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Cardiac Expression of the Ventricle-Specific Homeobox Gene *Irx4* Is Modulated by Nkx2-5 and dHand

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We report the isolation and characterization of the cDNAs encoded by the murine and human homeobox genes, *Irx4* (*Iroquois homeobox gene 4*). Mouse and human Irx4 proteins are highly conserved (83%) and their 63-aa homeodomain is more than 93% identical to that of the *Drosophila Iroquois* patterning genes. Human *IRX4* maps to chromosome 5p15.3, which is syntenic to murine chromosome 13. *Irx4* transcripts are present in the developing central nervous system, skin, and vibrissae, but are predominantly expressed in the cardiac ventricles. In mice at embryonic day (E) 7.5, *Irx4* transcripts are found in the chorion and at low levels in a discrete anterior domain of the cardiac primordia. During the formation of the linear heart tube and its subsequent looping (E8.0–8.5), *Irx4* expression is restricted to the ventricular segment and is absent from both the posterior (eventual atrial) and the anterior (eventual outflow tract) segments of the heart. Throughout all subsequent stages in which the chambers of the heart become morphologically distinct (E8.5–11) and into adulthood, cardiac *Irx4* expression is found exclusively in the ventricular myocardium. *Irx4* gene expression was also assessed in embryos with aberrant cardiac development: mice lacking *RXRα* or *MEF2c* have normal *Irx4* expression, but mice lacking the homeobox transcription factor *Nkx2-5* (*Csx*) have markedly reduced levels of *Irx4* transcripts. *dHand*-null embryos initiate *Irx4* expression, but cannot maintain normal levels. These data indicate that the homeobox gene *Irx4* is likely to be an important mediator of ventricular differentiation during cardiac development, which is downstream of Nkx2-5 and dHand.

Key Words: **cardiac development; homeobox gene; patterning.**

INTRODUCTION

The molecular signals that specify atrial or ventricular differentiation of primordial myocardial cells are largely unknown. Early in vertebrate development, bilateral regions of the splanchnic lateral plate mesoderm fuse to create a single linear heart tube (Olson and Srivastava, 1996; Fishman and Chien, 1997). This heart tube can be considered a series of domains, oriented from anterior to posterior, in which cells are fated to become the outflow tract (conus and conotruncus), right ventricle, left ventricle, common atrium, and sinus venosa (Yutzey and Bader, 1995; Mikawa and Fischman, 1996; Olson and Srivastava, 1996; Fishman and Chien, 1997). Cardiac looping displaces this anteroposterior conformation, and with further differentiation the atria and ventricles become morphologically distinct, creating four interconnected chambers.

Atrial and ventricular cells have unique ultrastructural, contractile, electrophysiologic, metabolic, and endocrine properties (McNutt and Fawcett, 1969; de Bold, 1985; Hume and Uehara, 1985; Bass *et al.*, 1993; Pawloski-Dahm *et al.*, 1998). These differences have been conserved throughout vertebrate evolution (Fishman and Chien, 1997; Fishman and Olson, 1997) presumably to enable the specialized roles each chamber plays in heart function (Fishman and Olson, 1997; Chen *et al.*, 1998; Fewell *et al.*, 1998; Pawloski-Dahm

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et al., 1998). Specification of atrial and ventricular chambers may be predicated on the regional expression of transcription factors that regulate promoter activity of genes expressed in particular compartments (Seidman *et al.*, 1988; Ross *et al.*, 1996; Wang *et al.*, 1996, 1998; Colbert *et al.*, 1997). Previously studied cardiac transcription factors are found throughout the developing heart and/or exhibit dynamic expression, characteristics that may be inconsistent for the establishment and maintenance of distinct atrial or ventricular phenotypes (Komuro and Izumo, 1993; Lints *et al.*, 1993; Edmondson *et al.*, 1994; Biben and Harvey, 1997; Srivastava *et al.*, 1997; Zou *et al.*, 1997; Dunwoodie *et al.*, 1998; Firulli *et al.*, 1998; Thomas *et al.*, 1998; Bruneau *et al.*, 1999).

Homeodomain-containing transcription factors of the *Iroquois* (*IRO*) family participate in patterning and tissue specificity in invertebrates (*Drosophila*) and vertebrates (*Xenopus*). *Drosophila IRO* genes *araucan* (*ara*) and *caupolican* (*caup*) define the prepattern of neural and vein precursors and regulate positional identity of sensory neurons (Gomez-Skarmeta *et al.*, 1996; Grillenzoni *et al.*, 1998). The third member of this family, *mirror* (*mirr*), defines the equator of the eye and participates in early embryonic segmentation (McNeill *et al.*, 1997). *mirr, ara,* and *caup* also act cooperatively in sensory organ and alula formation (Kehl *et al.*, 1998; Netter *et al.*, 1998). *Xenopus Xiro* genes are expressed in the early developing nervous system and appear to be involved in specifying neuronal precursors (Bellefroid *et al.*, 1998; Gomez-Skarmeta *et al.*, 1998). We have recently identified in chicken a new member of the *IRO* family, *Irx4*, that is expressed mainly in the ventricles of the developing heart and which is involved in regulating ventricular identity (Bao *et al.*, 1999).

To further define the role of Irx4 in cardiac development, we have cloned and characterized mouse and human *Irx4*. Analyses of the expression of *Irx4* during normal and aberrant cardiac embryogenesis indicate this homeobox gene demarcates cells destined for the ventricular compartment. We suggest that Irx4 signals ventricular cell specification and participates in ventricular chamber morphogenesis under the direction of a cardiogenic program regulated by Nkx2-5 and dHand.

METHODS

Isolation of Mouse and Human Irx4 cDNAs

A mouse embryonic day (E) 10 heart cDNA library (Stratagene) was screened by low-stringency hybridization using chicken *Irx4* (*cIrx4*) (Bao *et al.*, 1999). Partial sequences were obtained from several cDNAs. One of these had significant homology to *cIrx4* and was fully sequenced. Partial human *IRX4* cDNAs were isolated by hybridization of a human embryonic heart cDNA library (Clontech) with the mouse *Irx4* cDNA. Further sequence was obtained by performing reverse-transcriptase-mediated polymerase chain reaction (PCR) on human ventricle RNA using primers designed from the partial *IRX4* cDNAs. The 5′ end of the *IRX4* cDNA coding region was obtained by isolating a bacterial artificial chromosome (BAC) by PCR from a human genomic BAC library (Research Genetics) using the following primers, which are specific to *IRX4* and span an intron: HIRX4(116f), 5′-GCCGCTGCCGCCTACTACCCTTAC-3′; HIRX4(242r), 5′-AGGCCTTGAGCGTGCTGGTGGTCT-3′. Subclones were isolated and sequenced, using an automated sequencer (ABI).

Chromosomal Localization of IRX4

A CEPH-B yeast artificial chromosome (YAC) array (Research Genetics) was screened by PCR using the primers described above for the BAC screening. Hamster–human somatic cell hybrids encompassing entire chromosomes (National Institute of General Medical Sciences) and the Stanford G3 radiation hybrid panel (Research Genetics) were also screened by PCR.

Animals

Mice (Swiss Webster and C57Bl/6) were obtained from Taconic Farms, Inc. *Nkx2-5*$^{-/-}$ mice were generated as described (Tanaka *et al.*, 1999) and lack sequences encoding the entire homeobox protein. *Nkx2-5*$^{-/-}$ homozygous animals were generated by crosses of heterozygous mice. *RXRα*$^{-/-}$ mouse embryos, generously provided by Dr. H. M. Sucov (University of Southern California School of Medicine) were obtained by mating *RXRα*$^{+/-}$ mice as previously described (Sucov *et al.*, 1994). *Mef2c*$^{-/-}$ and *dHand*$^{-/-}$ embryos, generously provided by Dr. Eric Olson (University of Texas, Southwestern Medical School) were generated as described (Lin *et al.*, 1997; Srivastava *et al.*, 1997). Genotyping was performed by PCR on embryo tails or yolk sacs as previously described (Sucov *et al.*, 1994; Lin *et al.*, 1997; Srivastava *et al.*, 1997; Tanaka *et al.*, 1999). All animals were maintained according to institutional guidelines.

In situ Hybridization

Mouse embryos were harvested at various stages of development from timed matings between C57Bl/6 males and Swiss Webster females. Noon of the day of the vaginal plug was considered E0.5. Embryos were staged according to Kaufman (1992). Extraembryonic membranes were removed, and for embryos E9 and older, the brain was perforated and in most cases the pericardium was removed. Embryos were fixed overnight in 4% paraformaldehyde in PBS and dehydrated in methanol. Whole-mount *in situ* hybridization was performed on mouse embryos as previously described (Riddle *et al.*, 1993), except that mouse embryo powder was used to block the antibodies. All reagents were purchased from Boehringer Mannheim Biochemicals. For two-color whole-mount *in situ* hybridizations, fluorescein- and digoxigenin-labeled riboprobes were hybridized simultaneously to mouse embryos. The fluorescein-labeled probe was detected first using an anti-fluorescein alkaline phosphatase (AP)-coupled antibody and BCIP as substrate for the AP. Following color detection, embryos were fixed with 4% paraformaldehyde in PBS, and AP activity was destroyed by a 1-h incubation at 70°C. The digoxigenin-labeled probe was then detected as per the original protocol, using NBT/BCIP as the chromogenic substrate. Embryos were viewed using a dissecting microscope (Nikon). *In situ* hybridization was performed on sections of paraffin-embedded tissues as described (Tessarollo *et al.*, 1992; Tanaka *et al.*, 1999), using ^{33}P- or ^{35}S-labeled probes. Sections were counterstained with 0.1% toluidine blue or hematoxylin and eosin. Radioactive sections were viewed with a Zeiss Axiophot microscope using transmitted light to view the tissue morphology and

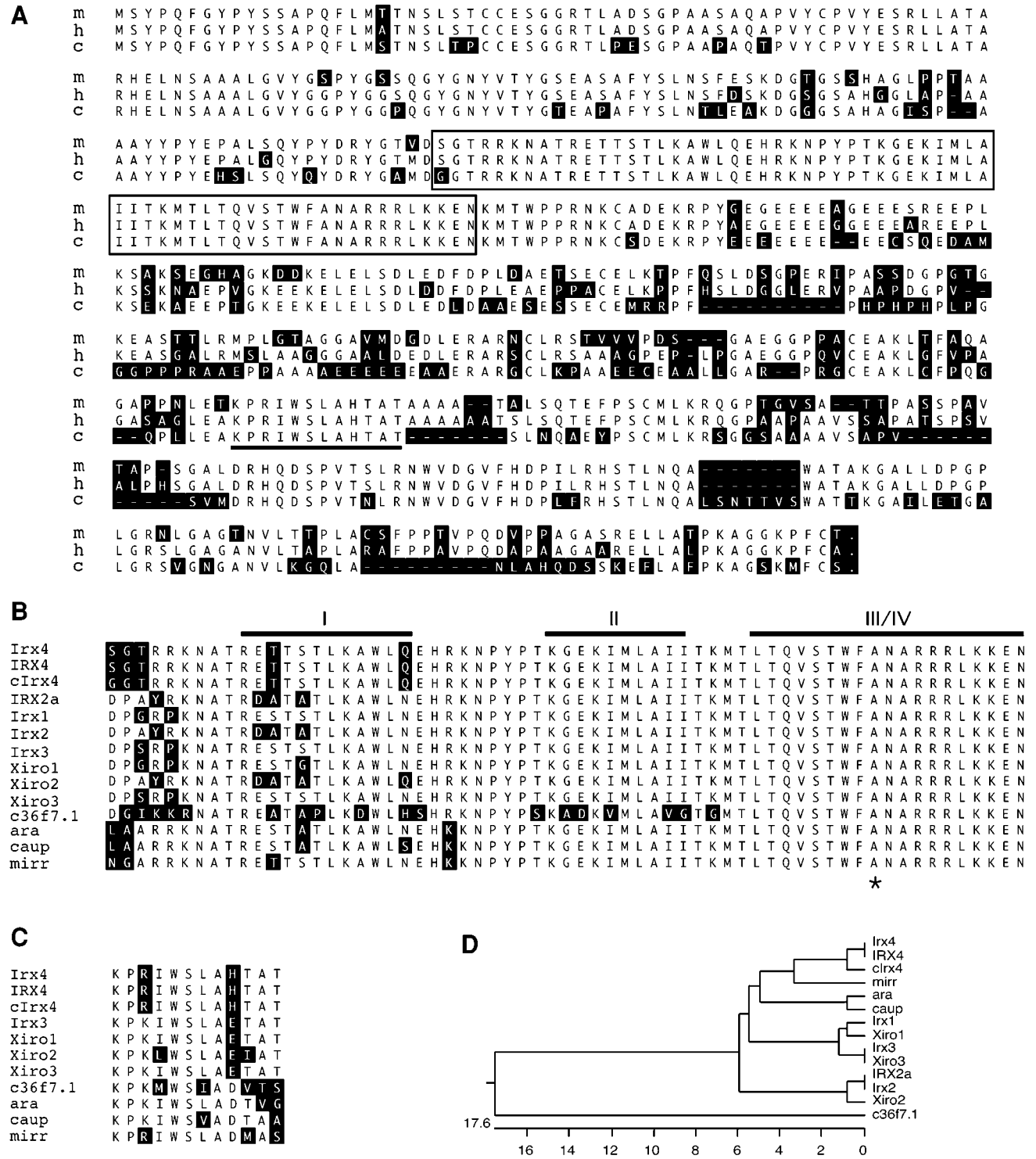
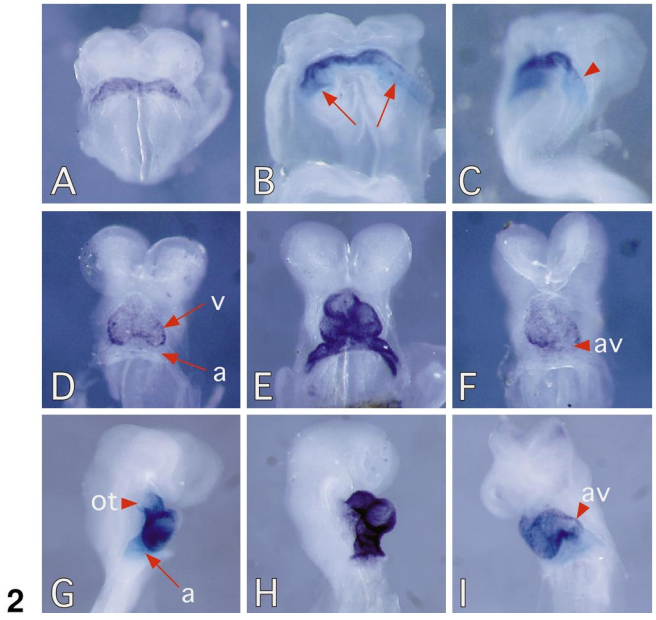


FIG. 1. Irx4 sequence and evolutionary conservation. (A) Alignment of mouse (m), human (h), and chicken (c; GenBank Accession No. AF091504) Irx4 amino acid sequences. Residues differing from the consensus are shaded and the homeodomain (boxed) and Iro box (underlined) are indicated. (B) Alignment of homeodomains found in IRO gene family members: human IRX2a (U90304); mouse Irx1 (Y15002), Irx2 (Y15000), Irx3 (Y15001); *Xenopus* Xiro1 (AJ001834), Xiro2 (AJ001835), Xiro3 (AF027175); *Caenorhabditis elegans* c36f7.1 (Z81045); and *Drosophila* araucan (X95179), caupolican (X95178), and mirror (U95021) (GenBank accession number provided in parentheses). Residues differing from the consensus sequence are shaded. The predicted location of the α helices (bars) and the alanine conserved in all IRO genes (asterisk) are shown. (C) Alignment of IRO box sequences from mouse, human, chicken, *Xenopus*, *C. elegans*, and *Drosophila* IRO genes. Residues differing from the consensus sequence are shaded. (D) Phylogenetic analysis of the IRO homeodomains. Sequences were analyzed in DNAStar using the Megalign software and the Clustal method. Mouse *Irx4* and human *IRX4* sequences have been deposited with GenBank under Accession Nos. AF124732 and AF124733, respectively.



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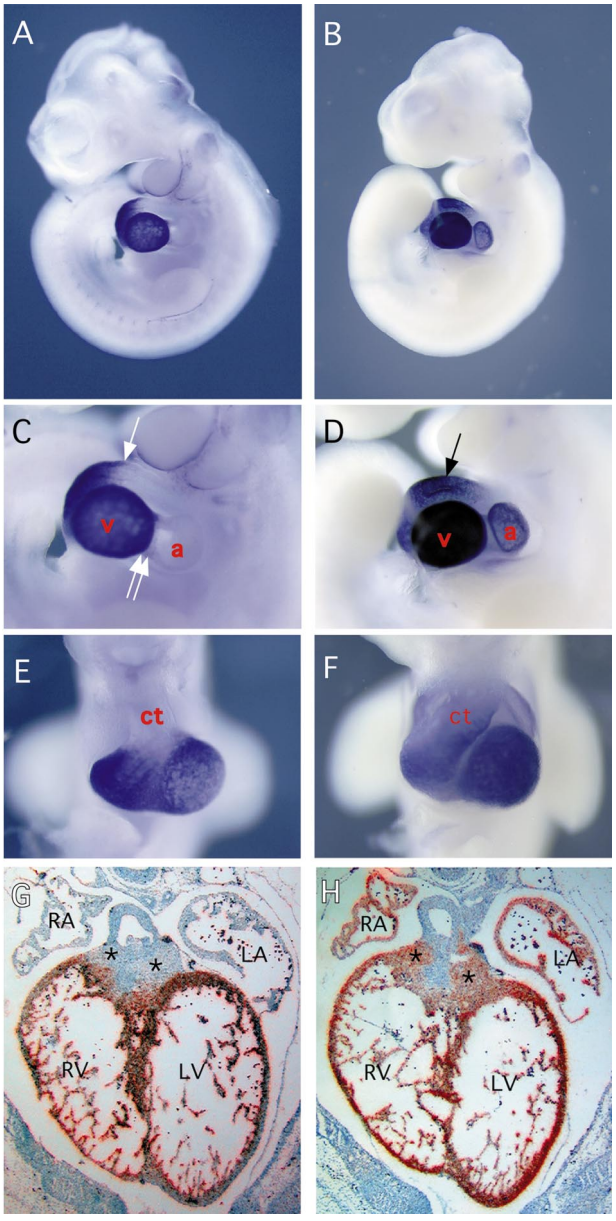

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FIG. 2. *Irx4* and *cMybp-C* expression in the early mouse embryo detected by *in situ* hybridization. (A) The *Irx4* probe (dark blue) labels the cardiac crescent of an E8 embryo (frontal view). (B) At the same stage as in (A) double *in situ* hybridization with *Irx4* and *cMybp-C* (turquoise) shows that *Irx4* expression is restricted to the anterior portion but absent from the posterior portion of the cardiac crescent (arrows). (C) A lateral view of the embryo pictured in (B). Arrowhead indicates the posterior border of *Irx4* expression. (D) At E8.5 (4 somites) *Irx4* is expressed in the ventricular (v) segment but absent from the posterior atrial (a) segment and the anterior outflow tract of the heart tube. (E) *cMybp-C* expression is found in all cardiocytes of the heart tube. (F) *Irx4* expression remains restricted to ventricular cells, above the atrioventricular (av) junction in the looped heart (E8.5, 7 somites). (G, H, and I) *In situ* hybridization of embryos following completion of heart looping (E8.5, 10–12 somites) shows that *cMybp-C* is expressed throughout the heart (turquoise in G and I; blue in H). Double labeling with *Irx4* (G and I) shows Irx4 expression in the atrioventricular junction but not the distal outflow tract (ot).

FIG. 3. Expression of *Irx4* (A, C, E, and G) and *cMybp-C* (B, D, F, and H) during the maturation of the developing heart. (A and B) Cardiac tissue exhibits the most abundant expression of *Irx4* (A) and *cMybp-C* (B) in the E10.5 mouse embryo. (C and D) Higher magnification of the cardiac region of embryos in (A) and (B), respectively, shows the boundaries of *Irx4* expression between the RV and outflow tract (single arrow) and the atrioventricular junction (double arrows). *cMybp-C* is expressed in the atria (a) and ventricle (v) and anteriorly into the outflow tract (single arrow). (E and F) Frontal views of the same embryos, showing a clear demarcation of *Irx4*, but not *cMybp-C* expression, between right ventricle and conotruncus (ct). (G and H) Radioactive *in situ* hybridization of adjacent transverse sections of a E13.5 mouse embryo shows restricted *Irx4* (G) and unrestricted *cMybp-C* (H) cardiac expression. Asterisks indicate the outflow tract myocardium.

refracted red light to view the radioactive signals or with dark-field illumination. Probes for *Irx4* were derived from an *Xba*I/*Sac*I fragment of the *Irx4* cDNA that does not contain the homeodomain. The probes for mouse *cardiac myosin binding protein-C* (*cMybp-C*) were transcribed from a partial (2.4-kb) *cMybp-C* cDNA (McConnell *et al.*, 1999). The probes for *α-cardiac actin* and *myosin light chain 2v* (*MLC-2v*) were as described (Tanaka *et al.*, 1999). All cDNAs were subcloned in pBluescript II SK(+) (Stratagene) and riboprobes were transcribed using T7 or T3 RNA polymerases.

Northern Blot Analysis

Total RNA was extracted from mouse embryos or tissues using Trizol reagent (Life Technologies). Fifteen micrograms of RNA per sample were size-fractionated and blotted to nylon membranes (Ausubel *et al.*, 1989). Hybridization was performed using ^{32}P-labeled DNA probes (Ausubel *et al.*, 1989). The mouse *Irx4* probe was a *Xba*I/*Sac*I fragment isolated from the full-length *Irx4* cDNA. A cDNA encoding a portion of *glyceraldehyde phosphate dehydrogenase* (*GAPDH*) served as a loading control probe.

RESULTS

Evolutionary Conservation of Irx4 Sequences

Mouse and human *Irx4* genes encode 515 and 519 amino acid proteins, respectively (Fig. 1A). Initiation codons for each gene were assigned based on favorable translational start sites (Kozak, 1996) that were directly preceded by in-frame stop codons. Both proteins are predicted to contain 63 amino acids that are homologous to homeodomains present in the TALE superclass of proteins (Burglin, 1997). Alignment of chick, mouse, and human Irx4 amino acid sequences indicates that these have been highly conserved throughout vertebrate evolution (Fig. 1A). The deduced amino acid sequences of mouse and human Irx4 are 83% similar; mouse and chicken Irx4 are 72% similar. Alignment with other mouse, *Xenopus*, and *Drosophila* IRO genes (Figs. 1B and 1C) show marked conservation of the homeodomain and of the IRO box, a 12-amino-acid motif of unknown function. Typical of TALE homeodomains is the presence of a small nonpolar residue at position 50, instead of the polar residue found in all other homeodomain-containing proteins (Burglin, 1997). An alanine is present at this position in Irx4 (asterisk in Fig. 1B) as in all other IRO genes, and the homeodomain is followed by acidic amino acid residues, the putative activation domain (Gomez-Skarmeta *et al.*, 1996, 1998; Bosse *et al.*, 1997; McNeill *et al.*, 1997; Bellefroid *et al.*, 1998). Phylogenetic analysis of the complete homeodomains of all IRO family genes isolated to date is shown in Fig. 1D.

The Human IRX4 Genomic Locus

The chromosomal location of the human *IRX4* gene was assigned to chromosome 5 by screening whole-chromosome hamster–human somatic cell hybrids (Methods and data not shown). Mapping was independently confirmed by screening the Stanford G3 radiation hybrid panel, resulting in a LOD score greater than 6.0 with marker *D5S678*, which is located on 5p15.3–ptel (http://www.ncbi.nlm.nih.gov/genemap). The *IRX4* gene was also identified to be encoded on the chimeric YAC 922E4, which contains STSs from chromosomes 4 (*WI-4413*) and 5 (*D5S392*) and less than 1 Mb of human DNA. We conclude that *IRX4* is located on chromosome 5p15.3, near *D5S392* and *D5S678*.

Cardiac Expression of Irx4 in Mouse Embryonic Development

Irx4 expression during development was assessed by whole-mount *in situ* hybridization on mouse embryos and compared to the expression pattern of *cMybp-C*, a sarcomere component that is ubiquitously found in cardiac myocytes. Unlike previously studied members of the IRO gene family, the main site of *Irx4* expression is the developing heart. At E7.5, when cardiac differentiation has begun in advance of heart tube formation, *Irx4* is weakly expressed in the bilateral primordia (not shown). As the cardiac crescent forms, cardiac *Irx4* expression increases (Fig. 2A). Therefore, the onset of Irx4 expression follows cardiac differentiation. Double *in situ* hybridization with an *Irx4* probe and another encoding cMybp-C reveals *Irx4* transcripts only in the anterior portion of the cardiac crescent (Figs. 2B and 2C). *Irx4* expression is absent from the anterior and posterior poles of the linear heart tube (Fig. 2D). Soon after looping is initiated (E8.5), the atrial and ventricular portions of the heart tube begin to be anatomically separated. During this stage and throughout the looping process, *Irx4* is restricted to the ventricular segments of the heart, including the atrioventricular (AV) junction and the posterior portion of the bulbus cordis, which is destined to become the right ventricle (RV) (Figs. 2F, 2G, and 2I). *Irx4* remains absent from the most anterior positioned cardiocytes, which will become the outflow tract (cf. Figs. 2F and 2G). Ventricular expression of *Irx4* is more pronounced at E10.5, when all chambers of the heart have become more clearly defined (Figs. 3A, 3C, and 3E). While *cMybp-C* expression is evident in myocytes located in all four chambers and the outflow tract (Figs. 3B, 3D, and 3F), right and left ventricular expression of *Irx4* abruptly stops at the AV junction and at the boundary of the RV and outflow tract. These expression patterns persist in the E13.5 embryo (Fig. 3G) and neonatal heart (Fig. 4A). In the E13.5 embryo, a gradient of *Irx4* is evident, with greater expression in endocardial than in epicardial cells (Fig. 3G; cf. *cMybp-C* expression in Fig. 3H). In the neonatal heart, *Irx4* expression is homogeneous throughout the ventricular myocardium (Fig. 4A). Analysis of adult mouse tissues by Northern analysis reveals continued high level ventricular expression of *Irx4* (Fig. 4B).

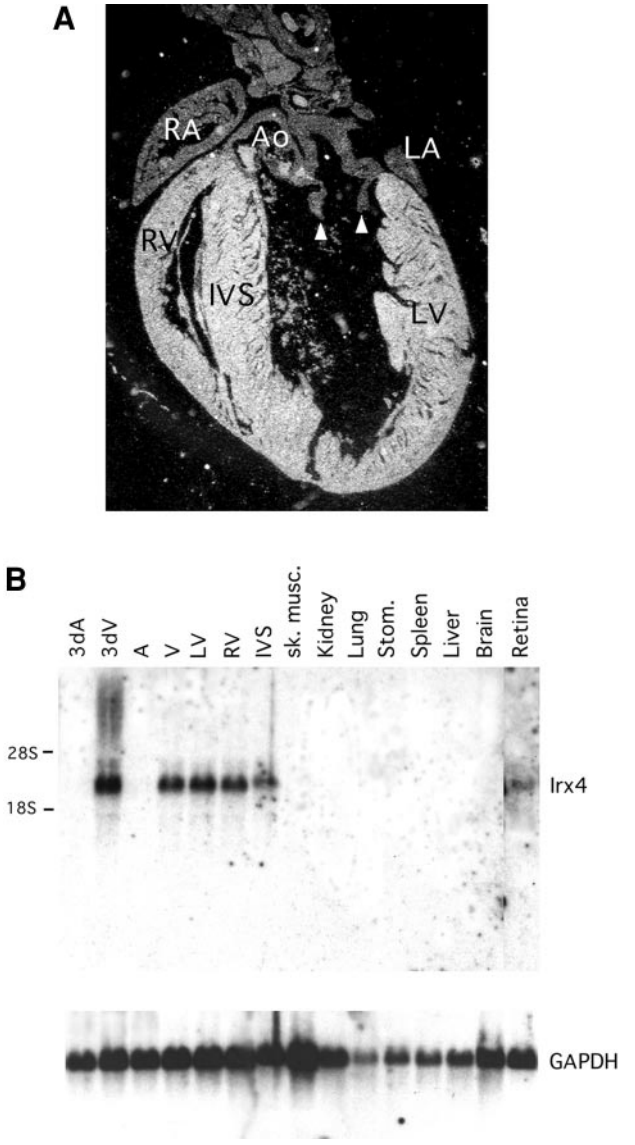


FIG. 4. Postnatal *Irx4* expression. (A) *In situ* hybridization of *Irx4* on a longitudinal section of a 3-day-old neonatal mouse heart. Arrowheads indicate mitral valve leaflets. Ao, aorta; IVS, interventricular septum; LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle. (B) Northern blot hybridization of total RNA (15 μg) from neonatal (3-day-old; 3d) atria (A) and ventricles (V) and adult tissues: left ventricle (LV), right ventricle (RV), interventricular septum (IVS), skeletal muscle (sk. musc.), kidney, lung, stomach (Stom.), spleen, liver, brain, and retina probed with *Irx4* and *GAPDH*. The lane containing retinal RNA was exposed twice as long as other lanes.

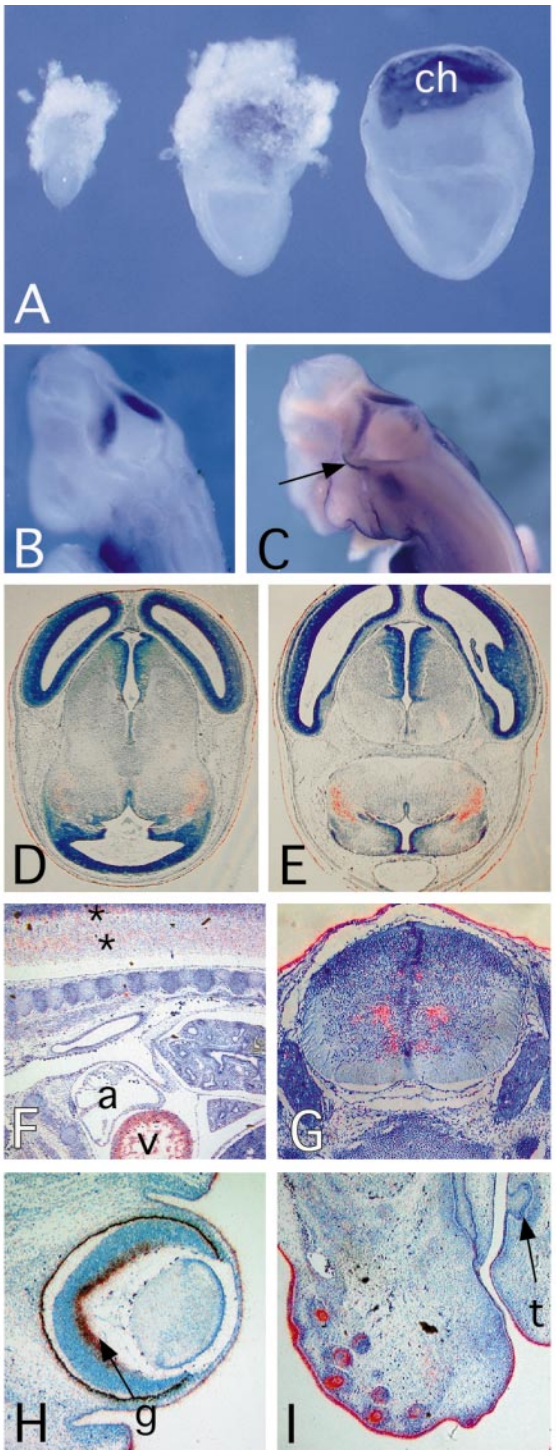
Extracardiac Expression of Irx4

Irx4 is also expressed at extracardiac sites during embryogenesis. *Irx4* transcripts are present in the chorion, beginning at E7 (Fig. 5A) and at E8.75, expression is detectable in the rostral rhombic lip of the developing hindbrain (Figs. 2I, 5B, and 5C). Later expression (E10.5) is also strong in neurons that will contribute to the pons. *Irx4* expression in the surface ectoderm is evident at this stage and is more pronounced in the ectoderm surrounding the branchial arches (Fig. 5C). In E13.5 embryos, expression in the CNS is localized to specific nuclei of the pons (Figs. 5D and 5E), in the spinal cord (Figs. 5F and 5G), and in the ganglion cell layer and pigmented epithelium of the retina (Fig. 5H). At E13.5, the ectodermal expression is manifested in keratinizing epithelium and vibrissae (Fig. 5I).

Irx4 Expression during Abnormal Cardiac Development

The vertebrate *tinman* homolog *Nkx2-5* (also known as *Csx*) is expressed throughout the heart, from the earliest stage of cardiac differentiation into adulthood (Komuro and Izumo, 1993; Lints *et al.*, 1993; Kasahara *et al.*, 1998). Mice lacking a functional *Nkx2-5* gene have embryonic defects in heart formation that include improper myocardial growth and differentiation (Lyons *et al.*, 1995; Tanaka *et al.*, 1999). Further, the normal ventricular (but not atrial) levels of transcripts for the genes encoding myosin light chain (MLC)-2v, atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), and SM22α are reduced in *Nkx2-5*$^{-/-}$ mice (Lyons *et al.*, 1995; Biben *et al.*, 1997; Tanaka *et al.*, 1999). We hypothesize that a ventricle-specific developmental program is disrupted in the absence of Nkx2-5. To determine if *Irx4* expression is dependent on Nkx2-5 in the ventricles, we analyzed *Irx4* expression in *Nkx2-5*$^{-/-}$ mouse embryos (Figs. 6A–6G). By whole-mount *in situ* hybridization or hybridization of embryo sections, *Irx4* is markedly decreased in hearts of *Nkx2-5*$^{-/-}$ mouse embryos ($n = 5$), although weak remnant expression remains in small patches localized mainly to the ventrolateral aspect of the ventricle. Hindbrain expression of *Irx4* is normal in *Nkx2-5* mutants, indicating a selective down-regulation of *Irx4* transcription in the heart. The down-regulation of *Irx4* in the *Nkx2-5*$^{-/-}$ mouse embryos does not appear to be due to decreased proliferation of ventricular cardiocytes, as *MLC-2v* expression is maintained throughout the myocytes derived from the ventricles (Fig. 6 and Tanaka *et al.*, 1999).

The bHLH transcription factor dHand (also known as Hand2/Hed/Thing-2) has been shown to be important for cardiac morphogenesis (Srivastava *et al.*, 1995, 1997). Mice lacking *dHand* fail to form a right (pulmonary) ventricle (Srivastava *et al.*, 1997; Thomas *et al.*, 1998). We examined *Irx4* expression in *dHand*$^{-/-}$ embryos and found transcript in the remnant left ventricle, albeit at considerably lower levels than wild-type controls (Fig. 7B). By E10, *Irx4* expression is not detectable above background levels in *dHand*$^{-/-}$ embryos (Fig. 7D). Cardiac expression of *MLC2v* was intact in E10 *dHand*$^{-/-}$ embryos (Fig. 7F), as was *Irx4* expression in the hindbrain (Fig. 7D), indicating that the down-regulation of *Irx4* is not due to general embryonic demise or impaired cardiac differentiation. We conclude that *Irx4* expression



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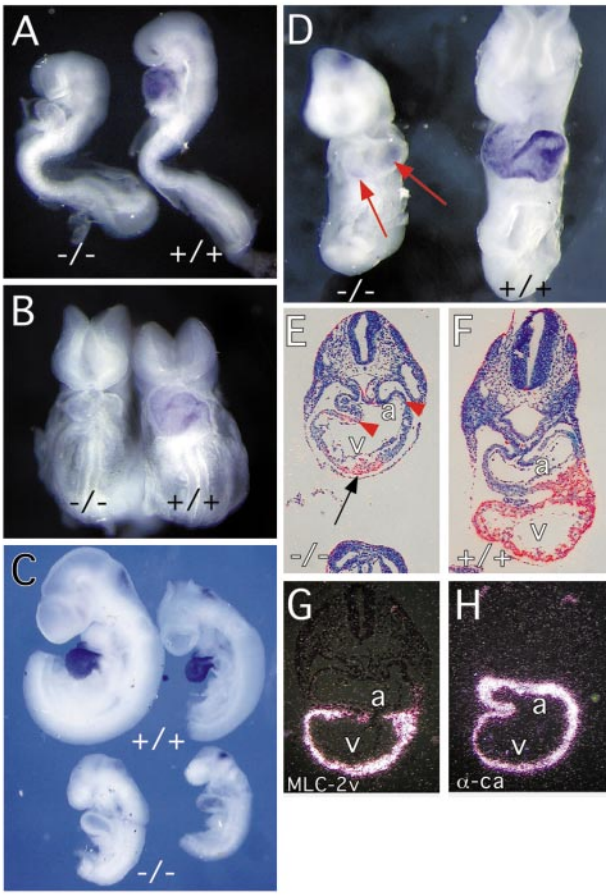

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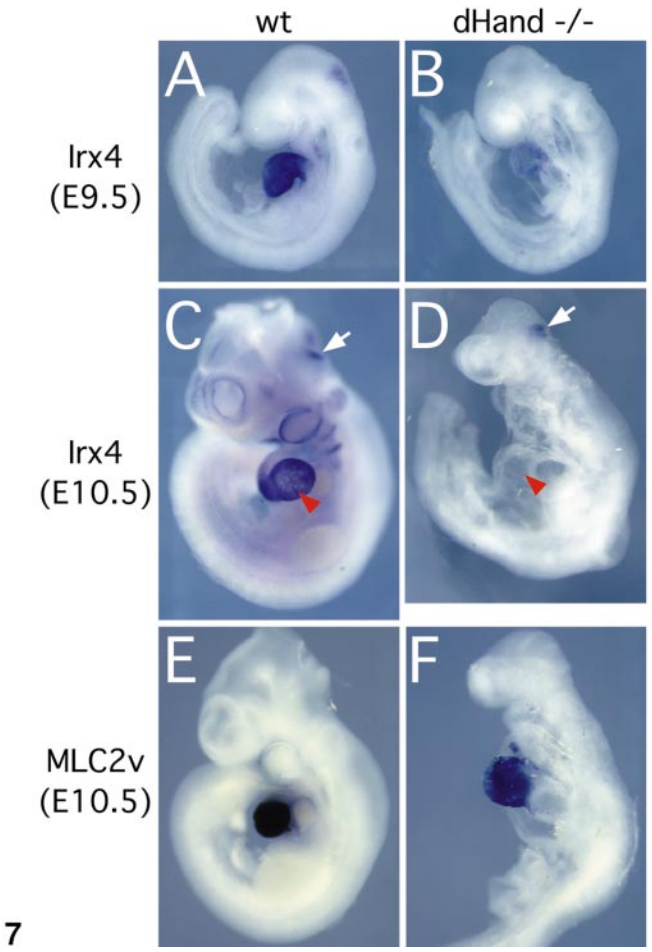
FIG. 5. Extracardiac *Irx4* expression in the mouse embryo detected by *in situ* hybridization. Irx4 expression is a blue color in A–C and a red signal in D–I. (A) Mouse embryos at E6.5, 7.0, and 7.5 (left to right) show increasing *Irx4* expression in the chorion (ch). (B) *Irx4* expression in the rostral rhombic lip is evident at E9.5. (C) At E11 *Irx4* is still expressed in the rhombic lip and is detectable in neurons that will contribute to the pons (arrow). (D and E) Transverse sections (D is more rostral than E) of E13.5 mouse embryos showing *Irx4* expression in discrete nuclei of the pons. (F) Parasagittal section at E13.5 shows *Irx4* expression in the spinal cord (asterisks) and cardiac ventricle (v) but not atria (a). (G) Spinal cord, transverse section. (H and I) *Irx4* is also expressed at E13.5 in the ganglion cell layer (g) of the retina (H) and in skin and vibrissae (I), but not in developing tooth bud (t).

FIG. 6. *Irx4* expression is regulated by Nkx2-5. (A) Whole-mount *in situ* hybridization of *Irx4* in *Nkx2-5*-deficient (−/−) and wild-type (+/+) littermate embryos at E8.5. (B) Frontal view of embryos in (A). (C) Attenuated expression of *Irx4* in *Nkx2-5*$^{-/-}$ embryos at E9.5. (D) Frontal view of two embryos pictured in (C). Arrows indicate remnant cardiac expression of *Irx4* in *Nkx2-5*-deficient embryos. Note that appropriate levels of *Irx4* are apparent in the developing hindbrain. (E–H) *In situ* hybridization of transverse sections from E9.5 embryos. (E) Foci of ventricular expression of *Irx4* (arrow) in an *Nkx2-5*$^{-/-}$ embryo compared to expression in wild-type (F) embryos. Arrowheads indicate the atrioventricular junction; a, atria; v, ventricle. (G and H) Expression of *α-cardiac actin* (α-ca) and *MLC-2v* in E9.5 *Nkx2-5*$^{-/-}$ embryos (dark-field view).



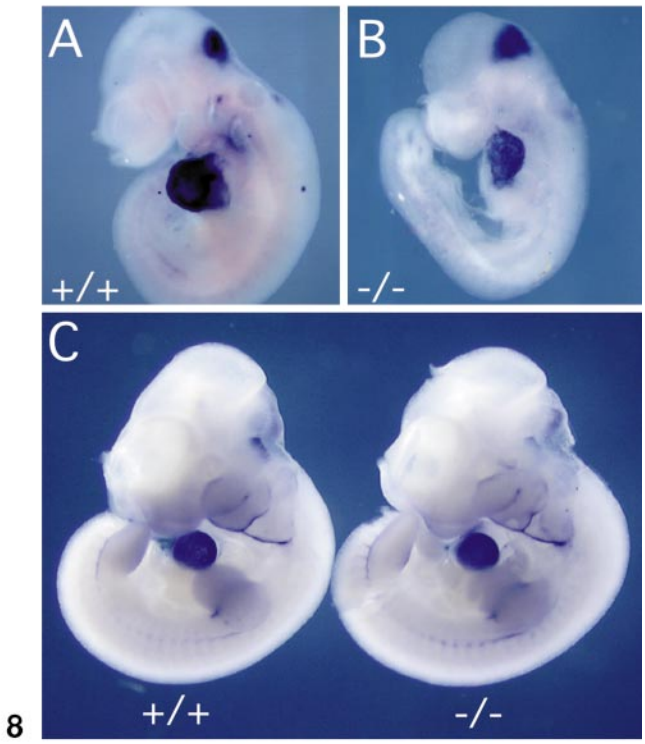


FIG. 7. *Irx4* expression is regulated by dHand-dependent pathways. (A–D) *Irx4* expression in wild-type (wt) embryos at E9.5 (A) and E10 (C) and in $dHand^{-/-}$ littermates (B and D). *Irx4* is weakly expressed in an E9.5 $dHand^{-/-}$ embryo (B) and is undetectable in the heart of an E10 $dHand^{-/-}$ embryo (D). Red arrowheads point to the left ventricle; white arrows indicate intact hindbrain expression in both wt and $dHand^{-/-}$ embryos. Apparent staining in the forebrain of the embryo pictured in (C) is due to probe trapping. *MLC2v* expression is unaffected in an E10 $dHand^{-/-}$ embryo (F); wild-type littermate is pictured in (E).

FIG. 8. *Irx4* expression is unaffected in $Mef2c^{-/-}$ and $RXR\alpha^{-/-}$ embryos. (A) *Irx4* expression in wild-type (+/+) embryos at E9.5 and in a $Mef2c^{-/-}$ littermate (B). (C) Whole-mount *in situ* hybridization of $RXR\alpha^{-/-}$ (−/−) and wild-type (+/+) embryos (E11) shows normal *Irx4* expression in both.

must be regulated in part by the morphogenetic program that is disrupted in $dHand^{-/-}$ embryos.

The Mef2 family of transcription factors is involved in regulating muscle-specific transcription (Black and Olson, 1998). $Mef2c^{-/-}$ embryos have severe defects in cardiogenesis; most notably these lack a right ventricle, and the expression of *ANF*, *MLC1a*, and *dHand* is reduced (Lin *et al.*, 1997). The similarity of phenotypes between the $Mef2c^{-/-}$ and the $dHand^{-/-}$ embryos, as well as the down-regulation of dHand in the $Mef2c^{-/-}$ hearts, suggests that Mef2c regulates *dHand*. We hypothesized that Mef2c might also indirectly regulate *Irx4* in the developing heart. *Irx4* expression was not affected in $Mef2c^{-/-}$ embryos (Fig. 8), indicating that a Mef2c-dependent pathway is not essential for cardiac expression of *Irx4*.

Mice lacking a functional *RXRα* gene have defects in cardiac development that result in hypoplastic ventricles (Kastner *et al.*, 1994; Sucov *et al.*, 1994; Dyson *et al.*, 1995). The ventricles of $RXR\alpha^{-/-}$ mice express the predominantly atrial gene *MLC2a* at levels above those found in wild-type animals, suggesting that they have acquired a phenotype resembling that of the atria (Dyson *et al.*, 1995). To determine if *Irx4* expression is regulated by RXRα-dependent pathways, we examined $RXR\alpha^{-/-}$ embryos at E11 (Fig. 8C). Ventricular expression of *Irx4* was unchanged in $RXR\alpha^{-/-}$ embryos, indicating that *Irx4* is not part of the RXRα-dependent transcriptional cascade that regulates *MLC2a* expression.

DISCUSSION

Irx4 is a novel homeobox gene of the *IRO* family of transcription factors that is restricted to the ventricular

compartment of the heart at all stages of development. Although also expressed in several other tissues during embryogenesis, the pronounced and highly selective location of *Irx4* transcripts in the heart implies a critical role for this transcription factor in specifying development and patterning of the ventricular myocardium.

Outside of the heart, *Irx4* is expressed is the developing chorion, hindbrain, skin, and retina. While *Irx4* functions in these tissues are largely unknown, several IRO genes have recently been identified with overlapping patterns of expression in the developing nervous system of the mouse (Bosse *et al.*, 1997) and frog (Bellefroid *et al.*, 1998; Gomez-Skarmeta *et al.*, 1998). CNS expression of *Irx4* is quite distinct from that of other vertebrate IRO genes and discretely localized to the hindbrain, spinal cord, and ganglion cell layer of the neural retina. Retinal expression of *Irx4* is restricted to the ganglion cell layer and pigment epithelium, possibly indicating a role for this gene in promoting differentiation of specific retinal cell types, as has been seen for other homeodomain genes such as the photoreceptor-specific gene *Crx* (Furukawa *et al.*, 1997).

In the heart, *Irx4* is the first cardiac transcription factor to be identified with early and exclusive expression in ventricular myocytes. The regulation of chamber-specific gene expression is not entirely conserved between avians and mammals (Lyons *et al.*, 1990; Yutzey *et al.*, 1994; Srivastava *et al.*, 1995, 1997; Biben and Harvey, 1997). The ventricle-restricted pattern of *Irx4* found in mouse is almost identical to that observed in chick (Bao *et al.*, 1999), with a slight difference being that *Irx4* appears earlier in the developing mouse heart than its chicken counterpart. This presumably indicates evolutionary conservation of important developmental functions. While these functions remain to be precisely defined, *Irx4* provides a novel molecular marker for identifying primordial ventricular cells and tracking their migration during cardiac morphogenesis. Our studies identified restricted expression of *Irx4* in the anterior portion of the cardiac crescent, a finding that indicates that anteroposterior segmentation and specific myocyte differentiation pathways are initiated before heart tube formation. The regionalized expression of *Irx4* occurs prior to chamber specificity of most cardiac genes (Zeller *et al.*, 1987; Lyons *et al.*, 1990; Kubalak *et al.*, 1994), with the sole exception of *MLC2v*, which appears to be regionalized at the same time as *Irx4*. Although previous explantation experiments in chicken (Yutzey *et al.*, 1995) suggested atrial and ventricular cell specification as early as gastrulation, compartments defined at this stage contain cells with a large degree of phenotypic plasticity (Satin *et al.*, 1988; Yutzey *et al.*, 1994). Chamber specificity of gene expression appears to be fixed later, occurring at least by E10.5 (Gruber *et al.*, 1998).

Irx4 expression also clearly demarcates cells that contribute to the RV versus the conus, thus revealing an early distinction between cells contained within the ventricular myocardium and those of the future outflow tract. Recent observations in transgenic mice support the presence of this boundary, in that a *MLC1/3f* enhancer promoted reporter gene expression in the ventricle, but not in the conus (Franco *et al.*, 1997). We hypothesize that demarcation of the boundaries of these regions may be important for regulation of the temporal expression of molecules such as *MLC1v* and *MLC2v*. Both of these are initially expressed in the ventricles and outflow tract, but later in development become excluded from the outflow tract (Lyons *et al.*, 1990; O'Brien *et al.*, 1993). Dynamic regulation of gene expression in these regions may be important for maturation of the outflow tract.

If *Irx4* shares the patterning functions of other *IRO* genes, its role in the ventricular compartment of the heart may be to regulate transcription of a specific gene program: induction of ventricular-specific genes and/or repression of atrial-specific genes in the ventricles. Our recent experiments in chick embryos support this model (Bao *et al.*, 1999). Overexpression of Irx4 throughout the heart results in the appearance of *ventricular myosin heavy chain* (*VMHC*) in the atria with concurrent down-regulation of *atrial myosin heavy chain* (*AMHC*). Expression of a dominant-negative form of Irx4 suppressed *VMHC* in the ventricles and induced *AMHC* in this chamber. Therefore Irx4 appears necessary and sufficient to impose a ventricular phenotype. We hypothesize that continuous expression within the mature ventricular chamber and throughout adult life may indicate that Irx4 also participates in ongoing regulation of processes that are unique to the ventricular myocyte.

The markedly attenuated expression of *Irx4* in the hearts of *Nkx2-5*$^{-/-}$ embryos provides genetic evidence that Nkx2-5 is necessary for cardiac expression of this member of the IRO gene family. Although several genes are down-regulated in *Nkx2-5*$^{-/-}$ embryos, a global cardiac differentiation program is not disrupted (Lyons *et al.*, 1995; Biben and Harvey, 1997; Biben *et al.*, 1997; Tanaka *et al.*, 1999), indicating that *Irx4* is part of a specific developmental pathway that is regulated by Nkx2-5. Notably, *Irx4* is absent from most, but not all, of the *Nkx2-5*$^{-/-}$ myocardium, unlike *ANF*, *BNP*, *myocyte enhancing factor 2c*, *SM22α*, *N-Myc*, and *MLC2v*, which are down-regulated in the entire ventricular *Nkx2-5*$^{-/-}$ myocardium (Lyons *et al.*, 1995; Biben *et al.*, 1997; Tanaka *et al.*, 1999). Down-regulation of *MLC2v*, *eHand*, and *cardiac ankyrin repeat protein* is also incomplete in certain portions of the hearts of *Nkx2-5* mutant embryos (Lyons *et al.*, 1995; Biben and Harvey, 1997; Zou *et al.*, 1997). While the mechanism by which foci of *Nkx2-5*$^{-/-}$ myocytes escape down-regulation of *Irx4* expression is unclear, these findings may indicate regional regulatory potential within the ventricular myocardium.

The observation that *Irx4* expression is initiated but not maintained in *dHand*$^{-/-}$ embryos indicates that pathways regulated by dHand are important for sustained high levels of expression of *Irx4* in the developing ventricular myocardium. These may be related to the impaired growth of the myocardium that is observed in the *dHand*$^{-/-}$ embryos. In this regard it is interesting to note that *dHand*$^{-/-}$ embryos

have normal *Nkx2-5* expression, but exhibit decreased levels of *GATA4* transcripts in the heart (Srivastava *et al.*, 1997). This indicates a specificity of defects in the remaining left ventricle and suggests a potential role for GATA4 in a genetic hierarchy that controls cardiac *Irx4* expression. GATA4 and Nkx2-5 synergize in activating certain cardiac genes (Durocher *et al.*, 1997; Lee *et al.*, 1998; Sepulveda *et al.*, 1998). Since *Irx4* expression is also perturbed in *Nkx2-5*$^{-/-}$ hearts, perhaps the decrease in *Irx4* mRNA levels in *dHand*$^{-/-}$ hearts is related to this interaction.

Nkx2-5, *dHand*, and *Irx4* are likely to be components of a transcriptional cascade, analogous to the hierarchical regulation of the *Xenopus* IRO (*Xiro*) genes. Neural induction factors activate *Xiro*, which in turn activates neural differentiation (Bellefroid *et al.*, 1998; Gomez-Skarmeta *et al.*, 1998). Since Nkx2-5 is also recognized to regulate expression of *eHand*, a pathway that governs patterning and growth of the heart may integrate these with other transcription factors or morphogen gradients. The early expression of *Irx4* is likely to participate in the definition of the boundaries for a ventricular phenotype, while such genes as *eHand* and *dHand* may be involved in the maturation of the ventricular myocardium. We propose that formation of specific cardiac compartments results from interactions between general cardiac transcription factors (such as Nkx2-5, MEF2s, GATAs) and overlapping, chamber-restricted regulators (such as Irx4, dHand, eHand), which specify the phenotypes and morphology of individual cells and chambers of the heart.

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