoBRL), according to the manufacturer's instruction. Co-transfection of a plasmid (pBXGAL-II) expressing the GAL4 DNA-binding domain and activation region II (amino acid 768-881) with pG_5ECAT served as a positive control for the activation of the CAT reporter gene, while transfection of a SV40-CAT plasmid served as a positive control for the CAT assay. Transfection of pG_5ECAT with pBXG1 was included as a negative control. Transfected cells were cultured for 36 hours and cell extracts were prepared for CAT assay according to the standard protocol ('Protocols and Applications Guide', Promega). The CAT activities were determined by thin-layer chromatography (TLC) and scintillation counting. Each experiment was repeated at least three times.

RT-PCR

To examine the expression of *Pitx2* in the early developing embryo, chick embryos from stages 4 to 31 were collected, and total RNA extracted. To examine the differential expression of *Pitx2* isoforms during the stages when heart looping direction is determined, head tissues, tissues containing only left LPM, and tissues containing right LPM and its adjacent extra-embryonic region from stage 8-10 embryos were isolated. Samples were treated with 2.25% trypsin/ 0.75% pancreatin on ice for 5 minutes prior to being transferred to a 1:1 solution of PBS and horse serum. Endoderm and ectoderm were then removed. Mesodermal tissues were collected and pooled together and total RNA extracted. Total RNA from same stage whole embryos was also extracted and used as positive control. Primers used for PCR detection of *Pitx2* isoforms were 5′-CGATAACGGGTAATGGAG-3′ (upper primer targeting to exon 2, for both *Pitx2a* and *Pitx2b*), 5′-CTCTCTCTTCCTCCATTT-3′ (lower primer targeting to exon 6, for all isoforms), and 5′-GTCCTCTCGCCGATGAGT-3′ (upper primer targeting to exon 4, for *Pitx2c*). PCR was carried out under the following conditions: 94°C, 1 minute; 62°C, 1 minute; 72°C, 1 minute (30 cycles). As a control, *Gapdh*, a constitutively expressed mRNA, was amplified from each sample using the following primers: 5′-AGAGGTGCTGCCCAGAACATCATC-3′ (upper primer) and 5′-GTGGGGAGACAGAAGGGAACAGA-3′ (lower primer). The PCR profile used was as follows: 94°C, 1 minute; 60°C, 30 seconds; 72°C, 1 minute (30 cycles).

RESULTS

Pitx2 isoforms

In our original screen of a chick embryonic brain cDNA library with a mouse *Pitx2* probe, two *Pitx2* cDNA isoforms containing a open reading frame were identified and named *Pitx2a* and *Pitx2c*, respectively. At the amino acid level, both *Pitx2a* and *Pitx2c* encode for a homeodomain-containing protein of 271 amino acids and 333 amino acids, respectively. The cDNA sequence of *Pitx2c* and its corresponding amino acid sequence have been reported in our previous studies (St Amand et al., 1998), while the amino acid sequence of *Pitx2a* is identical to that reported by Logan et al. (Logan et al., 1998). These two proteins differ by 77 amino acids at the 5′ region but are 100% identical from that point on, including 23 amino acids 5′ to the homeodomain, the homeodomain and the entire C terminus (Fig. 1). This region of identity also includes the fourteen amino acid motif in the C terminus, which is conserved among all members of the *Pitx/Rieg* family and some other selected homeodomain-containing transcription factors (Semina et al., 1998).

To confirm the presence of *Pitx2* isoforms in developing

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N-terminal region:
Pitx2c: M S C M K D P L S L E R L G A G N N K L C S S S P S
        S S S S S S S C H H Q Q P A L A M A T A L A P G Q A
        R S S L E A A K H R L E V H T I S D T S S P E A A
Pitx2a:                     M E S N C R K L V S A C V Q L

Common region:
E K E K S Q Q G K S E D A G P E D P S K K K R Q R R Q R T H
F T S Q Q L Q E L E A T F Q R N R Y P D M S T R E E I A V W
T N L T E A R V R V W F K N R R A K W R K R E R N Q Q A E L
C K N G F G P Q F N G L M Q P Y D D M Y P G Y S Y N N W A A
K G L T S A S L S T K S F P F F N S M N V N P L S S Q S M F
S P P N S I S S M S M S S S M V P S A V T G V P G S G L N S
L N N L N N L S N P S L N S A V P T P A C P Y A P P T P P Y
V Y R D T C N S S L A S L R L K A K Q H S S F G Y A S V Q N
P A S N L S A C Q Y P V D R P V .
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Fig. 1. Translated amino acid sequences of *Pitx2a* and *Pitx2c*. Amino acids that differ between the two isoforms reside in the N terminus and are compared. The homeodomain is shaded. The conserved 14 amino acid motif found in C terminus is underlined.

chick embryo, Northern blot analysis was performed on RNA samples isolated from various developmental stages (Fig. 2). Poly(A) RNA was isolated from stage 4, 8, 11, 21, 26 and 31 embryos and analyzed by Northern blot using ^{32}P-labeled probe generated from chick *Pitx2* exons 5 and 6, which are present in all isoforms. Two transcripts, approx. 1.6 and 2.3 kb, were detected in the samples from all the stages examined (Fig. 2A). To identify what specific isoforms these two transcripts are, RT-PCR was carried out on RNAs from the same stage embryos. It has been shown that the only difference between *Pitx2b* and *Pitx2a* is that *Pitx2b* has an additional 138 base exon 3 present between exons 2 and 5 (Schweickert et al., 2000). Therefore, an upper primer targeting to exon 2 and a lower primer targeting to exon 6 would cover both *Pitx2a* and *Pitx2b*. For *Pitx2c*, one pair of primers corresponding to exon 4, which is unique to *Pitx2c*, and exon 6 was used. As shown in Fig. 2B, only two PCR products, corresponding to the sizes expected for *Pitx2a* (303 bp) and *Pitx2c* (491 bp), respectively, were obtained. Sequencing analysis of these PCR products further confirmed the existence of *Pitx2a* and *Pitx2c*. *Pitx2b* is thus not present in the early chick embryos, at least at the stages examined. Similarly, it was noticed that that *Pitx2b* and *Pitx2a* were undetectable in zebrafish and *Xenopus*, respectively (Essner et al., 2000; Schweickert et al., 2000).

Pitx2c but not *Pitx2a* expressed in the left side LPM during left-right development in chick embryo

To determine the differential expression patterns of *Pitx2a* and *Pitx2c* during early chick embryogenesis, with particular interest in the establishment of the left-right axis, a 306 bp probe corresponding to the 5′ UTR and N-terminal coding region that are specific to *Pitx2c* was used. Since the sequence of 5′ UTR and the N-terminal coding region that are specific to the *Pitx2a* was too short (75 bp) to make an in situ hybridization probe, the expression pattern of *Pitx2c* could be only compared with that using full-length Pitx2 probe, as described previously (St Amand et al., 1998). By using whole-mount in situ hybridization, expression of *Pitx2c* was first detected at stage 5 (Fig. 3) with symmetrical expression in the hypoblast (data not shown). However, no expression was detected in the area opaca, as previously reported using a full-length *Pitx2* at this stage (St Amand et al., 1998). By stage 8, asymmetric expression, characteristic of *Pitx2*, became evident and restricted to the left LPM, as confirmed by sections through these embryos (Fig. 3). Expression was also detected symmetrically in the head mesenchyme at this stage, but again no expression was detected in the area opaca. At stage 10, asymmetric expression of *Pitx2c* remained in the left one half of the embryo, including left side heart tube as the two primitive heart tubes fuse (data not shown). At the stage of heart looping (stage 11), *Pitx2c* expression was still restricted to the left LPM and the left half of the fused heart tube (Fig. 3). Sections through these samples reveal restricted expression in the left half epimyocardium of the fused heart tube (Fig. 3) as previously seen using a full-length *Pitx2* probe (St Amand et al., 1998). We have previously reported the expression of *Pitx2* in the angioblasts of blood islands and the endothelia of the blood vessels in the extra-embryonic regions (St Amand et al., 1998). However, through all stages analyzed, expression of *Pitx2c* was not detected in the extra-embryonic tissue, or in the endothelia and blood islands (Fig. 3). Therefore, these results suggest that *Pitx2c* be involved in the left-right axis determination and dextral looping of the heart, but not in the vasculogenesis during chick embryonic development. However, these observations do not rule out the possibility that *Pitx2a* is also expressed in the left LPM and may contribute to left-right development. To examine whether *Pitx2a* is also expressed in the left LPM, RT-PCR for *Pitx2a* was performed using RNA extracted from the left LPM collected from stage 8-10 embryos. In addition, *Pitx2a* and *Pitx2c* expression was also examined by RT-PCR in the head and extra-embryonic mesoderm containing the right LPM. The results demonstrated that both isoforms are expressed in the head region, but that only *Pitx2c* transcripts were detected in the left LPM, while *Pitx2a* transcripts were found exclusively in the extra-

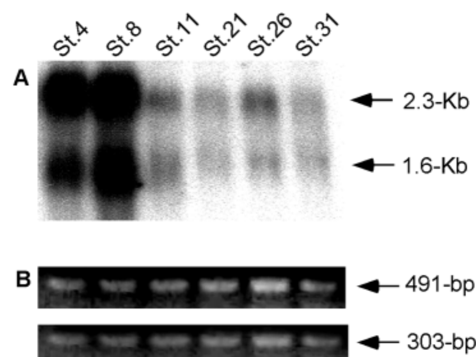

Fig. 2. Expression of *Pitx2a* and *Pitx2c* during early chick embryogenesis. (A) Northern blot detection of *Pitx2* isoforms in developing chick embryos. Two bands at about 2.3 and 1.6 kb were detected in Poly(A) RNA samples isolated from stages 4, 8, 11, 21, 26 and 31 embryos. (B) RT-PCR detection of *Pitx2a* (303-bp) and *Pitx2c* (491-bp) isoforms.

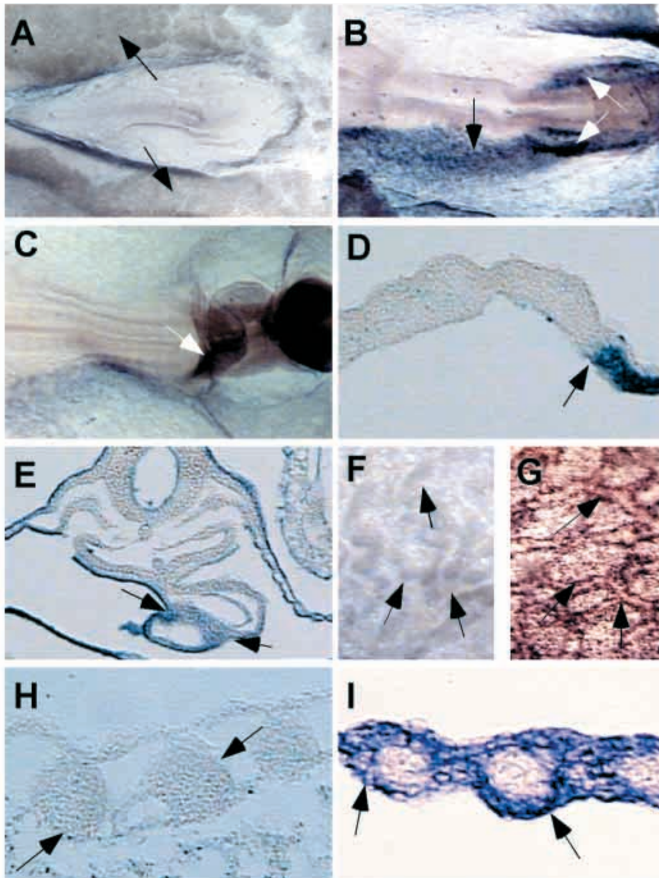

Fig. 3. Expression of *Pitx2c* in early developing chick embryos. (A) Ventral view of a stage 5 chick embryo. *Pitx2c* expression at this stage was detected symmetrically in the hypoblast but no expression was detected in the area opaca (arrows). (B) Ventral view of a stage 8 embryo showing the expression of *Pitx2c* in the left LPM (black arrow) as well as head mesoderm (white arrows). (C) Ventral view at stage 11 showing *Pitx2c* expression in the left LPM and the left heart tube (arrow). (D) Section through a stage 8 embryo showing restricted expression of *Pitx2c* to the left LPM. The arrow indicates the boarder between the segmental plate and the lateral plate mesoderm. (E) Section through a stage 11 embryo showing restricted expression to the left half epimyocardium of the fused heart tube (arrows). (F) Close up of area vasculosa of a stage 13 embryo showing that no expression of *Pitx2c* was detected in the blood vessels (arrows). (G) Close up of area vasculosa of a stage 13 embryo indicating *Pitx2* expression in the endothelia of the blood vessels (arrows) detected by a full-length *Pitx2* probe. (H) Section through extra-embryonic region of a stage 15 embryo revealing no expression of *Pitx2c* in the angioblasts (arrows) of blood islands. (I) Expression of *Pitx2* in the angioblasts (arrows) of blood islands of a stage 15 embryo detected by a full-length Pitx2 probe.

embryonic mesoderm (Fig. 4). Thus, *Pitx2c* contributes to *Pitx2* expression in the left LPM.

Both *Pitx2a* and *Pitx2c* encode a transcriptional activator

Prior to functional analysis of *Pitx2* isoforms in regulating left-right development, we asked whether both isoforms equally encode a transcriptional factor. To map the transactivation domain of *Pitx2* products, we generated a series constructs in which the C-terminal region (from the amino acid immediately after the homeodomain to the last amino acid) of the *Pitx2* and the N-terminal regions (from the second amino acid to the amino acid prior to the homeodomain) of *Pitx2a* and *Pitx2c* were fused in frame to the 147 amino acid DNA-binding domain of yeast GAL4. Transactivational activity of these domain was assayed by co-transfection of these constructs into cultured P19 cells with a CAT reporter plasmid containing five GAL4-binding sites and a basal promoter. As shown by TLC in Fig. 5, GAL4/Pitx2aN and GAL4/Pitx2cN fusion proteins did not activate CAT reporter gene expression, while GAL4/Pitx2C fusion protein activated CAT reporter gene expression. Similar results were obtained by scintillation counting (data not shown). Since the C-terminal region of *Pitx2* is identical in both *Pitx2a* and *Pitx2c*, we thus conclude that both *Pitx2a* and *Pitx2c* encode transcriptional activator and their transcriptional activation function resides within the C-terminal region.

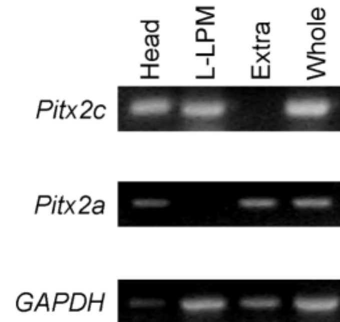

Fig. 4. RT-PCR detection of *Pitx2a* and *Pitx2c*. Both *Pitx2a* and *Pitx2c* transcripts were detected in the sample from stage 8 whole embryos (Whole), and were used as positive controls. *Pitx2a* transcripts were found in the head and the extra-embryonic mesoderm (Extra), but not in the left LPM (L-LPM). *Pitx2c* was detected in the head and left LPM but not in the extra-embryonic mesoderm. RT-PCT for *Gapdh* was included as an internal control.

Ectopic expression of *Pitx2a* and *Pitx2c* randomize embryonic heart looping

To analyze the function of *Pitx2* isoforms in the left-right specification processes in which *Pitx2* appears to be involved, RCAS-*Pitx2a* and RCAS-*Pitx2c* retroviruses were generated and used to direct ectopic expression of these isoforms to the right LPM of developing chick embryos, as described previously (Logan et al., 1998; Ryan et al., 1998). Stage 3+ or stage 4 chick embryos explanted in New culture were injected with either RCAS-*Pitx2a* or RCAS-*Pitx2c* retroviruses to the right LPM and cultured to stage 11 when heart looping completed. RCAS-alkaline phosphatase (*RCAS-AP*) retrovirus was used as a control. Only injected embryos that survived were harvested and scored for heart looping directions. Samples were randomly analyzed by whole-mount in situ hybridization to confirm viral misexpression. As shown in Fig. 6, ectopic expression of *Pitx2* in the right LPM was observed, indicating the efficiency of RCAS infection. The results demonstrated that the injection of control viruses had no effect on heart looping (0/10). However, ectopic expression of *Pitx2c* to the right LPM resulted in 56% (19/34) of embryos with leftward heart looping (Fig. 6; Table 1). This result was

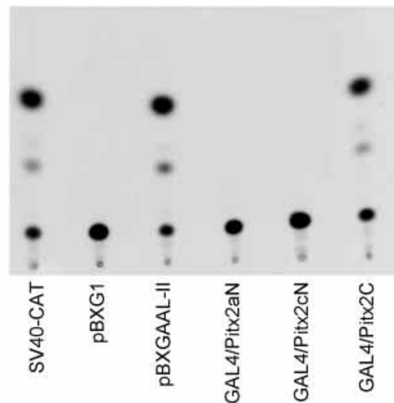

Fig. 5. Mapping of transactivation domain of Pitx2 proteins. P19 cells were transfected with the pG5ECAT reporter plasmid and the constructs indicated. SV40-CAT plasmid was transfected alone as a positive control for CAT assay. CAT activities were assayed by both TLC and scintillation counting. Results from both assays were the same, and only TLC results were shown here. Co-transfection of the CAT reporter vector with pBXG1 (containing GAL4 DNA-binding domain only) did not give rise CAT activity, while pBXGAL-II (expressing wild type GAL4) was able to activate CAT gene expression. Neither GAL4/Pitx2aN nor GAL4/Pitx2cN was able to activate CAT reporter gene expression, while the GAL4/Pitx2C activated CAT expression.

expectable, since *Pitx2c* is expressed in the left LPM. Interestingly, ectopic expression of *Pitx2a* to the right LPM also resulted in reversal of heart looping but at a slightly less efficient level (41%; *n*=17) (Fig. 6; Table 1). Thus when ectopically expressed, both isoforms are able to randomize the direction of embryonic heart looping. This similar effect of ectopic expression of the two *Pitx2* isoforms on heart looping could be explained by the fact that both isoforms encode transcriptional factors with similar transactivation activities, as demonstrated in Fig. 5.

Elimination of *Pitx2c* but not *Pitx2a* expression from left LPM randomizes heart looping

As an alternative approach to analyze functional differences between *Pitx2a* and *Pitx2c*, we performed a loss-of-function experiment using antisense oligonucleotide approach. Stage 6-7 chick embryos explanted in New culture were treated with antisense oligonucleotides and allowed to develop to stage 11 at which time they were collected and scored morphologically for alterations in heart looping direction. A random oligonucleotide was used as a control. Embryos treated with control oligonucleotide exhibited a 13% (3/23) effect on heart looping similar to previous report (Isaac et al., 1997).

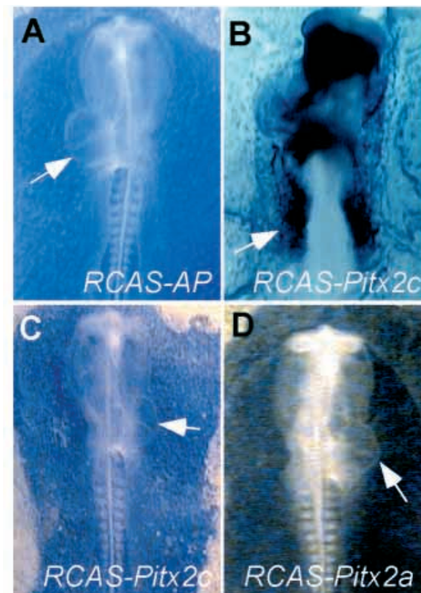

Fig. 6. Ectopic expression of *Pitx2a* and *Pitx2c* randomize heart looping. (A) Ventral view of a stage 11 chick embryo infected with RCAS-alkaline phosphatase (*RCAS-AP*) retrovirus at stage 4 exhibited normal rightward heart looping (arrow). (B) Ventral view of a chick embryo infected with *RCAS-Pitx2c* at stage 4 on the left LPM showed ectopic expression of *Pitx2* in the right LPM (arrow). (C) Ventral view of a stage 11 embryo infected with RCAS-*Pitx2c* at stage 4 exhibited a reversed heart looping (arrow). (D) Ventral view of a stage 11 embryo infected with RCAS-*Pitx2a* exhibited a leftward heart looping (arrow).

Treatment of embryos with antisense oligonucleotide specific to *Pitx2c* would be expect to eliminate *Pitx2c* expression in the left LPM. Random selection of these embryos for whole-mount in situ hybridization using a full-length *Pitx2* probe confirmed the absence of *Pitx2* expression in the left LPM (Fig. 7). However, expression of *Pitx2* in the head mesoderm was not significantly affected (Fig. 7). This observation also supports the idea that *Pitx2c* contributes to the expression of *Pitx2* in the left LPM. As expected, 43% (15/35) embryos treated with antisense oligonucleotide to *Pitx2c* exhibited a leftward heart looping (Fig. 7; Table 1). In contrast, embryos treated with antisense oligonucleotide specific to *Pitx2a* generated 19% (6/32) embryos with reversed heart looping (Table 1), a rate similar to that obtained after treatment with the control oligonucleotide. These results thus indicate that *Pitx2c* plays a crucial role in the regulation of left-right determination.

In order to confirm the results, we used another approach to perturb the function of *Pitx2* isoforms in the left LPM. We generated putative dominant negative RCAS-*Pitx2* constructs in

Table 1. Effects of overexpression or repression of *Pitx2* isoforms on heart looping

	Overexpression by retroviruses															Antisense oligos								
Type of experiment	*AP*			*Pitx2a*			*Pitx2c*			*Pitx2a-En^r*			*Pitx2c-En^r*			Random			*Pitx2a*			*Pitx2c*		
Looping direction	L	M	R	L	M	R	L	M	R	L	M	R	L	M	R	L	M	R	L	M	R	L	M	R
Number	0	0	10	7	0	10	19	1	14	2	2	21	9	1	9	3	0	20	6	3	23	15	0	20
Percentage (%)			(100)	(41)		(59)	(56)	(3)	(41)	(8)	(8)	(84)	(47)	(5)	(47)	(13)		(87)	(19)	(9)	(72)	(43)		(57)

L, left; M, middle; R, right. AP, alkaline phosphatase.

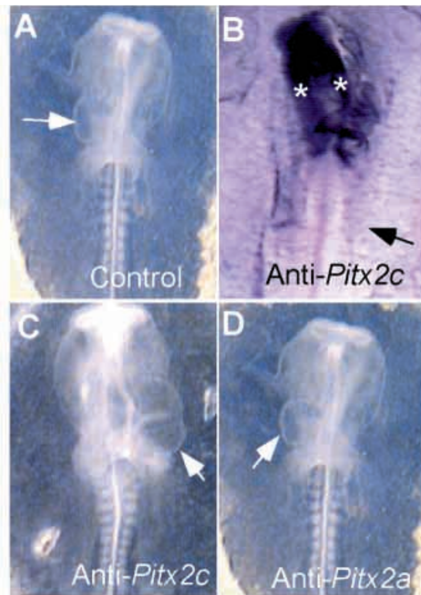

Fig. 7. Inhibition of *Pitx2c* but not *Pitx2a* by antisense oligonucleotides causes randomized heart looping. (A) Ventral view of a stage 11 embryo treated with control oligonucleotides (random sequence) showed normal rightward heart looping (arrow). (B) Ventral view of a stage 11 embryo treated with antisense oligonucleotides specific to *Pitx2c* showed elimination of *Pitx2* expression in the left LPM (black arrow). *Pitx2* expression in the head mesoderm (asterisks) was not significantly affected. (C) Ventral view of a stage 11 embryo treated with antisense oligonucleotides specific to *Pitx2c* exhibited a leftward heart looping (arrow). (D) Ventral view of a stage 11 embryo treated with antisense oligonucleotides specific to *Pitx2a* a displayed a normal rightward heart looping (arrow).

which the transactivation domain (C-terminal region) of *Pitx2*a and *Pitx2c* was replaced with the repressor domain of the *Drosophila* Engrailed protein (En^r). It has been demonstrated before that a fusion protein containing a DNA-binding domain, such as a homeodomain, and with repressor domain of Engrailed can interfere with transcriptional activation by the wild-type protein (Furukawa et al., 1997; Bao et al., 1999). Thus, both Pitx2a-En^r and Pitx2c-En^r should exert a dominant negative effect to block transcriptional activation by Pitx2a or Pitx2c proteins, respectively. RCAS-*Pitx2a-En^r* and RCAS-*Pitx2c-En^r* retroviruses were targeted to the left LPM of stage 3+ or stage 4 embryos placed in New culture. Injected embryos were allowed to develop to stage 11 and scored for heart looping direction. The expression of *Pitx2c-En^r* in retrovirus infected embryos was confirmed by whole-mount in situ hybridization using a probe specific to the *engrailed* repressor domain (Fig. 8A). As we expected, ectopic expression of RCAS-*Pitx2c-En^r* randomized heart looping. In 19 embryos infected with RCAS-*Pitx2c-En^r* retrovirus, nine of them (47%) exhibited a leftward heart looping (Fig. 8B; Table 1). In contrast, ectopic expression of RCAS-*Pitx2a-En^r* to the left LPM did not affect rightward heart looping (Fig. 8C), with only two out of total 25 (8%) infected embryos exhibiting a leftward heart looping (Table 1). These dominant negative studies provide additional evidence for the involvement of *Pitx2c* but not *Pitx2a* in the regulation of heart looping in developing chick embryo.

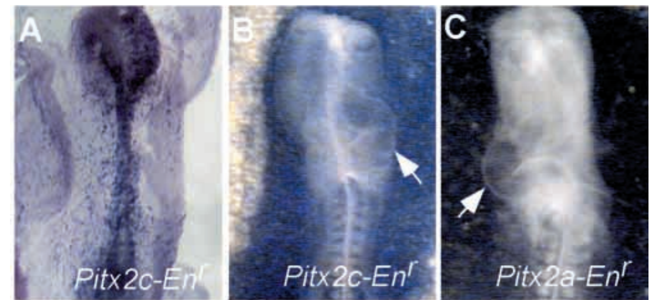

Fig. 8. Ectopic expression of dominant negative form of Pitx2c but not Pitx2a randomizes heart looping. (A) Dorsal view of a stage 11 chick embryo infected with RCAS-Pitx2c-Enr showed ectopic expression of Pitx2c-Enr in whole embryos using a specific probe to the Drosophila engrailed repressor domain. (B) Ventral view of stage 11 embryo infected with RCAS-Pitx2c-Enr showed a leftward heart looping (arrow). (C) Ventral view of a stage 11 embryo infected with RCAS-Pitx2a-Enr exhibited a normal rightward heart looping (arrow).

DISCUSSION

Pitx2 has been demonstrated to play a crucial role in the development of left-right axis and the dextral looping of the heart. Its left-right asymmetric expressions and regulation by Nodal are highly conserved in vertebrates, including zebrafish, frog, chick and mouse (Capdevila et al., 2000). As a transcriptional factor downstream from the Shh-Nodal signaling pathway, Pitx2 products may execute the left-right developmental program. Several isoforms of *Pitx2* have been isolated from human, mouse, chick, frog and zebrafish, but the distinct roles among these isoforms during embryogenesis are just begun to be elucidated (Essner et al., 2000; Schweickert et al., 2000). In this study, we examined the differential expression patterns of two Pitx2 isoforms, Pitx2a and Pitx2c, during early chick embryogenesis, and analyzed their function in the regulation of left-right development and heart looping.

By whole-mount in situ hybridization using a *Pitx2c*-specific probe, we showed that *Pitx2c* transcripts were detected symmetrically in the head mesoderm and asymmetrically in the LPM with restriction to the left LPM and the left side heart tube in the early chick embryo. However, *Pitx2c* expression was not found in the area opaca and extra-embryonic tissues where *Pitx2* transcripts were detected in the prospective angioblasts of blood islands and the endothelia of the blood vessels using a full-length *Pitx2* probe (St Amand et al., 1998). Using RT-PCR, we were able to confirm the in situ results and showed that *Pitx2a* is not expressed in the left LPM but is expressed in the head and extra-embryonic mesoderm. Thus, *Pitx2c* transcripts may primarily contribute to *Pitx2* expression in the left LPM and left side heart tube. This conclusion is also supported by the observations that no *Pitx2* transcripts could be detected in the left LPM after treatment with antisense oligonucleotides specific to *Pitx2c*. Consistent with our results, it was recently shown that *Pitx2c*, but not *Pitx2a* and *Pitx2b*, was asymmetrically expressed in the left LPM in both mouse and frog (Schweickert et al., 2000). However, it was reported that in the zebrafish, *Pitx2c* is expressed in the left dorsal diencephalon and developing gut while *Pitx2a* is restricted to the left heart primordium (Essner et al., 2000). Thus although

the function of *Pitx2* is conserved through vertebrates, different species may use different *Pitx2* isoforms in the regulation of left-right development.

Being a member of the *bicoid*-like homeobox gene family, *Pitx2* encodes a transcriptional factor that preferentially binds the *bicoid* homeodomain binding site and transactivates reporter genes containing this site (Amendt et al., 1998). In an effort to map the transactivation domains of Pitx2, it was shown that deletion of either N terminus or C terminus of human Pitx2a resulted in a decreased transactivation activity, but none of the deletions abolished transactivation activity completely (Amendt et al., 1999). It has been suggested that the both N-terminal and the C-terminal regions comprise transactivation domains (Amendt et al., 1999). However, by using a GAL4 DNA-binding/activation assay system, we have mapped the transactivation domain of Pitx2 exclusively to the C terminus. N termini from either Pitx2a or Pitx2c did not exhibit any transactivation activity in our assay system. We therefore suggest that the transactivation activity of Pitx2 resides in the C-terminal region that is identical in both Pitx2a and Pitx2c. The different conclusions drawn from previous results (Amendt et al., 1999) and our results are probably due to different assay systems used. Nevertheless, our results indicate that both *Pitx2a* and *Pitx2c* encode transcriptional activators.

It has been demonstrated previously that ectopic expression of *Pitx2* induces reversal of heart looping in the chick and *Xenopus* embryos (Logan et al., 1998; Ryan et al., 1998; Campione et al., 1999). However, in these studies, different *Pitx2* isoforms were not distinguished. In this study we have used both gain-of-function and loss-of-function approaches to examine the potentially distinct roles of these two *Pitx2* isoforms in the regulation of left-right development in term of heart looping direction. Interestingly, when ectopically expressed in the right LPM, both *Pitx2a* and *Pitx2c* equally randomized heart looping directions, despite the fact that *Pitx2c* is predominantly expressed in the left LPM. Similar results were observed in *Xenopus* in which ectopic expression of either *Pitx2a* or *Pitx2c* was able to reverse heart laterality (Essner et al., 2000). This could be explained by the fact that both *Pitx2a* and *Pitx2c* contain an identical C terminus where the transactivation activity resides and may activate same set of downstream genes or execute similar downstream functions, although these downstream genes or functions remain to be identified. In contrast, results obtained from loss-of-function analysis indicate a distinct role for these two isoforms in the regulation of heart looping. Elimination of *Pitx2c* transcripts in the left LPM by specific antisense oligonucleotide randomized heart looping. However, embryos treated with antisense oligonucleotide specific to *Pitx2a* did not exhibit randomization of heart looping. These loss-of-function results were further confirmed by an alternative approach. When misexpressed, Pitx2a-En^r or Pitx2c-En^r, both of which can function as dominant negative forms, would perturb endogenous Pitx2a or Pitx2c function, respectively, by interfering their transcriptional activity. Indeed, Pitx2c-En^r, but not Pitx2a-En^r, was able to randomize heart looping when ectopically expressed in the early embryo. The dominant negative form of *Pitx2a* seems not to interfere with endogenous Pitx2c function. This is probably due to the significant difference in the N terminus of the two isoforms, which may interact with distinct transcriptional factors for specific DNA binding and transactivation activity. Pitx2c-En^r could compete with endogenous Pitx2c for these co-factors more efficiently than Pitx2a-En^r. It was reported that Pitx2 protein can form homodimers (Amendt et al., 1999). Therefore, an alternative explanation for the different effects of the dominant negative forms of Pitx2a and Pitx2c is that Pitx2c-En^r forms dimers with endogenous Pitx2c, and perturbs its function. Together with the differential expression patterns of *Pitx2* isoforms, we conclude that *Pitx2c* plays a predominant role in the regulation of rightward heart looping in developing chick embryo.

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